Microsystem for biocatalytic production of biodiesel

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MICROSYSTEM FOR BIOCATALYTIC PRODUCTION OF BIODIESEL

DOCTORAL THESIS

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MIKROSUSTAV ZA BIOKATALITIČKU PROIZVODNJU BIODIZELA

DOKTORSKI RAD

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Mentor information

Bruno Zelić [redacted] In 1996, he completed his undergraduate studies in Chemical Engineering and received his Bachelor of Science in Chemical Engineering from the Faculty of Chemical Engineering and Technology, University of Zagreb. Bruno Zelić received his PhD in Chemical Engineering from the University of Zagreb in 2003. Since 2012, he has been a full professor at the Faculty of Chemical Engineering and Technology, University of Zagreb. His research interests are in the field of implementation of microreactor technology in biotechnology. More than 100 scientific and professional publications, 2 patents and more than 100 oral (plenary, key note, invited talks) and poster presentations at international conferences are testimony to his scientific work. Bruno Zelić was the Dean of the Faculty of Chemical Engineering and Technology, University of Zagreb from 2013 to 2017. From 2016 to 2017, he was a member of the Executive Board of European Federation of Chemical Engineering. He is currently Co-Editor-in-Chief of the journal Chemical and Biochemical Engineering Quarterly.

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ABSTRACT

Renewable fuels are recognized as an important energy source in today's energy market. Biodiesel, which is considered more environmentally friendly than fossil fuels, has attracted a lot of attention. Nowadays, biodiesel is produced industrially by transesterification of oils and fats of different origins. Transesterification, a reaction in which fatty acid esters are produced from oils or fats and alcohols in the presence of a catalyst, is the most widely used process for producing biodiesel.

However, the downstream processes used in industrial biodiesel production present a critical challenge and determine the final price of biodiesel. One of the biggest issues in biodiesel purification is the removal of glycerol, the by-product of a transesterification process.

To overcome the challenges of biodiesel production and purification, microsystems have been introduced as process intensification tools. Microsystems offer numerous applications, from microreactors to microextractors. The application of microsystems results in improved mass and heat transfer, higher reaction and extraction rates, lower costs and energy consumption, while producing much less waste. All these advantages can be used extensively in biodiesel production and purification.

The research conducted in this study was divided into several steps. Firstly, reaction conditions for the lipase-catalysed biodiesel synthesis were optimized. Edible and waste sunflower oil (acquired from deep frying of potatoes) were used as a substrate while methanol was used as the source of alcohol. Different microreactor configurations were analysed (different inlet strategy, different oil to methanol molar ratio, different residence times) in terms of getting FAME yield to meet the quality standard. The highest yield of 96.5 % at a residence time of τ = 20 min was obtained in the microreactor experiment using an emulsion of waste oil and commercial enzyme suspended in a water buffer as one inlet stream for a 2-stream inlet configuration.

After biodiesel was produced, purification was performed using two different technologies, extraction, and membrane filtration. Extraction was performed in microsystems using water or deep eutectic solvents (DESs). By using a ChCl:$Gly_{1:2.5}$ DES, free glycerol content in extract was less than 0.01 % (*w*/*w*) for the residence time of only 13.61 s. When biodiesel was purified by membrane filtration different membranes were used. Process was performed in an ultrafiltration module, where different membranes were tested for biodiesel purification, mainly glycerol removal. Polyacrilonitrile membrane showed average ultrafiltration efficiency (during 6 cycles) of 91.48 % with average free glycerol content in permeate of 0.006 % (*w*/*w*).

Process models for biodiesel production and biodiesel purification were developed. Different kinetic models were selected, based on which process models were developed. All process models were validated using independent experimental results.

Finally, integrated microsystems were developed, combining biodiesel production catalysed by lipase in a microreactor with biodiesel purification by either microextraction or ultrafiltration, connected in series. The best integrated microsystem was the set-up where 2-inlet feeding strategy for biodiesel production was combined with DES based microextraction. In this integrated system, for the residence time of 20 min, a FAME yield of 94 ± 3.1 % was achieved. Since the glycerol content in the purified biodiesel was lower than 0.02 % (*w*/*w*), biodiesel meets quality standards according to the standard EN 14214:2012+A2:2019.

Keywords: microsystems, biodiesel production by lipase catalysed transesterification, biodiesel purification by microextraction and membrane filtration, kinetics, mathematical modelling, integrated microsystem for the production and purification of biodiesel

PROŠIRENI SAŽETAK

Biogoriva se u posljednjih dvadesetak godina pokazuju kao potencijalno rješenje zahtjeva globalnog energetskog tržišta, pri čemu njihova primjena omogućuje postizanje ciljeva povezanih s održivim razvojem i zaštitom okoliša. Biodizel, kao jedan od predstavnika biogoriva, je u usporedbi s fosilnim dizelom ekološki prihvatljiviji, a uz to je netoksičan, biorazgradiv te se u primjeni kao gorivo odlikuje niskom emisijom stakleničkih plinova.

Uz procese direktnog umješavanja, pirolize i mikroemulzifikacije, najzastupljeniji postupak proizvodnje estera masnih kiselina (biodizela) je transesterifikacija različitih ulja i masti u prisutnosti alkohola (metanol, etanol, propanol, butanol). Transesterifikacija se najčešće odvija u kotlastim reaktorima uz prisutnost katalizatora. Katalizatori koji se koriste u reakciji transesterifikacije dijele se na homogene, heterogene i enzimatske. Kao homogeni katalizatori se najčešće koriste ili lužine (KOH ili NaOH) ili kiseline (sumporna kiselina). Glavni nedostatak procesa transesterifikacije kataliziranog lužinama je nastajanje sapuna što rezultira nižim iskorištenjem na esterima i stvaranjem emulzije. Osnovni nedostatak transesterifikacije katalizirane kiselinama je upotreba većih količina katalizatora. Osim toga, transesterifikaciju je moguće provesti učinkovito samo pri većim molarnim omjerima alkohol:ulje.

Korištenje enzima kao katalizatora u procesu transesterifikacije ima brojne prednosti u usporedbi s homogenim katalizatorima, čime se enzimatski katalizirana transesterifikacija može svrstati u procese zelene kemije. Enzimatska transesterifikacija provodi se pri blagim reakcijskim uvjetima s obzirom na temperaturu, tlak i pH-vrijednost. Kao supstrati u proizvodnji biodizela mogu se koristiti jestiva i otpadna ulja, a otpadna ulja mogu se upotrijebiti i bez predobrade. Štoviše, glicerol kao sporedni produkt procesa proizvodnje biodizela se kod enzimatski katalizirane transesterifikacije smatra visoke čistoće što je važna stavka u ekonomskoj opravdanosti ovakvog procesa proizvodnje biodizela. Jedan od najzastupljenijih enzima u industriji je enzim lipaza. Lipaza, enzim koji pripada skupini hidrolaza, može istovremeno katalizirati nekoliko reakcija kao što su esterifikacija, transesterifikacija i hidroliza. TIL lipaze (lipaze porijeklom iz *Thermomyces lanuginosus*) mogu se koristiti u obliku suspenzije ili imobilizirane, te se s obzirom na svoju visoku stabilnost široko primjenjuju u proizvodnji biodizela.

U industrijskim procesima proizvodnje biodizela, troškovi procesa povezanih s pročišćavanjem biodizela čine najveći udio u ukupnim troškovima, a mogu činiti 80 % proizvodnih troškova. Nakon proizvodnje biodizela transesterifikacijom, najčešće se za odvajanje glicerola od biodizela koristi dekantiranje. Nakon dekantiranja, određena količina glicerola (slobodni glicerol) zaostaje u biodizelu te su potrebni dodatni procesi njegova pročišćavanja kako bi se dobio biodizel koji zadovoljava odgovarajuće standarde kvalitete. Danas je poznato nekoliko metoda za pročišćavanje biodizela, kao što su mokro pranje i suho pranje, a sve veću primjenu nalaze i membranski procesi.

Proces mokrog pranja je najčešće korištena industrijska metoda pročišćavanja biodizela. Osnovni nedostatak ove metode je nastajanje velikih količina otpadnih voda, jer je približno 10 L vode potrebno za pročišćavanje 1 L biodizela kako bi se ispunili zahtjevi definirani standardima kvalitete. Kao alternativa korištenju velikih količina vode u procesu mokrog pranja sve više se koriste različita "zelenih otapala" kao što su ionske kapljevine (IL) i eutektička otapala (DES). Uz primjenu zelenih otapala sve više se za pročišćavanje biodizela koriste i membranski procesi. Tim se metodama značajno smanjuje i u nekim slučajevima potpuno eliminira korištenje vode pri pročišćavanju biodizela, te posljedično smanjuju ukupni troškovi i opterećenje okoliša tijekom proizvodnje biodizela.

Osim spomenutih nedostataka, tradicionalni procesi proizvodnje biodizela imaju i druge nedostatke kao što su dugo trajanje samog procesa, visoki proizvodni troškovi i potrošnja energije te niska učinkovitost. Zbog tih nedostataka nije moguće zadovoljiti potrebe tržišta za biodizelom. Kako bi se proces proizvodnje biodizela unaprijedio sve veću ulogu preuzimaju nove tehnologije, kojima se može povećati brzina reakcije, smanjiti molarni omjer alkohola i ulja u procesu transesterifikacije, te smanjiti potrošnja energije uslijed učinkovitijeg prijenosa tvari i energije.

Jedan od načina intenzifikacije procesa proizvodnje i pročišćavanja biodizela je provedba ovih procesa u mikrosustavima. Mikrosustavi su sustavi proizvedeni korištenjem mikrotehnologije i mikroinženjerstva. Osnovna strukturna jedinica ovakvog sustava je mikrokanal. Mikrosustav se sastoji od mreže mikrokanala s uobičajenim promjerom u rasponu od 10 do 500 μm. Ove male dimenzije mikrokanala omogućuju učinkovit prijenos tvari i energije, što uz kratko vrijeme zadržavanja doprinosi intenzifikaciji procesa. Druge važne prednosti mikrosustava su mala količina nastalih otpadnih procesnih struja i niža potrošnja energije. Također, mikrosustavi su kompaktni i jednostavni za korištenje, strujanje u mikrokanalima je najčešće laminarno, uz učinkovito miješanje i kratak difuzijski put molekula. Navedena svojstva mikrosustava ključni su razlozi za njihovu upotrebu jer procesi provedeni u njima rezultiraju visokim iskorištenjima i produktivnosti uz sigurne radne uvjete. Upravo su navedene karakteristike mikrosustava potencijalno rješenje problema koji se pojavljuju tijekom proizvodnje i pročišćavanja biodizela.

Razvoj integriranog mikrosustava za kontinuiranu proizvodnju i pročišćavanje biodizela osnovni je cilj ovog rada. Kako bi se uspješno razvio integrirani mikrosustav za kontinuiranu proizvodnju i pročišćavanje biodizela, provedena je serija istraživanja kojima su optimirani pojedini podprocesi neophodni za razvoj cjelovitog sustava. Na početku su određeni optimalni reakcijski uvjeti za sintezu biodizela procesom transesterifikacije kataliziranog enzimom lipaza u mikroreaktoru. U procesu transesterifikacije kao supstrati su korišteni jestivo suncokretovo ulje i otpadno suncokretovo ulje, dobiveno prženjem jestivog ulja, dok je kao alkohol korišten metanol. Ispitani su različiti tipovi mikroreaktora (stakleni i polimerni mikroreaktori) te različite konfiguracije mikroreaktora s obzirom na broj ulaznih procesnih struja (dva ulaza i tri ulaza), a proces transesterifikacije analiziran je pri različitim vremenima zadržavanja. Najveće iskorištenje na biodizelu od 96,5% postignuto je pri vremenu zadržavanja od τ = 20 min za reakciju transesterifikacije provedenu u mikroreaktoru s dva ulaza gdje je uz komercijalni enzim suspendiran u vodenom puferu kao supstrat korišteno otpadno ulje.

Nakon što su određeni optimalni uvjeti za provedbu reakcije transesterifikacije u mikroekstraktoru, provedena su istraživanja povezana s pročišćavanja biodizela pri čemu su korištene dvije metode, ekstrakcija i membranska filtracija. U procesima ekstrakcije, kao otapala korišteni su voda i različita eutektička otapala, a cilj ovih istraživanja bio je ispitati mogućnost korištenja eutektičkih otapala kao zamjene za vodu koja se koristi u industrijskim procesima pročišćavanja biodizela. Eutektička otpala su niskotemperaturna otapala koje odlikuje biorazgradivost i niska toksičnost, a pripravljaju se iz komponenti koje najčešće mogu biti prirodne tvari. Za pripravu eutektičkih otapala korišteno je nekoliko komponenti (kolin klorid, etilen glikol, glicerol, voda) u različitim rasponima omjera. Korištenjem eutektičkog otapala na bazi ChCl:Gly:H_2O kao otapala, provedeno je uspješno pročišćavanje biodizela, u kojem je udio slobodnog glicerola iznosio manje od 0,01 % (w/w), čime je zadovoljena vrijednost koju propisuju odgovarajući standardi kvalitete biodizela.

Pročišćavanje biodizela membranskom filtracijom provedeno je u ultrafiltracijskom modulu uz korištenje četiri membrane: polipropilenska (PP), polietersulfonska (PES), poliakrilonitrilna (PAN) i regenerirana celuloza (RC). PAN membrana pokazala se kao najučinkovitija za uklanjanje glicerola, a s gotovo konstantnom učinkovitošću (približno 91,48 %) korištena je u 6 ciklusa ultrafiltracije pri čemu je postignut udio slobodnog glicerola u permeatu od 0,006 % (w/w) što je znatno manje od vrijednosti koje propisuju standardi kvalitete biodizela.

Razvijen je matematički model procesa ekstrakcije pri kojem je simulirano pročišćavanje biodizela ekstrakcijom pomoću vode i eutektičkog otapala. Matematičkim modelom procesa ekstrakcije opisano je uklanjanje glicerola iz biodizela u mikroekstraktoru korištenjem stacionarnog 2D matematičkog modela procesa koji uključuje konvekciju u smjeru strujanja (x) i difuziju u dva smjera (x i y). Matematički model procesa ekstrakcije sastojao se od bezdimenzijskih parcijalnih diferencijalnih jednadžbi za opisivanje koncentracije glicerola u fazi biodizela i fazi otapala te odgovarajućih graničnih i početnih uvjeta.

Prilikom modeliranja procesa pročišćavanja biodizela membranskom filtracijom, mehanizmi čepljenja membrane opisani su Hermia modelima. Kako bi se dobio uvid u mehanizam koji dovodi do pada protoka u procesu membranske ultrafiltracije, korištena su četiri mehanizma čepljenja: potpuno čepljenje pora, unutarnje čepljenje pora, srednje čepljenje pora i formiranje kolača.

Na kraju je razvijeno nekoliko različitih integriranih sustava za proizvodnju i pročišćavanje biodizela. Kao prvi, razvijen je sustav u kojemu su se provedbom reakcije u DES-u osigurali uvjeti za sintezu i pročišćavanja biodizela u jednom stupnju, odnosno u jednoj mikroprocesnoj jedinici, ali ovaj sustav nije rezultirao zadovoljavajućim iskorištenjem biodizela. Integrirani mikrosustavi u kojima se proizvodnja biodizela katalizirana enzimom lipaza odvijala u mikroreaktoru, a pročišćavanje biodizela ekstrakcijom ili ultrafiltracijom u drugoj mikrojedinici spojenoj u seriju, rezultirala je procesom u kojemu je uz vrijeme zadržavanja od 20 minuta postignuto iskorištenje biodizela od 96,5 % i sadržaj glicerola u pročišćenom biodizelu manji od 0,02 % (maseni).

Ključne riječi: mikrosustavi, proizvodnja biodizela transesterifikacijom kataliziranom enzimom lipaza, pročišćavanje biodizela mikroekstrakcijom i membranskom filtracijom, kinetika, matematičko modeliranje, integrirani mikrosustav za proizvodnju i pročišćavanje biodizela

Contents

1. INTRODUCTION

Modern society is slowly but surely crossing every limit of progress in all possible spheres of life. However, progress also brings with it a great burden, namely the negative impact on the environment. With the increase in human population, the demand for energy is also continuously increasing. One of the most important sources of energy is fossil fuels (88 % of the world's total energy consumption), which are mainly obtained from non-renewable energy sources [1]. An energy source can be generally recognized as renewable if two very important principles are met: lower pollution potential and lower contribution to global warming. Biodiesel has found the widest use compared to the other biofuels (biogas and bioalcohols). It is recognized as a renewable, non-toxic, biodegradable and environmentally friendly alternative to fossil diesel [1,2]. Biodiesel is industrially produced by four main methods: blending, micro-emulsification, pyrolysis, and transesterification [3]. Transesterification has been shown to be the most efficient and economical method for producing biodiesel. In addition, a major advantage of transesterification is the wide variety of catalysts, which can be chemical or enzymatic [4]. To further emphasize the dominance of transesterification process, waste oils and fats can be used as a substrate for biodiesel production, which has been confirmed by various research groups [5-10]. Reduced environmental impact can be further emphasized by using enzymatically catalysed transesterification [4].

After selecting the transesterification type and determining the catalyst and substrate source for biodiesel production, it is important to clarify the scale at which the transesterification process will take place. Conventional biodiesel production scales have not been able to meet current market demands. Since scaling up was not suitable, downscaling was envisioned as a possible solution for intensifying all process steps of biodiesel production. Microsystems are recognized as one of the most promising process intensification technologies and their applicability in biodiesel production is gaining more and more attention [11].

Large volume of biodiesel produced is only “one side of the coin” when it comes to meeting the needs of the energy sector. Another important thing is the quality criteria defined by standards, which can only be achieved through the biodiesel purification. These criteria mainly refer to a high FAME (Fatty Acid Methyl Esters) yield and a low content of free glycerol, which is a by-product of biodiesel production. Wet washing (washing with water) is the most widely used industrial technique for biodiesel purification. By using this technique purity of biodiesel can easily achieve demands defined by standards [12]. However, the amount of water used in the purification of biodiesel by wet washing is so high that it is not environmentally and economically justified [13]. Another solution for purification can be extraction with deep

eutectic solvents (DESs). DES is a mixture of hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA). DES can be prepared from various compounds, each of which can have some kind of extractive property [14]. For this reason, DESs are not only used as catalysts or reaction media [15,16], but also find their application in the purification of biodiesel.

Based on all the information presented, the main objective of this research was to develop an integrated microsystem consisting of a microreactor for biodiesel production and a microextractor for biodiesel purification connected in series. The research also includes optimization of experiments for biodiesel production catalysed by lipase, preparation and application of different solvents for biodiesel purification by extraction, modelling of transesterification reaction catalysed by lipase and modelling of purification of biodiesel by extraction and by membrane filtration.

Research hypotheses are:

1. Determination of optimal reaction conditions for lipase catalysed production of biodiesel in a microreactor
2. Optimization of the biodiesel purification process by extraction and membrane filtration
3. Development of mathematical models, describing the processes of biodiesel synthesis and biodiesel purification in microsystems
4. Development of a fully integrated biodiesel production process on a microscale

Research hypotheses are confirmed by seven scientific papers regarding development of integrated microsystem for biodiesel production catalysed by lipase:

Paper 1: "Transesterification in Microreactors - Overstepping Obstacles and Shifting Towards Biodiesel Production on a Microscale" by M. Gojun, M. Bačić, A. Ljubić, A. Šalić and B. Zelić [17]

Paper 2: "Biodiesel purification in microextractors: Choline chloride based deep eutectic solvents vs water" by A. Šalić, A. Jurinjak Tušek, M. Gojun and B. Zelić [18]

Paper 3: "Purification of biodiesel produced by lipase catalysed transesterification by ultrafiltration: Selection of membranes and analysis of membrane blocking mechanisms" by T. Sokač, M. Gojun, A. Jurinjak Tušek, A. Šalić, B. Zelić [19]

Paper 4: "Continuous Integrated Process of Biodiesel Production and Purification—The End of the Conventional Two-Stage Batch Process?" by M. Bačić, A. Ljubić, M. Gojun, A. Šalić, A. Jurinjak Tušek, and B. Zelić [20]

Paper 5: "Kinetic Parameter Estimation and Mathematical Modelling of Lipase Catalysed Biodiesel Synthesis in a Microreactor" by M. Gojun, L. Pustahija, A. Jurinjak Tušek, A. Šalić, D. Valinger and B. Zelić [21]

Paper 6: "Model-to-model: Comparison of mathematical process models of lipase catalysed biodiesel production in a microreactor" by M. Gojun, A. Ljubić, M. Bačić, A. Jurinjak Tušek, A. Šalić and B. Zelić [22]

Paper 7: "Integrated microsystems for lipase-catalyzed biodiesel production and glycerol removal by extraction or ultrafiltration" by M. Gojun, A. Šalić and B. Zelić [23]

Aforementioned hypotheses were confirmed by these seven scientific papers published in internationally recognized journals (cited in Web of Science), and their allocation is graphically presented in Figure 1.

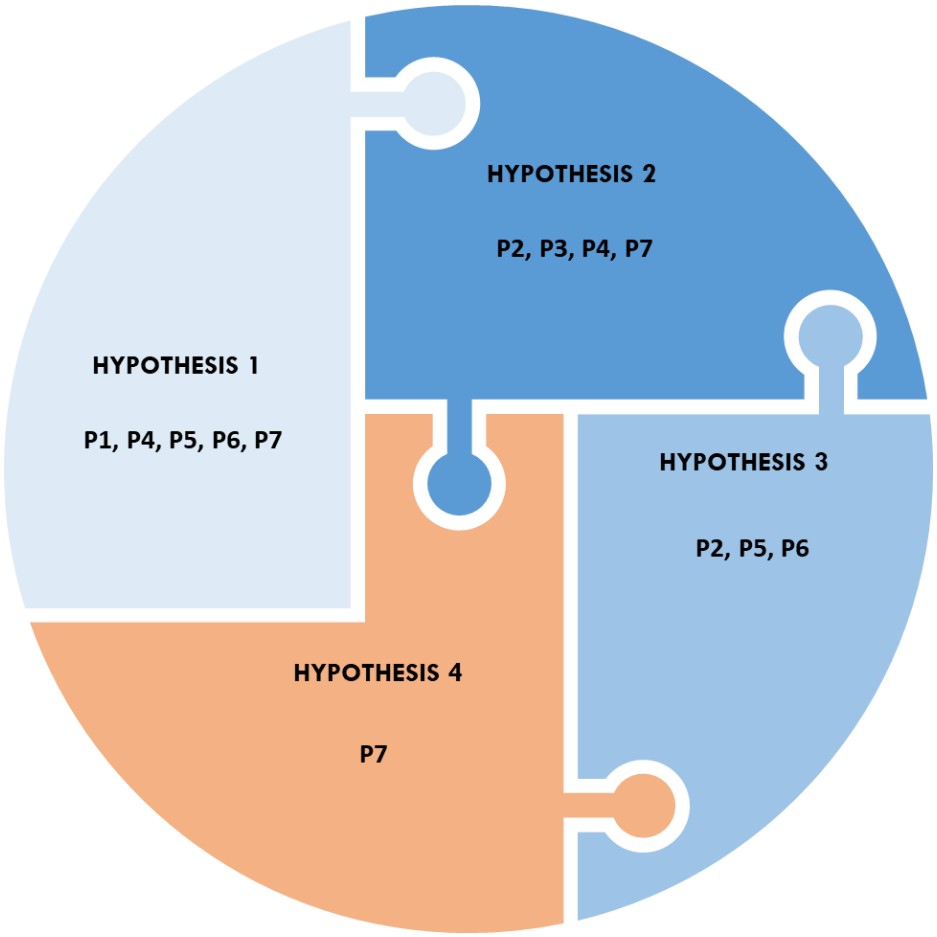

Figure 1. Relationship between hypotheses of thesis and corresponding scientific papers

2. LITERATURE REVIEW

To justify the scientific output of thesis, emphasis has been placed on two research topics which will together present one fully complementary result. The first topic is the research of biodiesel, its production and purification. The literature review will cover all important biodiesel production and purification processes directly aligned. Emphasis will be on the advantage of enzymatic production of biodiesel over chemical synthesis.

The second topic is microsystems and their advantages over mesoscale systems. A brief explanation of microsystems will be given, and different types of microsystems will be presented.

By combining these two research areas, main idea and motivation for thesis will be shown and compared to similar experiments described in the literature. To further explore reaction behaviour in microsystems, mathematical models of biodiesel production by transesterification and biodiesel purification by extraction and membrane filtration will be presented and explained.

2.1. Biodiesel

Biodiesel, a mixture of fatty acid alkyl esters, has emerged as a non-toxic and biodegradable alternative to petrol diesel. It is produced by four main production processes: blending, micro-emulsification, pyrolysis, and transesterification. Although all of the above processes have advantages and disadvantages, transesterification has proven to be the most widely used. A variety of transesterification processes can be carried out from triglycerides and some form of alcohols in the presence of various catalysts [3]. The alcohol used for transesterification process is usually methanol, due to its price and availability. Therefore, biodiesel is usually also recognized as FAME – fatty acid methyl esters.

Biodiesel used in internal combustion engines (as a mixture with fossil diesel) must meet very high quality criteria. According to the standard EN 14214:2012+A2:2019 [12], the mass content of esters in biodiesel must be higher than 96.5%, while the glycerol content must be lower than 0.02%. Even though the criteria are very strict today, that was not the case in the early days. Biofuels have come a long way since the first use of vegetable oil as a fuel by Rudolf Diesel himself. Today, it can be said that biofuels can be divided into four generations, based on the source of the biomass used. A brief classification is given in Figure 2. First and second generation feedstocks are mostly used for the production of biodiesel. Second generation waste oils are used especially in the transesterification process, which is the most widely used process to produce biodiesel [6].

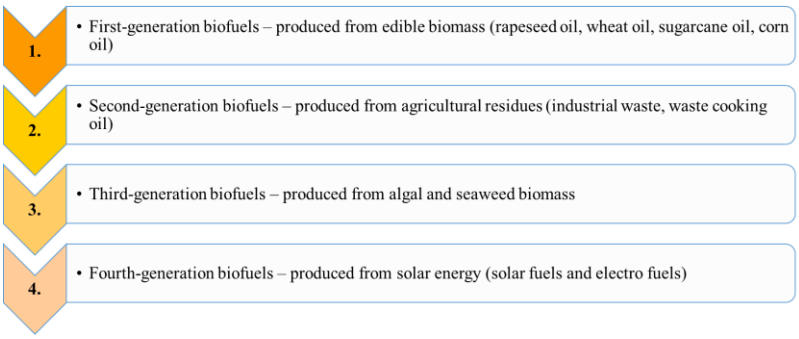

Figure 2. Classification of biofuels

The impact of people's daily lives on the environment has been thoroughly researched in recent decades. The advantage of biodiesel over fossil fuels is its minimal toxicity, biodegradability, and near zero-emission of aromatic compounds, sulphates, and other chemical components that negatively impact the environment, especially in relation to everyday transportation. In the last two decades, the number of scientific papers on biodiesel has increased significantly, as shown in Figure 3.

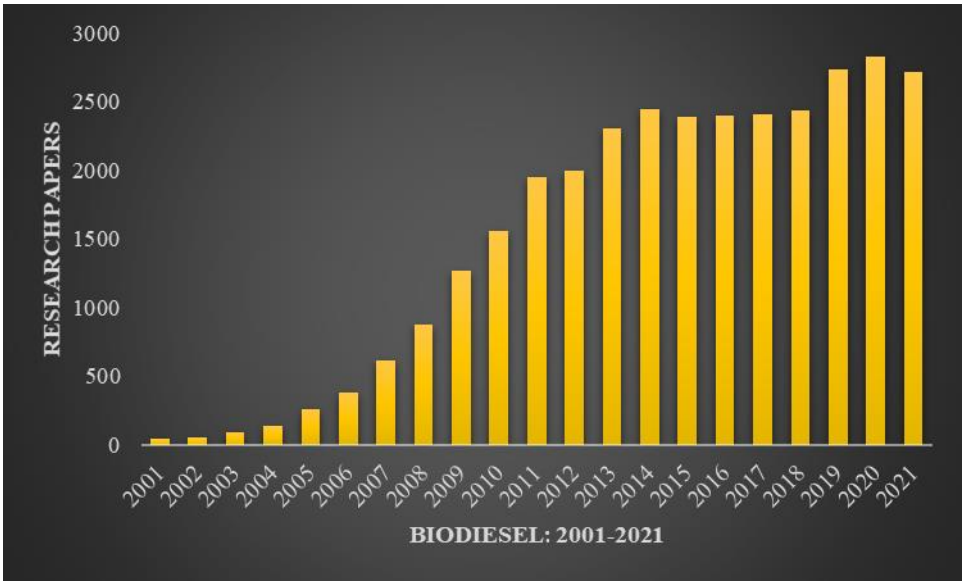

Figure 3. Scientific papers about biodiesel, annual data. Source: Scopus database

2.1.1. Biodiesel production

Nowadays, catalytic transesterification is the best-known method for biodiesel production on an industrial scale [5-7, 24,25], and it is usually carried out in batch reactors. As mentioned above, the advantage of transesterification is the possibility to use different biomasses and feedstocks (edible vegetable oils, animal fats, waste edible oils) [26] in combination with

different alcohols (mainly methanol, ethanol, propanol, or butanol) as substrates for biodiesel production.

Transesterification under energetically sustainable conditions is only possible in the presence of the catalyst. The feedstock used for biodiesel production usually determines the type of catalyst. Catalysts for transesterification processes can be divided into three main groups: homogeneous, heterogeneous and enzymatic catalysts. Homogeneous catalysts are used either in alkali-based processes (with KOH or NaOH as catalyst) or in acid-based processes (with sulphuric acid as catalyst). The major drawback of alkali-based transesterification processes is the undesirable saponification due to side reactions, which leads to lower ester yields and emulsion formation. On the other hand, acid-catalysed transesterification requires higher amounts of the catalyst. In addition, the process is efficient at higher alcohol-to-oil ratios. This leads to problems in the downstream processes, where several steps are required to purify the biodiesel after production. In summary, nowadays large amounts of wastewater are generated after chemical-catalysed biodiesel production, as the environmentally harmful wet washing is still the most widely used purification process [27].

2.1.1.1. Enzymatic transesterification

The use of enzymes in industrial processes has increased greatly in recent decades. One of the most widely represented enzymes in industry is lipase (EC 13.1.1.3.). Lipase, an enzyme belonging to the hydrolase group, can simultaneously catalyse several reactions such as esterification, transesterification, and hydrolysis [28,29]. The reason for this broad versatility is a dual hydrolytic and synthetic activity of lipases. Lipases from *Thermomyces lanuginosus* (TlLs) are basophilic and thermostable enzymes. TlLs can be purchased commercially as suspensions or in immobilised form. The high stability of TlLs has ensured their very wide use in biodiesel production [4]. The use of enzymes in the transesterification process has several advantages compared to bases and acids as catalysts, thus forming new green chemistry processes. This mainly refers to the performance under mild conditions, which include temperature, pressure, and pH. In addition to reaction conditions, substrates in biodiesel production can be both edible and waste oils, and waste oils can even be used without pre-treatment. Moreover, when lipases are used in transesterification glycerol as a by-product of biodiesel production is food-grade glycerol. Additionally, soap formation does not occur [28,30]. Finally, there is the possibility of reusing lipase during transesterification, which makes the whole biodiesel production process more efficient both environmentally and economically.

A list of the advantages and disadvantages of enzymatic transesterification compared to chemical transesterification can be found in Figure 4.

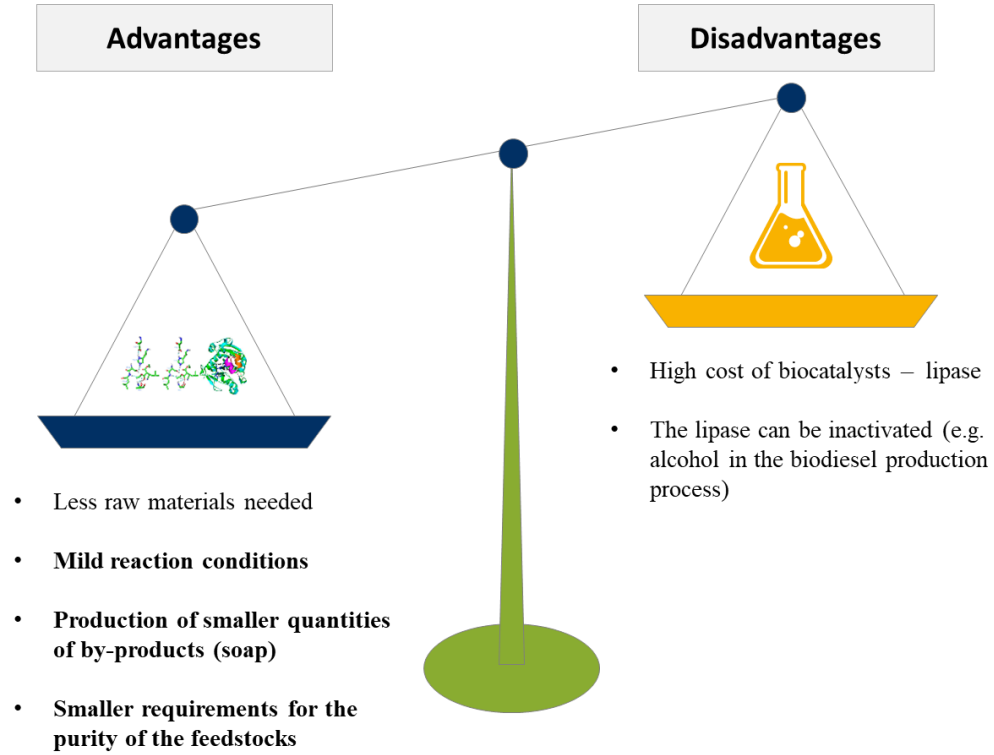

Figure 4. Advantages and disadvantages of enzymatic transesterification compared to chemical transesterification

2.1.2. Biodiesel purification

On an industrial scale, the cost of the downstream processes required to purify biodiesel accounts for the largest share of the total price of biodiesel, which can be as high as 80% [31]. After biodiesel production by transesterification, the most common separation process for separating glycerol from crude biodiesel is decantation [32]. Since a certain amount of glycerol (free glycerol) is still present in the biodiesel after this step, additional downstream processes are required. Nowadays, several methods are known for successful purification of biodiesel, such as wet washing, dry washing, and membrane separation processes [33]. A brief classification of biodiesel purification processes can be found in Figure 5.

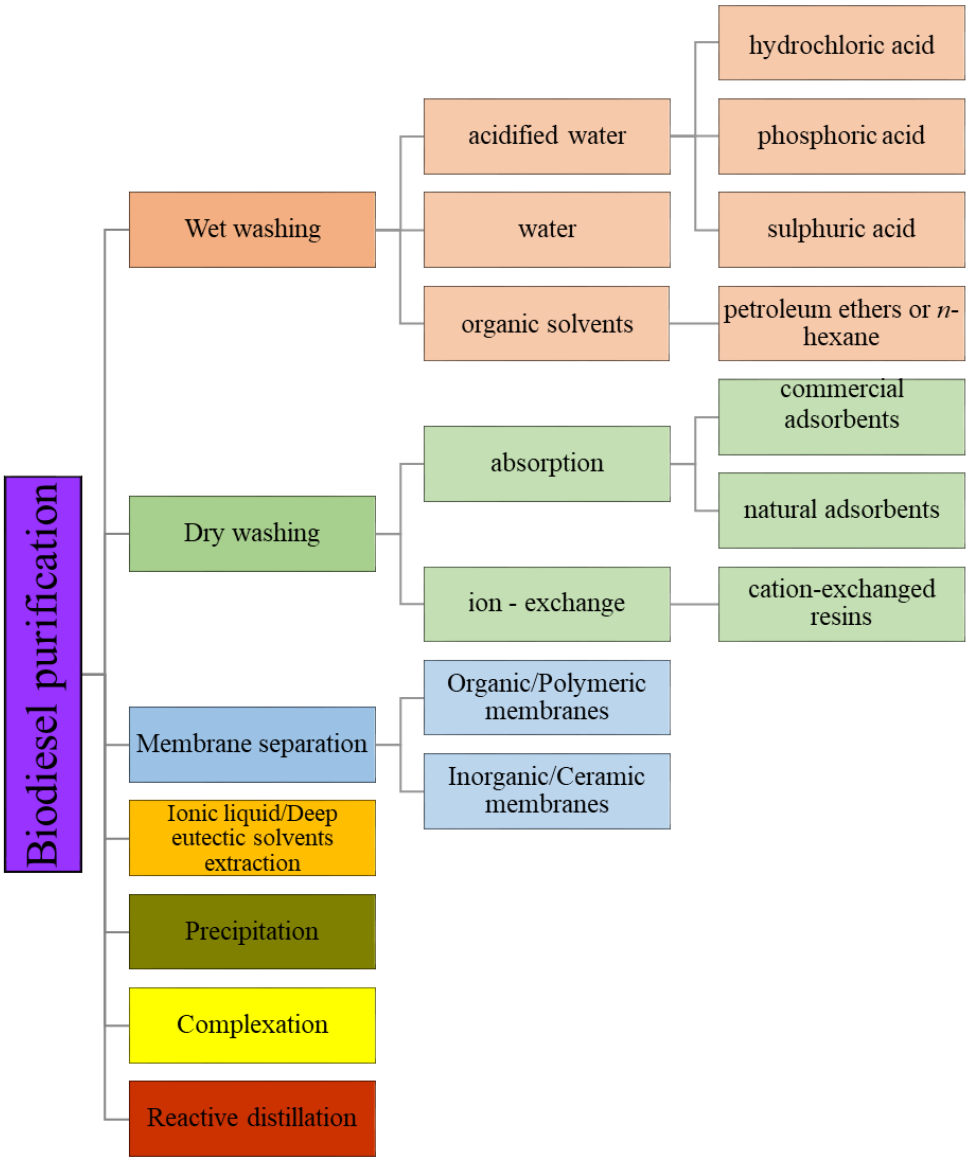

Figure 5. Different methods for the purification of the biodiesel

Wet washing is the most commonly used method. However, the main problem with water use is the generation of large amounts of wastewater, which requires additional purification methods [26]. According to Karaosmanoğlu et al. [34], 10 L of water is required to purify 1 L of biodiesel to meet the requirements of the standards. To cope with this economic and environmental setback, an alternative has been found in the application of green solvents, such as ionic liquids (ILs) and deep eutectic solvents (DESs) [35,36]. Another technology for biodiesel purification is separation by membranes. The application of membrane technology for biodiesel purification has been researched in the last decade. It has been confirmed that water consumption can be significantly reduced by alternative purification processes, resulting in a large reduction in process costs [37,38].

2.1.2.1. Deep eutectic solvents (DESs)

A DES is a binary or ternary mixture consisting of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) that form a hydrogen bond [39]. They are called green due to their high biodegradability and low toxicity. Moreover, they are prepared from cheap components such as choline chloride as HBA representatives, and various sugars, alcohols, and amides as naturally derived HBDs [40]. DESs have been utilized in numerous research areas: electrochemistry [41], polymer synthesis [42], organic chemistry [43], analytical chemistry [44], and as extraction medium [45,46]. Up to now, DESs have been successfully used for biodiesel purification [14,35,37,39,47-49], but only in discontinuous extractors/separators. Continuous biodiesel purification is the next step to further reduce purification cost and complexity of the overall biodiesel production.

2.1.2.2. Membrane technology

Membrane technology for biodiesel purification has received much attention in the last decade. It has been demonstrated that membrane technology can reduce water consumption during the biodiesel purification step, which has a significant impact on process costs [37,38]. Other confirmed advantages include process reliability and low energy consumption, as temperature and pressure conditions are more moderate [50]. In short, membrane technology is based on semipermeable barriers (membranes) that separate different types of a solution in a selective manner, allowing only limited passage for some components of the mixture [51]. Membranes are most commonly used for microfiltration (MF) and ultrafiltration (UF). The MF membranes separate fine particles in the size range of 0.1-10 μm. Ultrafiltration is a separation process in which the pore size of the membranes is in the range of 1-100 nm [52].

2.2. Microsystems

Microsystems are microscale systems fabricated using microengineering and microtechnology [53]. The basic structural unit is always a microchannel. The microchannel is located in a material/base called an element. The element and microchannel together form a chip. A larger structure is called a unit, which consists of the chip and the fluid lines responsible for the input and output streams. Finally, the combination of the unit and all other pieces of equipment (pumps, analytical systems, etc.) is called a microsystem [54]. A microsystem consists of a network of microchannels with the usual diameter in the range of 10 to 500 μm. These small dimensions of the microchannels allow efficient mass and energy transfer, which, with a short residence time, contributes to the intensification of the processes carried out. Other important

advantages are the low amount of process waste and lower energy consumption. Moreover, microsystems are compact and easy to implement, the flow of compounds through the microchannels is usually laminar, while efficient mixing and the short diffusion path of molecules are other advantages of microsystems [55]. The above properties of microsystems are good reasons for their use. They provide high utilization and productivity in carrying out the process, accompanied by safe working conditions [18,56].

2.2.1. Application of microsystems

Microsystems are used in various fields of chemical and pharmaceutical industry, biotechnology, and medicine [54]. The application of various microreactors to intensify chemical and biochemical production processes is being intensively researched, and the trend is increasing every year. Although the vast majority of reaction systems studied in microreactors are related to chemical synthesis, biocatalysis and biotransformations in a microreactor are receiving more and more attention [57]. In the last two decades, the application of microreactors in photochemistry has also been studied [58,59]. Figure 6. shows the number increase of scientific works on microsystems in the last two decades.

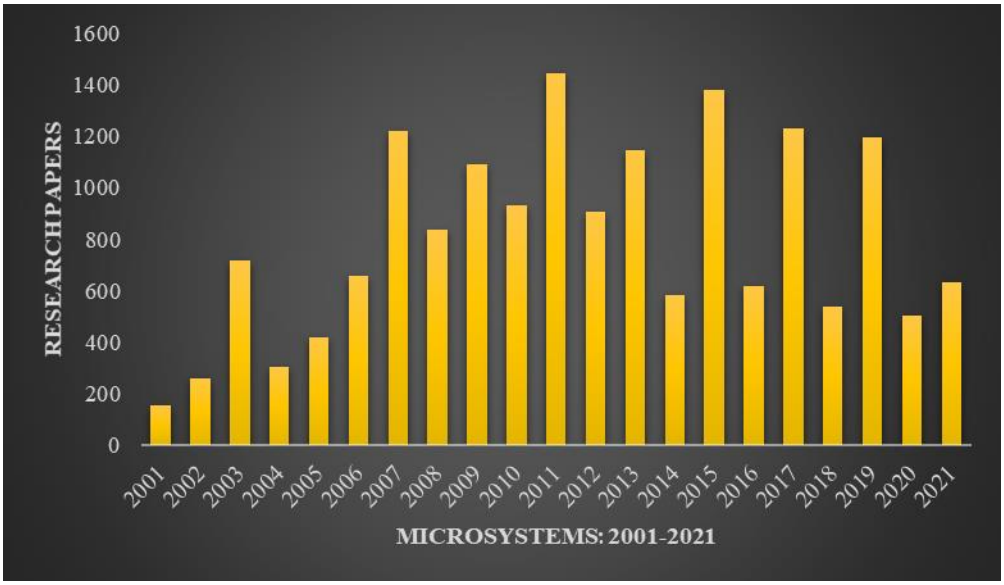

Figure 6. Scientific papers about microsystems, annual data. Source: Scopus database

For many years, the focus of microtechnology has been mainly on laboratory scale. Research has included process optimization, production, kinetic measurements, separation, and transfer of batch processes to continuous systems. In addition to laboratory research, interest has recently been slowly shifting to the development of modular systems, which should be the next step necessary if microsystems are to be used in industry [60]. The main idea is that these

modular systems include all production steps - from substrate preparation to clean products at the end of the process. The main advantage of modularity is that once all the required information and settings for each module are known, they are simply multiplied by the number of modules to be connected. In addition, modules ensure that the process can continue even if some modules stop working [55,57,60].

2.3. Biodiesel production in microsystems

Considering the increasing demand for biodiesel, existing conventional production processes needs to be improved to meet market demands. Therefore, conventional processes mainly related to batch production of biodiesel have recently been converted to continuous production processes. In addition, the production time must be shortened, therefore some kind of process intensification is required. Among the various process ideas, the use of microsystems has been found to be a good solution for biodiesel production [7].

The main objective of microsystems is to improve mass transfer, resulting in a shorter overall reaction time. Transesterification in microchannels significantly increases the dispersion of two phases between the reactants, oil/fat and alcohol, respectively. The ratio of oil to alcohol is one of the most important parameters and sometimes the decisive factor for the productivity of biodiesel. Since transesterification is an equilibrium reaction, an excess of alcohol is needed to shift the reaction towards the formation of alkyl esters [60]. In batch systems, a high excess of alcohol is acceptable for chemical transesterification, while for enzymatic transesterification this reactor system is not the first choice due to the inactivation of the enzyme by alcohols such as methanol and ethanol. The use of microsystems may be able to solve this problem. Microreactors have been successfully used for efficient micro-scale biodiesel production for some time [8,11,17,60].

2.4. Biodiesel purification in microsystems

As mentioned earlier, there are numerous biodiesel purification technologies, most of which are macro-scale batch processes. Regardless of which purification technology is used, it usually involves multiple purification steps, resulting in the need to install numerous additional pieces of equipment. Recently, micro-scale biodiesel purification has been explored. The first major advantage of microscale systems is certainly the size, which significantly reduces the space required for the purification equipment. Furthermore, if there are more purification steps, it means that only a few microchips are used. Another advantage is the transition from

discontinuous batch processes to continuous processes. The microscale allows much faster separation/extraction times because the diffusion path is very short. This phenomenon is due to a very high mass transfer rate in microchannels combined with a large surface area to volume ratio [52,61]. Microsystems designed for purification, usually known as microseparators/microextractors, have proven to be a favourable technique for the separation of chemical and biochemical products [62-66], including biodiesel [18].

2.5. Mathematical modelling

Even laboratory-scale research requires money, time, and manpower. To achieve sustainable processes, mathematical modelling can be an effective tool for process development. The biggest advantage of modelling is the use of simulations that can replace hours in the lab.

2.5.1. Enzymatic reactions

Enzymatic reactions can be successfully optimised by mathematical modelling. In short, in the development of enzyme-catalysed biodiesel production by transesterification, one of the most important steps is the determination of enzyme kinetics. In addition, knowledge of reactor type and dimensions, flow rates, and substrate concentrations are also important. Enzyme kinetic models, combined with mass balances, are an important component for the overall picture of enzymatic process design [67]. Research on biodiesel synthesis catalysed with lipase has been ongoing for two decades [9,68-70]. However, most of the kinetic studies deal with the esterification of free fatty acids [68-72]. In recent years, the kinetic model for lipase-catalysed transesterification has been established [21,22,76]. The first step in developing a mathematical model of the process occurring in a microreactor is to determine the kinetic parameters from the microreactor experiments. The already mentioned features of the surface area to volume ratio, the transport phenomena, the higher mass transfer rate and the shorter residence time distinguish the microscale from the conventional macroscale also in the determination of the parameters of the kinetic models [77].

The initial reaction rate method is usually used to estimate kinetic parameters. The initial reaction rate should be determined for different experimental setups (batch or continuous) and for different scales (micro or mesoscale). Since lipase can catalyse numerous reactions simultaneously (transesterification, esterification, hydrolysis) [10], estimation of kinetic parameters is required for both forward (transesterification) and reverse (hydrolysis) reactions.

The kinetics of biodiesel production by transesterification are modelled with a Bi-Bi Ping-Pong kinetics, Michaelis-Menten kinetics and Hill kinetics [78]. These models are selected for

several reasons. Michaelis-Menten kinetics is usually used in a reaction with two substrates, Bi-Bi Ping-Pong kinetics is present in biodiesel synthesis, while Hill kinetics showed some interesting results in research conducted by Šibalić et al. [79].

In addition, four mathematical process models were presented and usually used to describe the transesterification process catalysed by lipase in a microreactor. The first model used is a 2D mathematical process model. It includes the kinetics of the reaction, diffusion in two directions (x and y) and convection in the flow direction (x) [21,64,80]. The second model used is a 1D model previously described by Jurinjak Tušek et al. [81]. In this model, the approximation of the microreactor with two parallel plug flow reactors is described. The third model is also a 1D model described by Tušek et al. [82]. In this model, it is assumed that there are no radial velocity fluctuations and no axial dispersion. It is also assumed that the mass transfer coefficient is insignificant.

The Bi-Bi Ping-Pong process model, as the fourth model, is based on the assumption that the most important step is the esterification of fatty acids. The model further investigated by Liu et al. [76] states that there are no mass transfer limitations, various substitutions of mono-, di-, triglycerides and fatty acids are treated as a single constituent, methanol is considered as the main inhibitor of the enzyme, and the limiting step of the reaction is fatty acid esterification and glyceride hydrolysis.

2.5.2. Mathematical model of biodiesel purification in a microextractor

Glycerol separation in a microextractor was described by Jurinjak Tušek et al. [18] using a 2D mathematical model. The model includes diffusion in two directions (x and y) and convection in the flow direction (x). The mathematical model for steady-state conditions in a microextractor consists of dimensionless partial differential equations for the glycerol concentrations in both phases: in the biodiesel and DES /water phases [18].

2.5.3. Modelling of blocking mechanisms in the process of dead-end ultrafiltration

During the process of membrane filtration, the reduction of permeate flux is determined by several mechanisms: the formation of a precipitation layer, the clogging of pores, and the concentration polarization. To better describe the mechanism of flux decrease, Hermia developed a fouling model that includes four basic types of fouling: the complete blocking model, the intermediate blocking model, the standard blocking model, and the cake layer model [83]. These four models are distinguished by the assumption that molecules of different sizes

enter the pores of the membrane to form different fouling. Since the purification of biodiesel takes place in an ultrafiltration module, these four models can be used to predict the fouling mechanism. Several different factors could be determined prior to the purification process: membrane type, pH, temperature, water content, and transmembrane pressure (TMP) [19]. Cycles of semi-continuous and discontinuous ultrafiltration are usually used to compare selected membranes.

3. DISCUSSION

The goal of this thesis is to present the development of an integrated system for lipase catalysed biodiesel production on a microscale. To develop integrated system on a microscale, several process steps should be optimised independently, and finally combined into fully functional microsystem. The individual steps of process development are addressed in some of the seven scientific papers that form the basis for this discussion. The first step of the process development relates to the determination of the optimal reaction conditions for the lipase catalysed synthesis of biodiesel in a microreactor. In addition, various biodiesel purification methods were investigated on a microscale. This mainly relates to the removal of excess glycerol. To fully understand the reaction mechanism of biodiesel production by enzyme catalysed transesterification and the separation behaviours in both extraction and membrane filtration, mathematical modelling was used. The mathematical modelling served as a tool for process optimization and, if possible and applicable, for predicting a part of the results for independent experiments performed later. Finally, an integrated microsystem was developed that included the biodiesel production and purification steps. The integrated microsystem, as well as all the process steps previously developed, were based on the production of biodiesel that meets quality standards, while respecting the principles of green and sustainable chemistry.

3.1. Biodiesel synthesis

3.1.1. Biodiesel synthesis in batch reactors

As a basis for the research, lipase catalysed transesterification of sunflower oil (both edible and waste) to biodiesel was carried out in four batch experiments (Table 1, Paper 1 [17]). These experiments were conducted because there is limited data in the literature on biodiesel production by lipase catalysed transesterification [10]. The goal is to ultimately compare the performance of batch and microreactor systems to confirm the idea that the use of microsystems intensifies biodiesel production.

Four combinations of batch experimental setups for biodiesel production were based on two different oil sources and two different reaction media (parameters were kept constant in all experiments: oil to methanol molar ratio 1:3.4, enzyme concentration $\gamma_{E,0}$ = 0.1 mg/mL). Two different oil sources, edible and waste sunflower oil, were used as substrates. Lipase-catalysed transesterification was carried out in two reaction media, an aqueous buffer and ChCl:$Gly_{1:3.0}$ DES. DES was proposed as the reaction medium because its composition can ensure stability and activity for enzymes such as lipase, even in the presence of high excess of methanol used as the second substrate [84]. All experiments were performed for a total time of 48 hours, and

commercial lipase, Lipolase 100L, was used as catalyst in all of them. As can be seen (Table 2, Paper 1 [17]), the highest FAME yield was obtained with edible sunflower oil as substrate in the experiment where an aqueous buffer was used as reaction medium. A slightly lower yield was observed in experiments with waste sunflower oil, probably due to impurities present in this substrate. In experiments with ChCl:$Gly_{1:3.0}$ DES, the yield was rather low, which can be explained by the lack of water in the system (Experiments 3 and 4, Table 2, Paper 1 [17]). According to Merza et al. [85], higher FAME yields for lipase-catalysed transesterification can be obtained when at least 1 % (w/w) water is added to pure anhydrous DES. In the experiments carried out in this research, the water content in the reaction medium was determined to be 0.7 %, which is the main reason for the lower value of FAME yield for the transesterification reaction carried out in DES as the reaction medium.

3.1.2. Biodiesel synthesis in a microreactor – results and guidelines for future experiments

After initial batch experiments, several microreactor experiments were set up to find the optimal system for biodiesel production by lipase-catalysed transesterification. The aim of this research step was to obtain similar FAME yields at shorter (residence) time compared to the yields obtained in batch experiments, thus confirming the intensification of transesterification. A two-inlet strategy was used to feed substrates into a microreactor. The experimental setup is shown in Figure 7 (Figure 1, Paper 5 [17]).

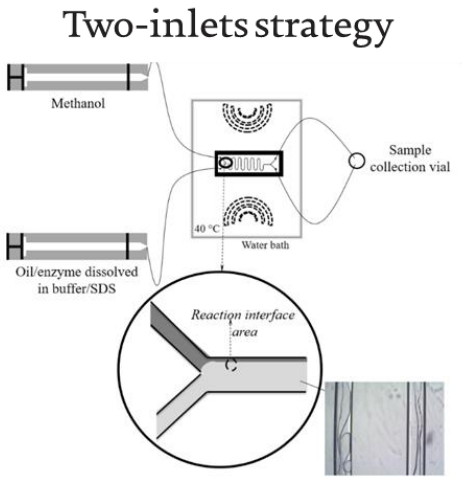

Figure 7. Two-inlet strategy used for supply of substrates into a microreactor

In these microreactor experiments, a two-inlet strategy was used and different process conditions were tested, depending on the oil source, reaction medium and source of lipase (both commercial and raw lipase produced by solid-state fermentation were used). All experiments

(Experiments 5-12, Paper 1 [17]) were performed for the same range of residence times (τ = 0.5-30.62 min), with the same initial substrate ratio (the molar ratio of oil to methanol 1:3.4.) and for the same lipase concentration ($\gamma_{E,0}$ = 0.1 mg/mL), so that the comparison was easier. The main process parameters compared were FAME yield and volumetric productivity.

Influence of Oil Origin on FAME Yield

The first two experiments performed in a microreactor were Experiments 5 and 6 (Table 1 – Table 4., Paper 1 [17]). These experiments were conducted under the same process conditions as batch experiments 1 and 2. Sunflower oil was used as substrate in experiment 5 and WCO (waste cooking oil) was used in experiment 6. The comparison of the FAME yield for these two experiments is shown in Figure 8. Obviously, for the same residence time the FAME yield is higher when WCO was used in transesterification. One explanation for the highest FAME yield in the experiment with WCO (I =33 % for residence time τ = 30.62 min) is the cracking of the long chains in the oil during frying and the resulting shorter chain fatty acids present in the reaction medium during transesterification [17].

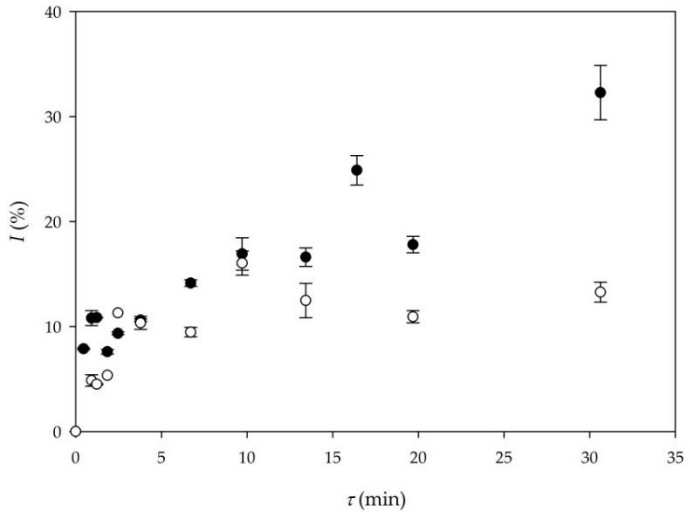

Figure 8. Comparison of FAME yield depending on oil origin (○ – edible oil (Experiment 5), ● – WCO (Experiment 6)) (Figure 2, Paper 1 [17])).

Influence of Enzyme Origin on FAME Yield

To make the overall process more economical raw lipase was used. Raw lipase was produced by solid-state fermentation of *Thermomyces lanuginosus* on by-products from cold-pressed oil production. After solid-state fermentation lipase was extracted from fermentation medium by water, partially purified, and used as catalyst for biodiesel production. The results of transesterification catalysed by raw lipase were compared with those obtained for biodiesel

production catalysed by commercial lipase. As shown in Figure 9 (Figure 4, Paper 1 [17]), there is a significant difference in FAME yield when commercial lipase and raw lipase [22] were used. The main reason for this is the much higher initial activity of the commercial enzyme (about 200 times higher) compared to the raw lipase. At this point, the raw lipase is not suitable for biodiesel production without additional optimization of the purification steps. An additional approach to make the process more economical would be to immobilise or recycle the commercial lipase.

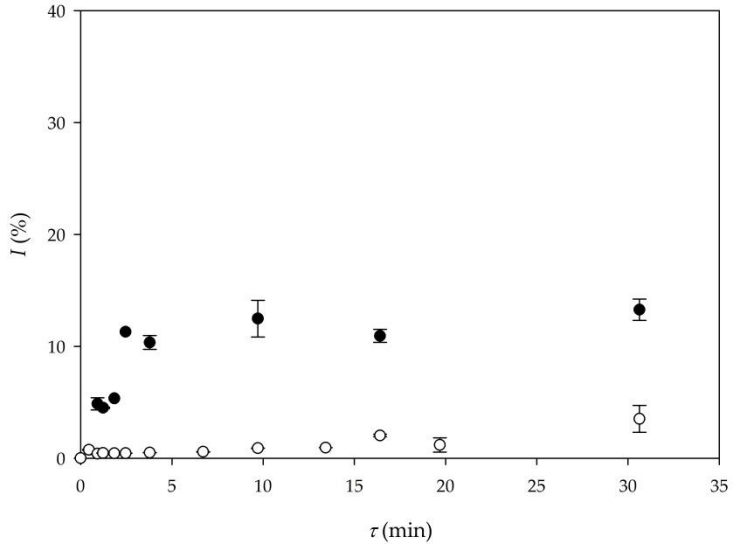

Figure 9. Comparison of FAME yield according to lipase origin (● – commercial lipase Lipolase 100L (Experiment 5), ○ – raw lipase produced by solid-state fermentation (Experiment 9)) (Figure 4, Paper 1 [17])

Influence of the Reaction Medium on FAME Yield

Transesterification in a microreactor using DES as reaction medium was performed under the same conditions as in the batch experiments (Experiments 3 and 4, Paper 1 [17]). The first reason was to obtain more information about the reaction performance in a microreactor, and the second reason was to test the possibility of simultaneous glycerol removal using the DES present in the system [17].

A possible process duality could prove to be a valuable process optimization technique for biodiesel production in microreactors. In a separate study [18], ChCl:$Gly_{1:3.0}$ DES was efficiently used to remove glycerol from biodiesel. Based on these results, a DES with the same composition was used for biodiesel production. While the amount of DES was 50 % (*v*/*v*) in the previously conducted experiments, only 10 % (*v*/*v*) of DES was used in this experiment.

Although the efficiency achieved was not sufficient to meet the biodiesel purity standards in terms of glycerol removal [12], this clearly indicates that in future optimization, an integrated system for biodiesel production and simultaneous glycerol removal on a single microchip could be a solution for further process intensification.

Figure 10 shows the comparison between the yields obtained in Experiment 6 (buffer) and Experiment 8 (DES). As can be seen in Figure 10, in the experiment where a buffer served as the reaction medium (Experiment 6), a higher FAME yield was obtained. In the experiment were DES was used as the reaction medium (Experiment 8), about 15 % of the FAME yield was obtained for the residence time of 30 minutes.

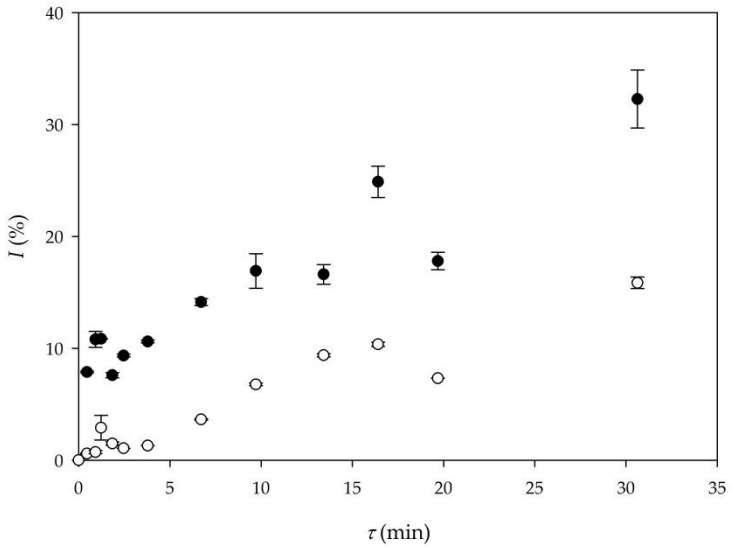

Figure 10. Comparison of FAME yield in different reaction mediums (● – buffer as a reaction medium (Experiment 6), ○ – DES as a reaction medium (Experiment 8)) (Figure 3, Paper 1 [17])

Summarized results of FAME yield and calculated volumetric productivity for all conducted experiments are given in Table 1 (Table 4, Paper 1 [17]).

Table 1. Comparison of the biodiesel production processes performed in a batch and in different types of microreactors (Table 4, Paper 1 [17])

Experiment	t (h)	I (%)	Q_p (kg/ (L·d)	Reference
1	48	91.34	0.45	
2	48	70.22	0.35	
3	48	6.44	0.03	
4	48	5.23	0.02	
	τ (min)			
5	30.62	13.26	9.87	
6	30.62	32.28	20.88	[17]
7	30.62	12.42	5.76	
8	30.62	15.85	7.35	
9	30.62	3.51	1.63	
10	30.62	20.08	9.31	
11	30.62	1.76	0.81	
12	30.62	5.45	2.53	
13	30	97.81	69.88	[26]
14	19.8	32.72	35.42	[21]
	t (h)			
15	8	40	1.78	[85]

It is noted that the highest FAME yield is obtained in the batch system (Experiment 1, 91.34 %). However, comparing the volumetric productivity, the best results are obtained in Experiment 6, where 20.88 kg/ (L·d) of biodiesel was produced. This clearly confirms that the use of microreactors for lipase-catalysed transesterification leads to an intensification of biodiesel production. In addition, waste sunflower oil was used as substrate in Experiment 6, confirming the aforementioned applicability of waste oil in enzymatic transesterification. However, when comparing these results with those of Šalić et al. [26], FAME yield and volumetric productivity were three times lower. The main difference between the results is the consequence of the high methanol excess used in the study of Šalić et al. [26].

The high methanol excess used in the lipase-catalysed transesterification was a direction for conducting further experiments and resulted in high FAME yields. Because of the relatively short residence time typical for experiments conducted in microreactors, the enzyme and methanol should be in contact for only a very short time, potentially reducing the negative effects of methanol on enzyme activity, which is typical effect when lipase-catalysed transesterification is carried out in batch reactors. Moreover, by creating an emulsion between the oil and the enzymes, which is the basic concept for the experiments with the 2-feed strategy, the enzyme is partially protected from the negative effects of the methanol, since the reaction takes place only at the interface of the two phases.

Moreover, the results obtained with DES were not as good as those obtained in a buffer medium, but this research topic remains open for further experiments using different DESs, different DES water contents and different ratios of substrate to DES.

In summary, the further optimization of the biodiesel production in the continuation of the research took place in two directions:

1. increasing the excess methanol in the reaction medium,
2. exploration of different DESs and their application for biodiesel synthesis.

3.1.3. Biodiesel synthesis in a microreactor – methanol excess

Although lower molar ratios of substrates are more favourable for carrying out enzymatic biosynthesis, the oil to methanol molar ratios used in this study were varied in the range of stoichiometric 1:3.4 to huge excess 1:90, with some experiments carried out for molar ratios of 1:10 and 1:30. As can be seen from the results shown in Figure 11 (Figure 5, Paper 6 [22]), the yield of FAME increased with increasing methanol concentration in the system, and in the process where a large excess of methanol was used, the yield was over 90 % for the residence time of only 40 minutes.

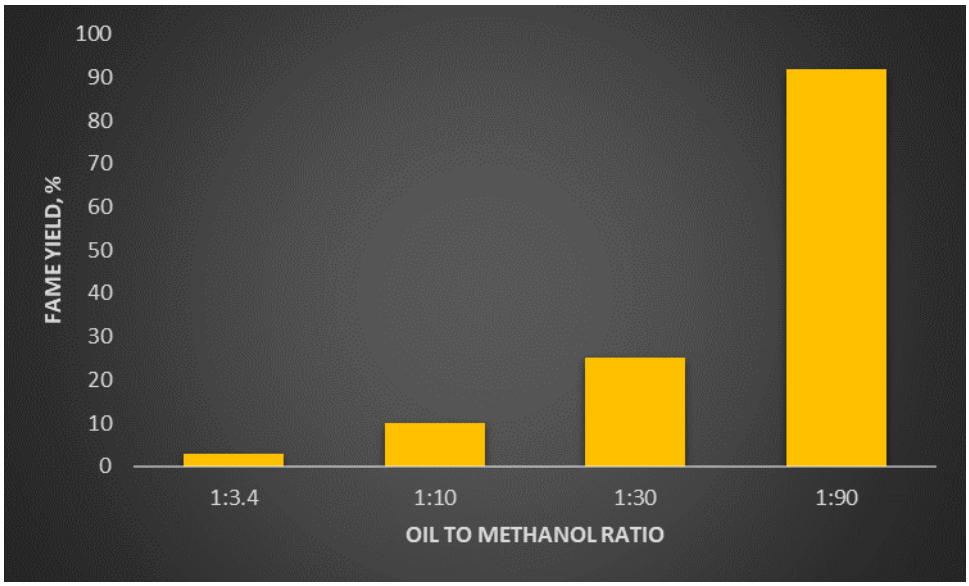

Figure 11. Influence of different oil to methanol molar ratios on FAME yield (Figure 5, Paper 6 [22])

In comparison, when the same reaction was carried out in a batch reactor [17,86], 24-48 hours were required to obtain the same yield. It is also important to note that the reaction in the batch reactor was performed with a 30-fold lower methanol concentration (molar ratio 1:3.4) to avoid inhibition by methanol. Experiments conducted in a microreactor allow for a very high excess of methanol, resulting in the FAME yields that meet the quality standards specified in the EN 14214:2012+A2:2019 standard [12]. At a molar ratio of oil to methanol of 1:90, FAME yield of 92.7 ± 4.6 % was achieved.

3.1.4. Biodiesel synthesis in a microreactor catalysed by lipase immobilized on magnetic particles

The idea of this experiment is to preserve lipase during continuous biodiesel production in a microreactor. In all experiments presented above, lipase was used in free form and consequently was washed out of a microreactor during continuous biodiesel production. Therefore, lipase was immobilized on magnetic nanoparticles and then fixed at a specific location in the microreactor using the magnetic field generated by a permanent magnet. In this way, the enzyme was fixed in a microreactor during the continuous process of transesterification. Since lipase is not present in the outlet stream, the purification step after biodiesel synthesis should be easier and simpler to perform. The schematic of the experimental setup is shown in Figure 12.

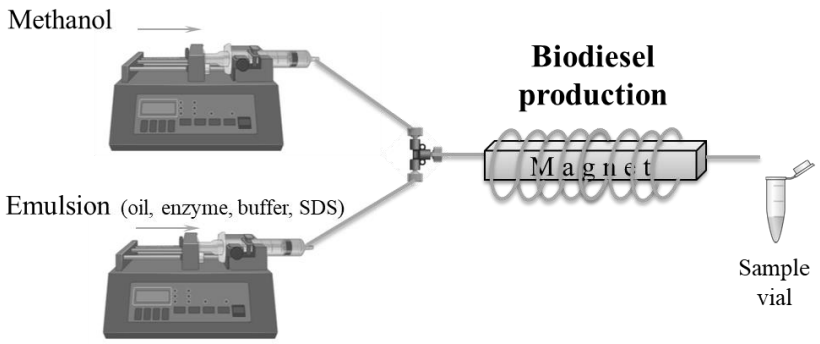

Figure 12. Two-inlets strategy for biodiesel production catalysed by enzyme immobilized on magnetic nanoparticles (Figure 2d, Paper 7 [23])

Experiment was performed with lipase immobilized on magnetic nanoparticles in a microreactor in a magnetic field at different residence times. The major drawback in this experiment was the activity of the immobilized lipase. After immobilization on magnetic nanoparticles, the lipase activity retained only 57 % of its original activity (S.A. = 412.11 ± 6.31 U/mg). This resulted in a maximum FAME yield of 35 %, with a relatively short residence time of 3.5 minutes. Unfortunately, the FAME yield decreased with longer residence times. Nevertheless, the initial results obtained in a microreactor in which transesterification was catalysed by lipase immobilized on magnetic nanoparticles were promising and a good basis for further optimization of this process.

3.2. Biodiesel purification

As already explained in detail, biodiesel production by transesterification is only the first stage to obtain biodiesel that meets the required quality standard [12]. Regardless of the type of transesterification process, various impurities such as free glycerol, alcohol, catalyst, and soap (in some reactions) must be removed from the produced biodiesel. Free glycerol presents the largest share of impurities in crude biodiesel, and its removal is in the focus of the research. After transesterification, the most common separation process for removing glycerol from crude biodiesel is decantation, followed by additional downstream purification processes that are required. These can be generally divided into wet and dry washing processes. To ensure sustainability of biodiesel purification, the focus was put on extraction with DESs and membrane filtration. In this way, waste streams from biodiesel purification should be minimized. It is also expected to reduce the operating time.

3.2.1. Biodiesel purification by DESs

To address this economic and environmental drawback of water washing [34], an alternative was found in the application of green solvents such as ionic liquids (ILs) and DESs [35,36]. After biodiesel production by lipase-catalysed transesterification of fresh sunflower oil and waste cooking oil, various DESs were used on a microscale for the extraction process. The results were compared with the purification of biodiesel by wet washing in a microextractor. The DESs used in this study were choline chloride:ethylene glycol (ChCl:Etgl) DES and choline chloride:glycerol:water (ChCl:Gly:H_2O) DES. ChCl:Etgl was prepared in a molar ratio of 1:2.5, while ChCl:Gly:H_2O was prepared in different molar ratios without (1:1; 1:2; 1:2.5 and 1:3) and with the addition of water (1:1:0.5; 1:1:1 and 1:1:2) (Table 1, Paper 2 [18]).

To establish the reference point of this study, water was used as a solvent in a microextractor. The separation was performed for different flow rates at a flow ratio of biodiesel:water = 1:5.5. The results are shown in Figure 13a-c (Figure 5, Paper 2 [18]) in terms of glycerol concentration, extraction efficiency and free glycerol content.

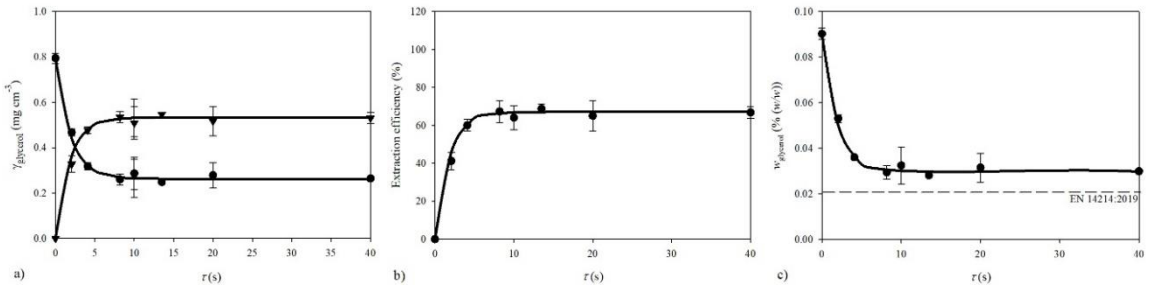

Figure 13. Influence of the residence time on the extraction of glycerol from the biodiesel using water as a solvent (wet washing): (a) glycerol concentration, (b) extraction efficiency, (c) free glycerol content and (▬ mathematical model, ● biodiesel phase, ▾ water phase) (Figure 5, Paper 2 [18])

The separation efficiency was 75.19 % for a residence time of only 8.3 s. This resulted in insufficient glycerol separation, and at the end of the process the amount of glycerol remaining was higher than the recommended values according to the European standard EN 14214 [12]. The reason why the separation efficiency was not higher than 75 % is probably because the capacity of the water extraction was too low to perform a complete extraction in one step.

After using water as a solvent for biodiesel purification in a microextractor experiment, new experiments were performed using unpurified biodiesel and ChCl:Etgl-based DESs as solvents. As can be observed, DES provided much more efficient glycerol extraction than water and almost all of the glycerol was extracted from the biodiesel. An initial extraction efficiency of 98.35% was obtained when DES was used as solvent. On the other hand, the maximum efficiency was obtained at a much higher residence time compared to the experiment conducted with water as solvent (τ = 174 s for DES and τ = 8.2 s for water as solvent). Therefore, research was continued to see if the extraction time could be reduced using a different type of DES (ChCl:Gly:H_2O). The purification of the biodiesel was carried out using ChCl:Gly1:2.5. As can be seen from Figure 14 (Figure 9, Paper 2 [18]), the glycerol was almost completely removed from the biodiesel with a residence time of only 13.61 s. This was a significant improvement compared to the ChCl:Etgl based DES, where the same efficiency was achieved after 180 s. According to Hayyan et al. [11], the glycerol content in a DES has a greater tendency to attract more glycerol into the DES and form a higher glycerol ratio DES, which explains the much shorter extraction times compared to the ChCl:Etgl based DESs.

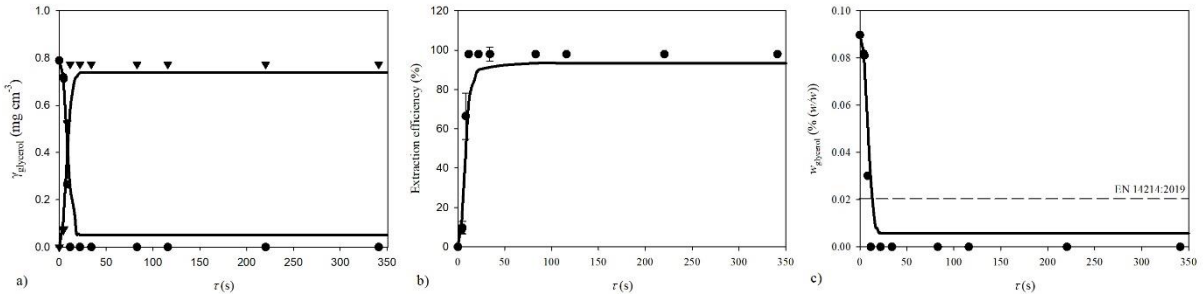

Figure 14. Influence of the residence time on the extraction of glycerol from the biodiesel synthetized from WCO using ChCl:Gly1:2.5 based DES (a) glycerol concentration, (b) extraction efficiency, (c) free glycerol content (▬ mathematical model, ● biodiesel phase, ▾ DES phase) (Figure 9, Paper 2 [18])

It is important to note the results obtained for biodiesel synthesised from WCO when purified with a ChCl:Etgl based DES. Even though the residual concentration of glycerol after the decantation was higher than that obtained after synthesising biodiesel from edible sunflower oil, the efficiency was the same, resulting in purified biodiesel that met quality standards (Figures 7 and 8, Paper 2 [18]).

The removal of glycerol during biodiesel purification by extraction with any of investigated DESs has several advantages compared to the biodiesel wet washing process with water. In particular, higher extraction efficiency and a significantly lower amount of solvents used are the main advantages of the DES application for biodiesel purification. In addition, there is less waste to be treated at the end of the process. Moreover, since it is possible to separate the DES and the biodiesel phase at the end of the microchannel, the recirculation of the DES is possible, which, in combination with its regeneration, justifies the application of DESs for biodiesel purification [18].

3.2.2. Biodiesel purification by membrane filtration

Biodiesel purification was performed in a semi-batch module shown in Figure 15 (Figure 1, Paper 3 [19]) using membranes (45 mm diameter). A total of four membranes were tested and compared: polypropylene (PP), polyethersulfone (PES), polyacrilonitrile (PAN), and regenerated cellulose (RC). The initial volume of biodiesel in a membrane module was 35 mL in each experiment. The process was continued until the remaining volume of biodiesel in the membrane module was 5 mL. Semi-continuous and discontinuous processes were performed to test the reusability of the membranes. In the discontinuous process, the membrane was removed from the module at the end of the first filtration cycle, washed with ethanol, and reused. In the semi-continuous process, the amount of 5 mL of biodiesel remaining in a module after the first cycle was removed and 35 mL of new crude biodiesel was fed into the system without washing the membrane. Cycles were repeated until permeate flux decreased significantly.

The membranes were evaluated based on permeate flux and glycerol content in the permeate. The results obtained show that the PAN membrane is the most efficient for glycerol removal. It was efficiently reused in 6 cycles and in each cycle of ultrafiltration the efficiency was 91.48 % with an average free glycerol content in the permeate of 0.006 % (*w*/*w*).

3.3. Mathematical modelling

After the initial researches dealing with biodiesel production and purification, focus was shifted towards mathematical modelling. Motivation behind it was further process development in terms of saving numerous hours of additional experiments in laboratory. With the mathematical modelling of biodiesel production and biodiesel purification, optimization of process conditions can be made.

Therefore, various mathematical models of biodiesel production by lipase-catalysed transesterification have been developed. For the biodiesel purification step, a model of extraction by different solvents (water and DESs) and a model of fouling mechanisms for membrane filtration were developed.

3.3.1. Modelling of biodiesel production by lipase-catalysed transesterification

Development of lipase-catalysed biodiesel production process was based on the results of our initial study [21]. To describe the process more realistically than in initial study, kinetic parameters of the reverse reaction – biodiesel hydrolysis, were also estimated in this study. Three kinetic models were selected to describe lipase catalysed biodiesel production by transesterification where oil and methanol were used as substrates: Michaelis-Menten, Hill and Bi-Bi Ping-Pong kinetic models. Flow chart of methodology used for mathematical modelling of lipase catalysed biodiesel production by transesterification is shown in Figure 15 (Figure 1, Paper 6 [22]).

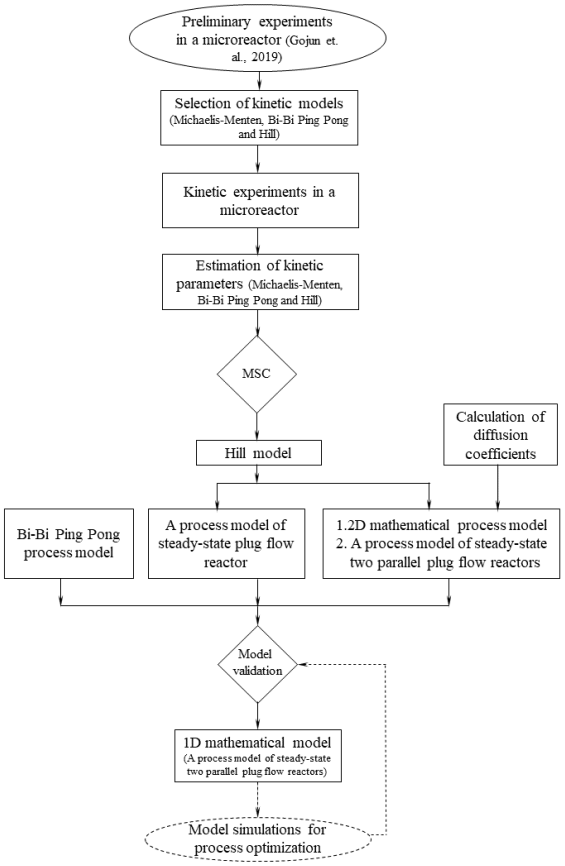

Figure 15. Flow chart of methodology used for mathematical modelling of lipase catalysed biodiesel production by transesterification (Figure 1, Paper 6 [22])

In the initial research [21], kinetic parameters were estimated based on the assumption that the enzyme follows Michaelis-Menten kinetics. Based on kinetics and type of microreactor used, a 2D mathematical transport model was developed. Biggest simplification of the process model used in initial research was the assumption that there is no reverse reaction (hydrolysis).

As already mentioned, lipase can simultaneously catalyse hydrolysis, esterification and transesterification. Due to this property, it was necessary to estimate the kinetic parameters of both reactions (transesterification and hydrolysis) that occur in a biodiesel synthesis process. The influence of reactants and products concentrations on the reaction rate were measured to estimate the kinetic parameters of the proposed kinetic models. As can be seen on Figure 16 (Figure 4, Paper 6) [22]), the dominant reaction in biodiesel production process is transesterification with an approximately 12 times higher maximal reaction rate in comparison to reverse reaction – biodiesel hydrolysis.

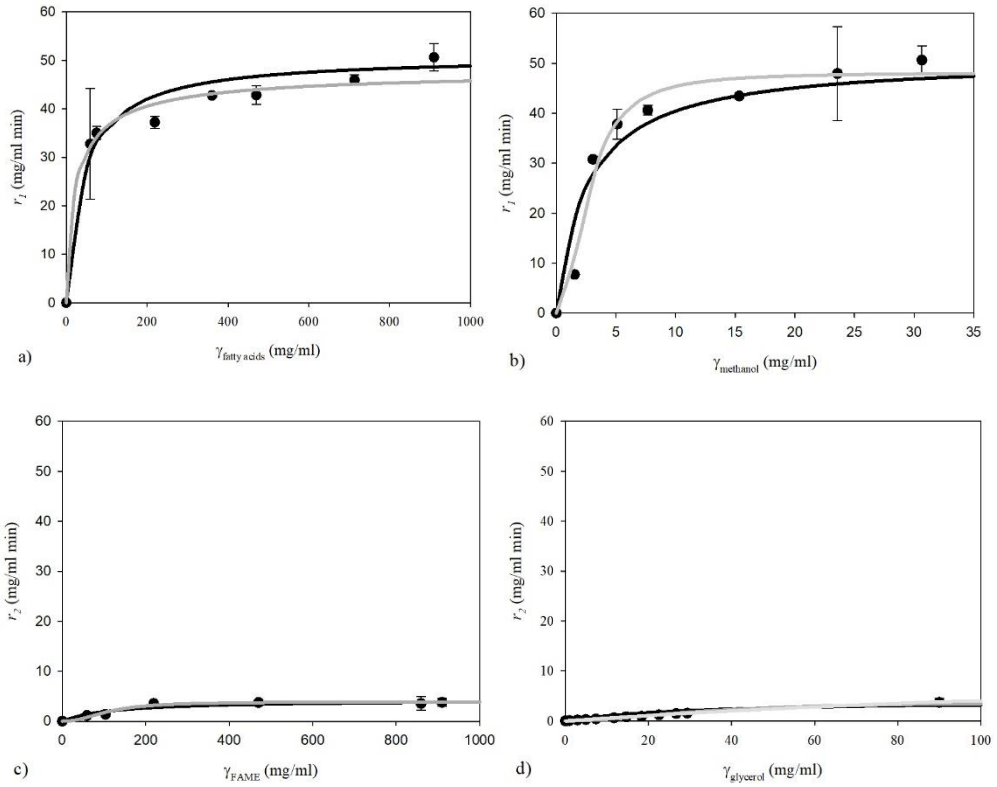

Figure 16. Kinetics of biodiesel synthesis and FAME hydrolysis: dependence of the reaction rate on initial concentration of (a) fatty acids (b) methanol, (c) FAME, (d) glycerol (▬ Michaelis-Menten model, = = Bi-Bi Ping-Pong model, ▬ Hill model, • experimental data) (Figure 4, Paper 6 [22])

To describe the biodiesel production process as detailed as possible, three kinetic models were selected for modelling of both forward and reverse reaction. Bi-Bi Ping-Pong and Michaelis-Menten kinetic models were chosen because they are the mostly used models for describing the kinetics of multiple substrate enzymatic reactions. Hill kinetic model was selected because it showed some promising results in research conducted by Šibalić et al. [79].

As can be seen from Figure 16., all kinetic models (Michaelis-Menten and Bi-Bi Ping-Pong kinetic models are overlapping) described the results of kinetic measurements well. The model selection criterion (MSC) was used to estimate which kinetic model described the obtained experimental data the best. The most appropriate model will be the one with the largest MSC, which makes the Hill kinetic model as the best one (Table 3, Paper 6 [22]).

Four different mathematical process models were proposed: Bi-Bi Ping-Pong, one 2D and two 1D process models (a process model of steady-state two parallel plug flow reactors and a

process model of ideal plug flow reactor). Model validation was performed using data from independent experiments, in which oil to methanol ratio was altered. Briefly, the oil to methanol ratio was increased starting from 1:3.4 and ending with 1:90. Positive influence of increasing methanol excess in the reaction medium during transesterification was described elsewhere, contact between alcohol and triglycerides was enhanced [91] and purer product - biodiesel was obtained [92].

As can be seen from the results shown in Figure 17 (Figure 5, Paper 6 [22]), FAME yield increased with increasing the methanol concentration in the reaction medium. The yield was over 90 % for the residence time of only 40 min for the experiment with highest oil to methanol ratio (1:90).

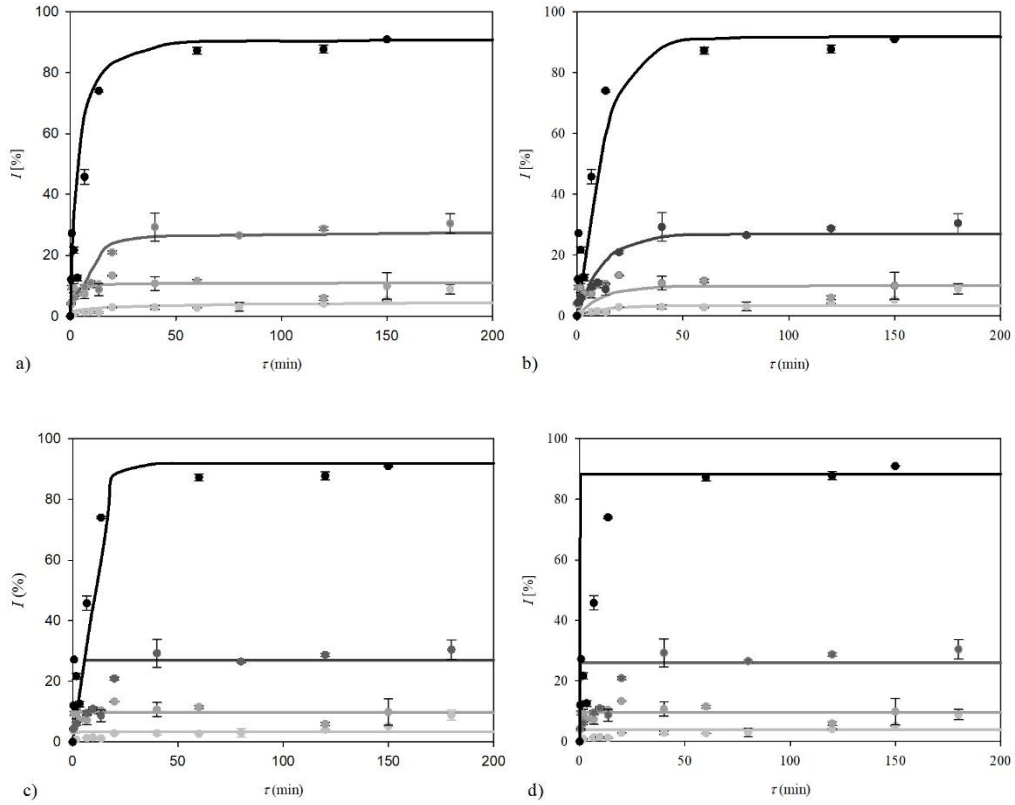

Figure 17. Process model validation of biodiesel synthesis in a microreactor a) 2D mathematical process model, b) a process model of steady-state two parallel plug flow reactors, c) a process model of ideal plug flow reactor and d) Bi-Bi Ping-Pong mathematical process model (▬ mathematical model, ● 1:3.4 oil to methanol molar ratio, ● 1:10 oil to methanol molar ratio, ● 1:30 oil to methanol molar ratio, ● 1:90 oil to methanol molar ratio) (Figure 5, Paper 6 [22])

The comparison of model efficiency was evaluated based on R^2 as presented in Table 2 (Table 5, Paper 6 [22]).

Table 2. R^2 of analysed mathematical models (Table 5, Paper 6 [22])

Mathematical model	Oil to methanol ratio			
	1:3.4	**1:10**	**1:30**	**1:90**
2D mathematical model	0.535	0.603	0.890	0.933
A model of steady state two parallel plug flow reactors	0.330	0.653	0.891	0.942
A model of ideal plug flow reactor	0.108	0.454	0.508	0.910
Bi-Bi Ping-Pong model	0.148	0.253	0.216	0.264

By increasing the oil to methanol ratio, the value of R^2 for all four analysed models was increased. The highest numerical value of R^2 for all four analysed oil to methanol ratios was obtained for the steady-state two parallel plug flow reactor process model, followed by the 2D process model and the ideal plug flow reactor process model. The lowest R^2 values were obtained for the Bi-Bi Ping-Pong model. Based on the obtained results, it could be concluded that the Bi-Bi Ping-Pong mathematical process model is not accurate for describing biodiesel synthesis in a microreactor, but perhaps could be used for rough predictions. Other models proposed in this study are better suited for more refined and accurate predictions.

3.3.2. Modelling of biodiesel purification by extraction

All investigated DESs have the overall advantage when compared to the biodiesel wet washing process using water [18]. The high extraction efficiency was supported by mathematical model of biodiesel purification by extraction (Figure 14). By using a ChCl:$Gly_{1:2.5}$ based DES, glycerol was almost completely removed from the biodiesel for the residence time of only 13.61 s (glycerol content < 0.01 % (*w*/*w*)). In Figure 14 (Figure 9, Paper 2 [18]) extraction efficiency and glycerol concentration and content are shown for the ChCl:$Gly_{1:2.5}$ based DES.

The glycerol separation in a microextractor was described with 2D model including convection in the flow direction (x) and diffusion in two directions (x and y). The mathematical model for steady-state conditions in a microextractor was composed of dimensionless partial differential equations for glycerol concentrations in biodiesel and DES (water) phase and corresponding boundary and initial conditions.

2D model provides good description regarding glycerol concentration, extraction efficiency and free glycerol content, for both biodiesel/water system and biodiesel/DES system.

3.3.3. Modelling of blocking mechanisms in the process of dead-end membrane filtration

When working with membranes, fouling, the deposition of substances on the membrane surface, is one of the biggest problems. It causes deterioration of all membranes and is a major economic burden on the processes in which membranes are used. In addition to pore blocking, there are two other processes that can lead to a reduction in permeate flux: concentration polarisation and the formation of a precipitation layer [93]. To gain better insight into the mechanisms that lead to a decrease in flux in dead-end filtration at constant pressure, the Hermia model of dead-end filtration at constant pressure was used. All four basic fouling models were considered: the complete blocking model, the intermediate blocking model, the standard blocking model, and the cake layer model. The estimated model constants (k_c, k_s, k_i, and k_g) and flux at t = 0 min were determined along with the correlation coefficients (R^2) (Table 2, Paper 3 [19]). The predominant fouling mechanism for each membrane was analysed by fitting all four models to the experimental data and based on the obtained R^2, R^2_{adj}, and F-values for each model. From the analysis of the results, it appears that the predominant fouling mechanism changes during the filtration cycles. Also, different fouling mechanisms may occur during the same ultrafiltration run, which may be the reason for the discrepancy between experimental and predicted data for certain operating conditions.

In Figure 18 the experimental and model simulation results of biodiesel ultrafiltration performed by polyacrylonitrile (PAN) membrane through 6 cycles of reuse are shown (Figure 4, Paper 3 [19]). Intermediate blocking was prevalent after the first filtration cycle and a complete cake layer was present in the following five filtration cycles. In addition, a significant decrease in flux is observed in the first few minutes of each cycle.

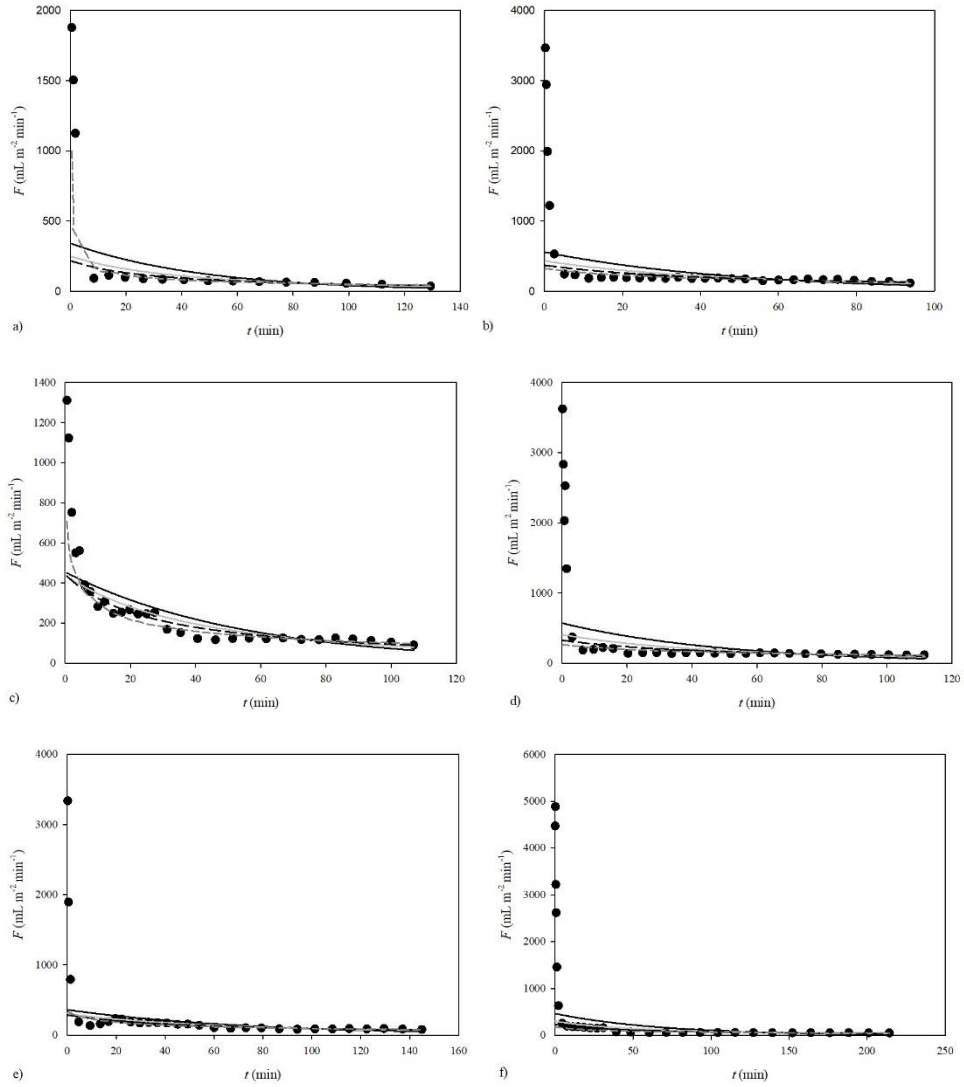

Figure 18. Permeate flux for biodiesel ultrafiltration using polyacrylonitrile membrane in six (a-f) semi-continuous process (● permeate flux, Hermina's model: ▬ complete blocking, ▬ standard blocking, – – – intermediate blocking, complete cake) (Figure 4, Paper 3 [19])

3.4. Development of integrated microsystems for biodiesel production

As mentioned above, the increase in methanol concentration led to an increase in FAME yield in the reaction medium. It was observed that in the process where a large excess of methanol was used, the FAME yield exceeded 90% when the residence time was between 20 and 40 min [22,26]. This was only possible in a microreactor configuration with a 2-inlet strategy, where a mixture of oil and methanol (emulsion prepared by adding emulsifier SDS) was used as one inlet stream and methanol as the second inlet stream.

Purification of the produced biodiesel is mainly related to the removal of glycerol in microextractors [18] using either water [20] or DESs as solvents. Glycerol- and ethylene glycol-based DESs were mainly used in experiments where DES was used as solvent [18,39]. As an alternative to avoid the addition of different solvents into the product stream, purification of biodiesel by membrane ultrafiltration could be used [19].

3.4.1. Simultaneous (one-pot) biodiesel production and purification

Further research was conducted with the idea of developing an integrated system that would combine biodiesel production and biodiesel purification in a single pot. The main feature of this system was the dual role of DESs, allowing the simultaneous use of DES as reaction and extraction media [20].

In short, DESs (ChCl:Gl and ChCl:EtGl) were used as reaction and extraction media in this integrated process. To optimize the integrated one-pot biodiesel production and purification, the experiments were conducted according to the experimental design (Table 2, Paper 4 [20]). The integrated process of biodiesel production and purification was carried out in both, batch and microreactor, respectively. The parameters that were changed were the mass ratio of a reaction phase (oil, methanol, enzyme and water) and the DES phase, the water content and the molar ratios of the DES components. The effects of the three separate variables (water content (X_1), DES composition (X_2), and mass ratio of phases (X_3)) on biodiesel yield (Y) were evaluated by applying the Box-Behnken design.

Experiments conducted with ChCl:Gly resulted in higher biodiesel yield and glycerol extraction efficiency. A phase mass ratio of 1:1, a water mass fraction of 6.6 %, and a ChCl:Gly molar ratio of 1:3.5 were determined to be optimal process conditions. When the reaction was carried out in a batch reactor under the optimal conditions, the process resulted in a yield of 43.54 ± 0.2 % and a glycerol extraction efficiency of 99.54 ± 0.19 % (t = 2 h). Unfortunately, the free

glycerol content was higher than specified in the international standards (w_{Gly} > 0.02 %); therefore, the process was carried out in a microsystem to improve mass transfer. Since the yield remained approximately the same (45.33 ± 1.74 %) and the free glycerol content was lower than the standards (w_{Gly} = 0.0019 ± 0.003 %), the microsystem proved to be a good direction for future process optimization.

The major drawback of one-pot biodiesel production and purification performed in a microreactor was the low FAME yield of 43.5%, which does not meet quality standards [12].

3.4.2. Integrated system with glycerol removal by DES based extraction

Integrated system with DES as extraction medium is comprised of two microsystems units: microreactor responsible for biodiesel production and microextractor responsible for biodiesel purification. Basic scheme is given in Figure 19 (Figure 2, Paper 7 [23]).

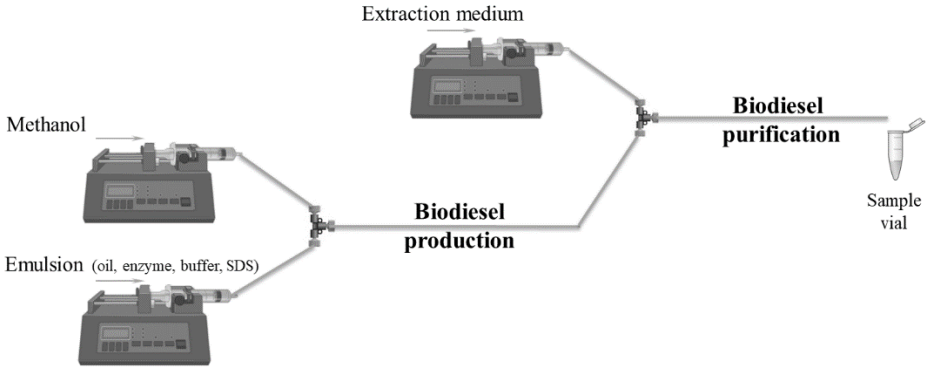

Figure 19. Scheme of the integrated microsystem composed of biodiesel production in a microreactor and microextractor for biodiesel purification by DES

In the study conducted by Šalić et al. [18], ChCl-Gly DES has been confirmed as best extraction medium for biodiesel purification. However, in that study, crude biodiesel produced previously in batch reactor was used in the purification. In another study performed by Šalić et al. [26], first proposal of an integrated microsystem was presented, consisting of two microchips connected in series, for biodiesel production directly aligned by biodiesel purification. In first microchip biodiesel production by lipase catalysed transesterification occurred, with the usage of 2-inlet strategy. Emulsion (a mixture of oil and lipase combined with emulsifier SDS) was one inlet, and high methanol excess was second inlet. Reason for using high methanol excess to get high FAME yield was described previously [22]. A second microchip was responsible for biodiesel purification by means of extraction with ChCl-Gly DES.

Even though it was promising, this system had some drawbacks. A major drawback was the inability to reuse the lipase. That problem can be solved either by enzyme recirculation or enzyme immobilization. In this work, integrated system with immobilized lipase was proposed. Moreover, improvement of the first integrated system shown in Šalić et al. [26] will be presented.

3.4.3. Integrated systems with glycerol removal by membrane ultrafiltration

While extraction was successful as a purification method, a different approach to glycerol removal based on membrane ultrafiltration was investigated. The integrated system was based on the studies of Sokač et al. [19], where the PAN membrane was successfully used for glycerol removal, resulting in glycerol concentrations in the purified biodiesel that met the quality standard. As described earlier, after biodiesel production in a microreactor, the reaction mixture was fed into the separation unit shown in Figure 20 for glycerol removal (Figure 3b, Paper 7 [23]). To ensure that the biodiesel was successfully passed through the membrane as an upper stream, the flow ratio of upper stream to lower stream was maintained at 4:1 to achieve a uniform flow of purified biodiesel.

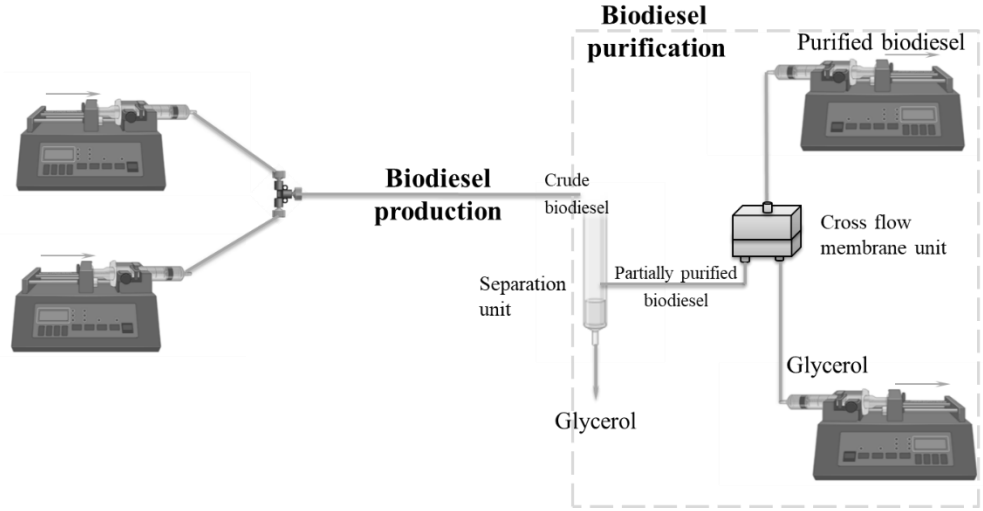

Figure 20. Scheme of the integrated microsystem composed of biodiesel production in a microreactor and microextractor and unit for biodiesel purification by membrane ultrafiltration (Figure 3b, Paper 7 [23])

3.4.4. Integrated systems – results and concluding remarks

According to the standard [12], in order to present a successful result from the integrated system, two guidelines must be met: the content of FAME in the biodiesel must be higher than 96.5 %, while the glycerol content must be lower than 0.02 % (w/w). In order to fully cover the possibilities of adjusting the integrated system, a total of eight experiments were conducted. All of these experiments have been covered in previous research [17-22,26], but it was important to investigate them in a fully integrated microsystem. In Experiments 1-7, free lipase was used as catalyst while Experiment 8 was the only one in which immobilised lipase was used. The process conditions are given in Table 3 (Table 1, Paper 7 [23]).

Table 3. Process conditions used for biodiesel synthesis for experiments performed in integrated systems (Table 1, Paper 7 [23]).

Experiment	Strategy	PTFE tube diameter	Oil to methanol ratio	Oil source
1	3-inlets	500 μm	1:3.4	Edible oil
2	3-inlets	500 μm	1:90	Edible oil
3	3-inlets	500 μm	1:90	Edible oil
4	2 x 2-inlets	500 μm	1:90	Edible oil
5	2-inlets	1000 μm	1:90	Edible oil
6	2-inlets	1000 μm	1:90	Waste oil
7	2-inlets	1000 μm	1:90	Edible oil

Experiment 8, the only one with immobilised lipase, had the same process conditions as Experiment 5. Two main strategies were used to feed in substrates into the microsystem: the 3-inlet strategy and the 2-inlet strategy. In the 3-inlet strategy, one inlet is divided into two inlets, so oil was feed through first inlet, lipase was feed through second inlet, while methanol was feed as third separate inlet. When considering the diameter width of the microchannel, 2 dimensions were used: 1000 and 500 μm. Two molar ratios of oil to methanol and two oil sources were used (edible sunflower oil and waste cooking oil). Figure 21 shows the FAME yield (Figure 7, Paper 7 [23]) after biodiesel production while Figure 22 (Figure 8, Paper 7 [23]) shows the glycerol content after biodiesel purification for all eight experiments.

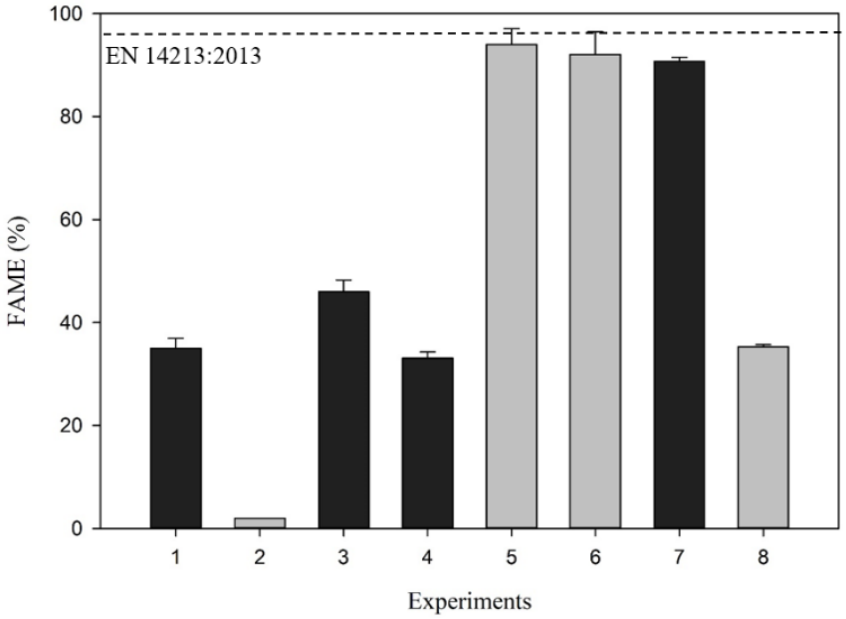

Figure 21. FAME yield accomplished for different integrated systems (biodiesel purification by: ■ - ultrafiltration, ■ - DES based extraction) (Figure 7, Paper 7 [23])

The highest FAME yield was obtained in the experiments in which the feeding strategy with 2 inlets was combined with DES based purification (Experiments 5 and 6). Considering the confidence intervals, the FAME yield obtained in these experiments was within the range defined by the biodiesel quality standards. Similar results were obtained in the experiments where the 2 inlet strategy was combined with ultrafiltration (Experiment 7). Obviously, the feeding strategy with 3 inlets is not a suitable method to obtain a high FAME yield, despite the method used for biodiesel processing (Experiments 1-3). The same is for the feeding strategy with 2 x 2 inlets. Consequently, the low FAME yield obtained during production cannot be increased by the integrated purification. Although promising, the integrated biodiesel production based on lipase immobilized on magnetic nanoparticles needs further optimization (Experiment 8).

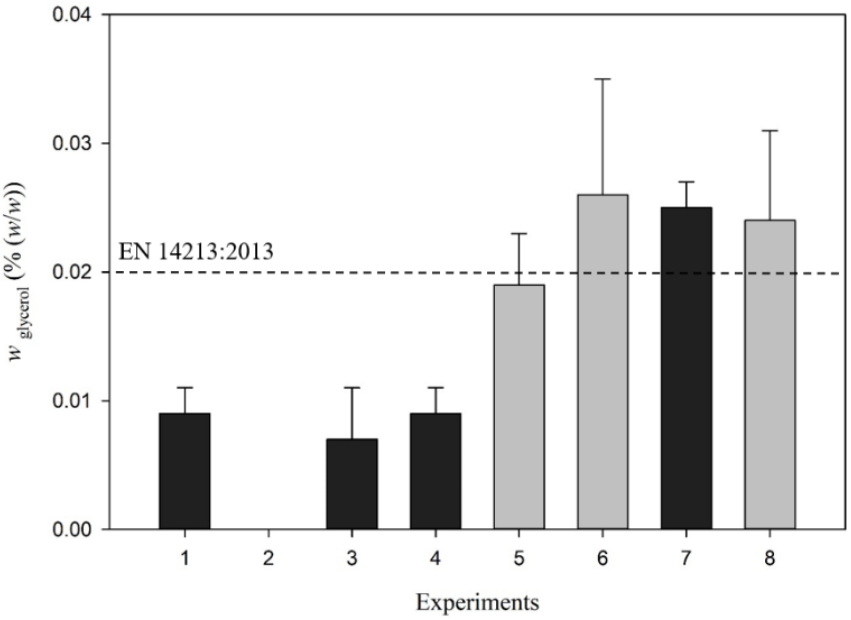

Figure 22. Glycerol content in biodiesel for different integrated systems (biodiesel purification by: ■ - ultrafiltration, ■ - DES based extraction) (Figure 8, Paper 7 [23])

The glycerol content (*w*/*w*) in the different integrated experiments is also analysed (Figure 23 – Figure 8, Paper 7 [23]). Obviously, the highest biodiesel purities were obtained in the experiments with low FAME yields. On the other hand, both purification methods were quite successful, even in the integrated experiments where the FAME yield was high.

Experiment 5 was identified as the best integrated microsystem. The highest FAME yield of 94 ± 3.1 % and glycerol content below 0.02 % (*w*/*w*) were obtained in the integrated system in which the 2-inlet strategy was combined with the DES based extraction.

4. CONCLUSIONS

- Enzymatic transesterification in microreactors is a viable option for biodiesel production when sunflower waste oil serves as the primary substrate. The highest yield of 96.5 % at a residence time of τ = 20 min was obtained in the microreactor experiment using an emulsion of waste oil and commercial enzyme suspended in a water buffer as one inlet stream for a 2-stream inlet configuration.
- The results obtained using DES as the reaction medium in enzymatic transesterification were not as good as those obtained in a buffer medium, but this area of research will be explored further. While DESs have already been confirmed as excellent extraction media, their role in synthesis has yet to be confirmed.
- Although promising, biodiesel production using lipase immobilized on magnetic nanoparticles needs further optimization. The most important goal is to preserve the original lipase activity of free lipase after the process of immobilization.
- Different kinetic models were used for the description of biodiesel synthesis in a microreactor catalysed by lipase. The Bi-Bi Ping-Pong mechanism, the most commonly used, was compared to Michaelis-Menten and Hill kinetic mechanisms. Based on the model selection criterion, the Hill model was proposed as the best kinetic model for biodiesel production catalysed by lipase.
- Amongst the four proposed mathematical process models in this research, titled the 2D mathematical process model, the process model of steady-state two parallel plug flow reactors, and the process model of steady-state plug flow reactor and Bi-Bi Ping-Pong mathematical model, the 2D mathematical process model and the process model of steady-state two parallel plug flow reactors showed good agreement between experimental results and model simulation.
- Glycerol removal in biodiesel purification by extraction was found to be very efficient using a ChCl:Etgl based DES and a ChCl:Gly:H_2O based DES as solvents. All DESs studied have the overall advantage compared to the biodiesel wet washing process with water. The glycerol separation in a microextractor was described with 2D model in biodiesel and DES/water phase.
- Four different ultrafiltration membranes: polypropylene (PP), polyethersulfone (PES), polyacrilonitrile (PAN), and regenerated cellulose (RC) were tested for the removal of glycerol from biodiesel produced by lipase-catalysed transesterification. PAN membrane is most efficient for glycerol removal. It was efficiently reused for 6 times

and in every cycle of ultrafiltration efficiency was around 91.48% with average free glycerol content in permeate of 0.006 % (*w*/*w*).

- Hermia's model was used to analyse the blocking mechanism. Working with the PAN membrane in discontinuous mode, intermediate blocking was the predominant mechanism after the first filtration cycle, while complete cake layer was the predominant mechanism for the following five filtration cycles.
- The first attempt at an integrated biodiesel production and purification process using DESs was a one-pot system. In this system, DES served as the reaction and extraction medium. The highest FAME yield and extraction efficiency was obtained in experiments with the ChCl:Gly:H_2O DES. Optimal conditions: Mass ratio of the phases 1:1, the mass fraction of water 6.6 % and a molar ratio of ChCl:Gly 1:3.5. Although the glycerol content was within the limits, FAME yield of 43.5 % was obtained, which was not sufficient according to the standard.
- Several integrated microsystems have been developed consisting of biodiesel production and purification. The best integrated microsystem was the setup in which the 2-inlet feeding strategy for biodiesel production was combined with DES based extraction and connected in series. In this integrated microsystem, a FAME yield 94 ± 3.1 % and a glycerol content in the purified biodiesel of less than 0.02 % (*w*/*w*) were achieved with a residence time of 20 minutes.
- The integrated microsystem, which consists of production and purification steps connected in series, proves to be a viable solution for intensifying the biodiesel production process.

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APPENDIX

Paper 1

M. Gojun, M. Bačić, A. Ljubić, A. Šalić, B. Zelić, Transesterification in microreactors - overstepping obstacles and shifting towards biodiesel production on a microscale, Micromachines 11 (2020) e457,

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Paper 2

A. Šalić, A. Jurinjak Tušek, M. Gojun, B. Zelić, Biodiesel purification in microextractors: choline chloride based deep eutectic solvents vs water, Sep. Purif. Technol. 242 (2020) e116783,

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Paper 3

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Paper 4

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Paper 5

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Paper 6

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Paper 7

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Paper 1

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Transesterification in Microreactors—Overstepping Obstacles and Shifting Towards Biodiesel Production on a Microscale

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Abstract: Biodiesel, which was earlier used only as an alternative fuel, is now an indispensable component of commercial diesel. Conventional production processes are unable to cope with the increasing demand for biodiesel, and therefore more and more work is being done to intensify the existing processes. The intensification of the biodiesel production process, taking into account the environmental and economic factors, is based on increasing productivity. One way to achieve that is by reducing the volume of production units. The application of the enzymatic reaction path, while reducing the volume of process equipment to the micro-level, has significantly magnified the productivity of the biodiesel production process, which is primarily due to better mass transfer in microsystems. Additional breakthrough is the use of deep eutectic solvents (DES) instead of buffers for enzyme stabilization. In this study, a lipase from *Thermomyces lanuginosus* (TIL) (both commercial and produced by solid-state fermentation) was used as a catalyst for biodiesel production. Edible and waste sunflower oil, as well as methanol, were used as substrates. The reaction mediums were buffer and DES. The transesterification reaction was carried out in a batch reactor and the emphasis was made on different microreactor configurations. The highest yield of 32% for residence time of only τ = 30 min was obtained in the microreactor system with an emulsion of waste oil and a commercial enzyme suspended in a buffer. This indicates that enzymatic transesterification could be a valuable reaction path for dealing with waste oils. Furthermore, biodiesel synthesis in DES showed somewhat lower yields, but by increasing the water content in the system, the reaction could prove much better results. In the end, the effects of reaction conditions on the volumetric productivity of the process were analyzed.

Keywords: lipase catalyzed transesterification; biodiesel; microreactors; deep eutectic solvents

1. Introduction

Biodiesel, a mixture of fatty acid methyl esters (FAME), is a biodegradable and non-toxic alternative fuel to petrol diesel. When compared to fossil biodiesel, it excels in its bio-degradability, minimal toxicity and a near zero-emission of aromatic compounds, sulfates and other chemical components which have a negative effect on the environment [1–3].

The usage of alternative fuels emerged more than a hundred years ago, when Rudolf Diesel used vegetable oil as fuel in his engine [4]. During the first part of the 20th century, vegetable oils were used instead of diesel fuels only occasionally. Until the mid-1970 s, alternative and renewable fuel sources had hardly any economic and ecological impact. Due to climate change, mostly comprising of air pollution and global warming caused by CO_2 and declining petroleum reserves (which imminently leads to an increase in crude oil prices), the development of alternative fuel sources got full attention,

was backed up by the government, the general public, researchers and industries [5]. Biodiesel has been identified as one of the most prominent options for partially reducing the use of conventional fossil fuels, which resulted in the fact that 60% of new cars and trucks operating in Europe run on biodiesel [6]. State of the art internal combustion engines can use pure B100 biodiesel (100% biodiesel), but the more common use in Europe is a mixture of fossil diesel and biodiesel with the most common being B7 (7% biodiesel) [7].

Industrial production of biodiesel can be carried out by four methods: blending, micro-emulsification, pyrolysis and transesterification [8]. Biodiesel, regardless of the production method, must meet the quality standards according to EN 14,214 [9]. Each of the biodiesel production methods has its advantages and disadvantages and transesterification has proven to be the most efficient and the most economical method. The transesterification process path can be carried out as homogeneous or heterogeneous, according to the nature of the catalyst. Furthermore, based on the nature of the catalyst, transesterification can be chemical or enzymatic [10]. Different oils and fats of vegetable and animal origin can be used in the transesterification process and the thing that makes this process particularly acceptable in terms of environmental impact is the possibility of using waste oils and fats [11].

The use of enzymes in industrial processes is increasingly prevailing. One of the most represented enzymes in the industry is lipase (EC 13.1.1.3.). Lipase belongs to the group of hydrolases and can simultaneously catalyze a number of reactions such as transesterification, hydrolysis and esterification [6,12]. Although lipase enzymes from yeast of the genus Candida are the most represented in research and are used in various fields, ranging from the production of low-energy chemicals to biodiesel [11], some authors cite lipases from *Thermomyces lanuginosus* (TlL) as very effective catalysts [10]. TlL lipases are basophilic and thermostable enzymes and are commercially available as suspensions or in an immobilized form. One of the processes in which TlL lipases have found application is the production of biodiesel, where the high stability of TlL has a significant impact on process efficiency [9]. Since it catalyzes the transesterification reaction, the use of lipase has recently been increasing for biodiesel production. Several reasons can be pointed out: the use of edible and waste oils as raw materials, whereby waste oils can be used without pre-treatment; the transesterification reaction is carried out under mild process conditions. Furthermore, the lipase can be reused in the process because it is easy to separate other components of the reaction mixture, which makes the whole biodiesel production process more efficient, both economically and environmentally. Glycerol produced as a by-product is of high purity and can be used as a feedstock in other processes without further processing [6,13].

Given the increasing demand for biodiesel, there is a need to improve existing conventional production processes in order to catch up with the needs of the market. Therefore, biodiesel batch production processes are recently being replaced by continuous processes using heterogeneous catalysts to intensify the process and carry it out with a small number of process steps, which leads to a small amount of waste process streams. Microreactors have been recognized as one of the most significant technologies that usually result in process intensification and among other processes in which they are used, they have also shown their applicability in biodiesel production [14].

Microreactors are reactor systems on a microscopic scale that have been produced, partially or completely, using microtechnology and microengineering [15]. A microreactor consists of a microchannel network of the usual diameter of 10–500 μm. The small dimensions of the microchannels enable efficient mass and energy transfer, which, with a short retention time, contributes to the intensification of the conducted processes. The small size of a microreactor leads to a small amount of waste process streams and lower energy consumption, since it involves the use of small quantities of reactants and catalysts. In addition, microreactors have some other advantages, of which the most important ones are compactness and ease of implementation, the laminar flow of process streams, efficient mixing and the short diffusion path of the molecules [16]. The aforementioned characteristics of microreactors also give actual reasons for their use, which include the high utilization and productivity achieved during the implementation of the process, including safe working conditions [11,17].

Additional distinction, as well as an advantage, of microreactors is the scale-up of the conducted processes. In comparison to the classical scale-up approach, the process steps in microreactors are magnified by connecting the process units in parallel or in series [18]. Thus, the long and costly approach to scale-up process characteristic for meso- and macro-reactors can simply be overcome by connecting individual microreactors. In that way, the overall capacity of the process is increased and the characteristics of each microreactor that make up such a system remain the same. Despite all of the aforementioned advantages of microreactor systems, their use for carrying out enzymatically catalyzed reactions is not as widely represented in the literature [19].

Despite all of the aforementioned advantages, the commercialization of the enzymatic process for biodiesel production faced a major obstacle—the high cost of the enzyme and methanol inhibition [20]. One possible way to tackle this obstacle is the usage of deep eutectic solvents (DES) as reaction mediums. They have been suggested because of their enhancement of mass transfer, as well as the reduction of glycerol accumulation and methanol inhibition. Furthermore, they are prepared in mild reaction conditions and from cheap substrates [21].

In this study, a TlL (both commercial and produced by solid-state fermentation) was used as a catalyst for biodiesel production. Edible sunflower oil and waste oil were raw materials used for biodiesel production coupled with methanol, while reaction mediums were buffer and DES. Different experiments were set up, including both batch processes and microreactor experiments, respectively. In all the experiments, FAME yield was monitored as the most important indication for experimental set up, which could be further explored. In the end, the comparison of biodiesel synthesis within different types of microreactors and in a batch reactor was given. Based on yield and volumetric productivity, the best system for biodiesel production was proposed.

2. Materials and Methods

2.1. Materials

Chemicals

Edible sunflower oil (Zvijezda, Zagreb, Croatia) was bought in a supermarket. Waste cooking oil (WCO) used in this research was collected after deep frying of potatoes. F.A.M.E. mix GLC-10, the commercial lipase from *Thermomyces lanuginosus* (Lipolase 100L), isoamyl alcohol, iso-octane and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Handels GmbH (Vienna, Austria). Chloroform and acetonitrile were purchased from Fisher Chemicals (Loughborough, United Kingdom). Tris (hydroxymethyl) aminomethane (TRIS), methanol, HCl and *n*-heptane were purchased from BDH Prolabo (VWR, London, United Kingdom). Dipotassium hydrogen phosphate (K_2HPO_4) and ammonium sulfate were purchased from Merck (Darmstadt, Germany). KOH and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Lach:ner (Prague, Czech Republic). Sodium hydrogen carbonate was purchased from Kemika (Zagreb, Croatia). 4-nitrophenyil-acetate was purchased from Acros Organics (Fischer Scientific, Geel, Belgium).

2.2. Methods

2.2.1. Lipase Production and Purification

Solid-state fermentation of by-products from cold-pressing oil production was used for the production of lipase from fungi *Thermomyces lanuginosus.* Lipase production and purification is described elsewhere [22].

2.2.2. Lipase Assay

Enzyme activity was determined by a test described elsewhere [23]. Only difference was use of 0.0375 mol/L 4-nitrophenyl acetate in assay. Briefly, 100 µL of the sample was added to 3900 µL of 50 mmol/L TRIS-HCl buffer pH 8 and homogenized. 1 mL is of sample is needed to start the test; 950 µL

of previously made mixture was added to UV-cuvette and reaction was started with the addition of 50 µL of 0.0375 mol/L 4-nitrophenyl acetate (dissolved in acetonitrile). For determination of the enzyme activity, spectrophotometer (Shimadzu UV–1601, Kyoto, Japan) was used. The change of absorbance was measured at 400 nm, with the total determination time of 20 s. To confirm repeatability, all measurements were performed in triplicate. The results showed no significant difference on 95% confidence interval.

2.2.3. Emulsion Preparation

The preparation of water-in-oil emulsion started with addition of buffer (containing the enzyme) to oil in 8:1 ratio, followed by the addition of SDS as the selected emulsifier (γ = 0.1 mg/mL). The mixture was then mixed on the laboratory shaker (Tehtnica, Vibromix 313EVT, Prague, Czech Republic) at 600 rpm for 25 min.

2.2.4. Deep Eutectic Solvent (DES) Preparation

Anhydrous DES used in this work was the combination of choline chloride (20.16 g) and glycerol (38.88 g) in a molar ratio of 1:3.0 [21]. After weighing, the above-mentioned masses were placed in a beaker and stirred on a magnetic stirrer (200 rpm) at 50 °C. The process was carried out for approximately 60 min until a homogeneous, colorless and transparent liquid (T = 25 °C, ρ = 1.2250 g/mL, η = 0.078 Pa s) is obtained. DES was then cooled to room temperature and stored.

2.2.5. Determination of Free Fatty Acids (FFA) in Oil (Chemical Synthesis)

Concentration of FFA in sunflower oil, was determined by dissolving edible sunflower oil (60 mg) in 4 mL of iso-octane, after which 200 µL of 2 mol/L KOH methanol solution was added. Mixture was rapidly shaken for 30 s and then left on room temperature. When the mixture became transparent and with visible layer of glycerol decanted at the bottom of the flask, 1 g of sodium hydrogen carbonate was added in order to neutralize the mixture. In order to determine FFA, upper layer of the mixture was separated and analyzed by gas chromatography, applying the same method which is briefly described in Section 2.2.6.

2.2.6. Measurement of Fatty Acid Methyl Esters (FAME) and Glycerol Concentrations

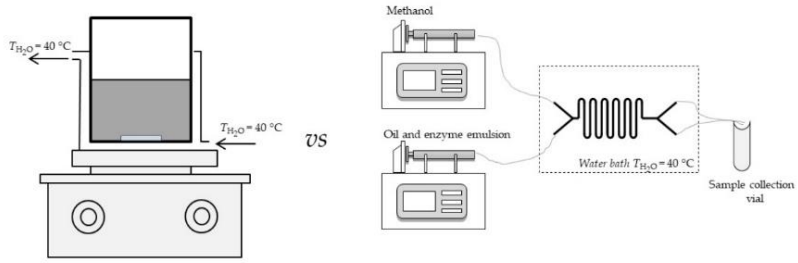
FAME and glycerol concentration were determined according to the method described by Budžaki et al. [24] on a gas chromatograph (Shimadzu GC-2014, Tokyo, Japan) equipped with FID and Zebron ZB-wax GC capillary column (length 30 m, I.D. 0.53 mm and film thickness 1.00 µm, Phenomenex, Torrance, CA, USA). Carrier gas in this method was nitrogen, at rate of 1.97 mL/min. With the total determination time of 15 min, measurement starts at the temperature of 180 °C for 1 min, with column heating up to 230 °C, at rate of 5 °C/min. F.A.M.E. mix GLC-10 was used as a standard for identifying peaks for corresponding esters of fatty acids. Retention times of FAME compounds are as follows: 7.74 min for palmitic, 10.590 min for stearic, 10.867 min for oleic, 11.575 min for linoleic and 12.615 min for α-linoleic (linolenic). The retention time for glycerol was 9.02 min, applying the same method. To confirm repeatability, all measurements were performed in triplicate. On 95% confidence interval, the results showed no significant difference.

2.2.7. Macro-Scale Biodiesel Production—Batch Reactor Experiments

Setup for biodiesel production in batch reactors, from edible and waste cooking sunflower oil using Lipolase 100L as catalyst, was previously described by Budžaki et al. [24]. In order to ensure a sufficient amount of methanol in the one-step reaction, the molar ratio of oil to methanol was 1:3.4. All experiments were performed for two days (48 h) with constant stirring (600 rpm). The initial lipase concentration (dissolved in phosphate buffer pH 7.4 (2 experiments) and DES (2 experiments)), was

the same in all four experiments ($\gamma_{E,0}$ = 0.1 mg/mL). Overview of performed experiments is given in Table 1.

Table 1. Experimental setup and different reaction conditions during oil transesterification catalyzed by lipase performed in a batch reactor and in a microreactor system.



	Oil Type		Enzyme		Solution	
	Sunflower Oil	**WCO**	**Commercial**	**Partially Purified**	**Buffer**	**DES**
			Batch Reactor			
Experiment 1	+		+		+	
Experiment 2		+	+		+	
Experiment 3	+		+			+
Experiment 4		+	+			+
			Microreactor			
Experiment 5	+		+		+	
Experiment 6		+	+		+	
Experiment 7	+		+			+
Experiment 8		+	+			+
Experiment 9	+			+	+	
Experiment 10		+		+	+	
Experiment 11	+			+		+
Experiment 12		+		+		+

2.2.8. Micro Scale Biodiesel Production—Microreactor Experiments

A glass microreactor with two inlets (length: width: depth = 332 mm: 500 µm: 50 µm with an internal volume of 8.3 µL; Micronit Micro-fluidics B.V., Enschede, Netherlands) was selected for the transesterification process on a micro scale. Total of eight different experiments were performed (Table 1).

In the fifth experimental setup, emulsion which was formed from sunflower oil and enzyme dissolved in buffer (potassium phosphate buffer pH 7.4 (c = 0.01 mol/L), in 9:1 ratio), with the addition of emulsifier (SDS), was placed into one syringe, while the second syringe was filled with methanol. In the sixth experiment, sunflower oil was replaced by WCO. In the seventh experiment, enzyme was dissolved in DES (in 1:9 ratio) and mixed with edible sunflower oil and SDS (to form a stabile emulsion). This mixture was placed into one syringe, while the second syringe was filled with methanol. In the eight experiment, experimental conditions were same as in seventh with the exception of edible sunflower oil that was replaced with WCO. Experiments nine (9) to twelve (12) were similar to experiments five (5) to eight (8) but instead of commercial enzyme lipase, lipase produced by solid-state fermentation of by-products from cold-pressing oil and partially purified was used. Syringes were placed on pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA) and connected with silica/PTFE tubes to both microreactors. All experiments were performed at 40 °C in order to obtain optimal enzyme activity. A water bath with a heat regulation system (Thermomix 1420, Braun, Germany) was used to secure required temperature.

In order to determine the influence of DES water content on biodiesel yield and volumetric productivity additional set of experiments were performed in a microreactor. As in previous experiments performed with DES, the first experiment in this part of investigation was carried out with anhydrous DES while in other experiments different amounts of water were added to obtain different water content (% *w*/*w*) in the process ranging from 1 to 8%. Those experiments were conducted in a PTFE microreactor (length: width: depth = 1.2 m: 500 μm: 50 μm with an internal volume of 236 μL) for residence time of 30 min.

For all experiments, an oil: methanol: enzyme ratio 10:1.24:1 was kept constant by altering the flow ratios. The influence of total flow rate ranging from Φ = 0 μL/min to Φ = 200 μL/min) on measurement of fatty acid methyl esters (FAME) formation was monitored. The reaction at the exit of the microreactor was stopped by placing outgoing silicate tubes in an organic solvent (Marmur solution—chloroform: isoamyl alcohol = 24:1, placed in vials and cooled on ice). This combination consequently led to enzyme deactivation [11].

2.2.9. Data Processing

Enzyme operational stability decay rate constant (k_d) was described by first-order kinetics (Equation (1)).

$$\frac{d_{relative\ activity}}{dt} = -k_d \cdot relative\ activity \quad (1)$$

The experimental data were the basis for the estimation by nonlinear regression analysis. The least-squares method implemented in the Scientist®3.0 software package (Micromath®, Saint Louis, MO, USA) was used.

3. Results

3.1. Transesterification Process in a Batch System

As mentioned earlier, transesterification of oil into biodiesel was conducted in separate batch experiments 1–4 (Table 1). These experiments were conducted because there is no available literature data (with the exception of biotransformation using a commercial enzyme and edible sunflower oil [24]) and in order to ultimately compare the performance of batch and microreactor systems.

A total of four different process conditions were tested. The main goal was to investigate different oil sources (edible and waste sunflower oil) as substrates for biodiesel production, as well as the possibility of using DES as a replacement for an aqueous buffer as the reaction medium [24]. DES was proposed since its composition can provide stability and activity for enzymes such as lipase in deep eutectic mixtures, even under high concentrations of molecules (such as methanol) that can denature the enzyme. In all experiments, for a total time of 48 h, commercial lipase Lipolase 100L was used as a catalyst. In one of our previous experiments [24], this time has been proven as sufficient for biodiesel synthesis by enzymatic transesterification with the high conversion of oil.

The obtained results are presented in Table 2. As it can be observed, the highest biodiesel yield was obtained in experiment 1, where the primary substrate used was edible oil and an aqueous buffer was used as a reaction medium. Somewhat lower yield was noticed in experiment 2, where waste oil was used as a substrate. This can be explained by possible impurities contained in waste oil, which probably had some influence on enzyme activity during the transesterification process.

Table 2. Comparison of biodiesel yield obtained in batch experiments.

	Time, h	**Biodiesel Yield, %**
Experiment 1	**48**	**91**
Experiment 2	**48**	**70**
Experiment 3	**48**	**6**
Experiment 4	**48**	**5**

In the experiments (3 and 4) where DES was used instead of an aqueous buffer, a significantly lower biodiesel yield was noticed. This can be explained by the lack of water in the process (only 0.7% (*w*/*w*) of water was in the reaction medium). According to Merza et al. [25], adding just 1% (*w*/*w*) of water to pure ChCl:Gly DES, thus forming ChCl:Gly:H_2O DES, higher biodiesel yields can be achieved. Since the commercial enzyme is 73% water, premise was that the water content in Lipolase 100L would be sufficient for enhanced lipase activity. Same researchers report that adding up to 4% (*w*/*w*) of water is preferable in the system to maintain lipase activity.

Enzyme operational stability was monitored during all batch processes. In all experiments, samples were taken from the reactor and enzyme activity was measured. Initial enzyme activity was taken as the reference point, and the relative activity for every sample was calculated according to the reference point. The results for enzyme operational stability in all batch experiments are shown in Figure 1. As it can be seen, when an enzyme is dissolved in buffer, there is a decrease in enzyme activity in the first 5 h. There are several reasons why enzyme deactivation occurred, such as mechanical forces, formation of glycerol and water content [23]. Additionally, methanol used in the reaction is one of the representatives of low chain alcohols and it has a significant impact on enzyme activity. According to literature [26], methanol causes the decrease of enzyme activity by stripping bounded water from the enzyme and by penetrating into the enzyme active sight. In our previous papers [11,24], we demonstrated that a higher concentration of methanol (90% (*v*/*v*) solution) completely deactivates the enzyme in 25 h. In this research, the initial concentration of methanol was 30.6 mg/mL, which caused deactivation at the beginning of the process. During the reaction, due to the methanol consumption, the enzyme operational stability did not change significantly, and it was almost constant after the first 5 h.

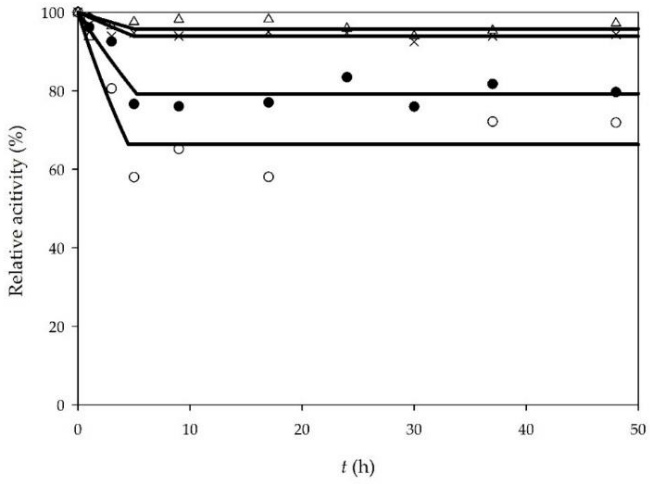


Figure 1. Lipase stability in batch processes (○—edible oil, enzyme dissolved in buffer, •—waste oil, enzyme dissolved in buffer, Δ—edible oil, enzyme dissolved in deep eutectic solvent (DES), ×—waste oil, enzyme dissolved in DES).

In order to determine the enzyme operational stability decay rate, Equation (1) was used. For the first 5 h of experiments, if sunflower oil was used as a substrate, the enzyme operational stability decay rate was $k_{d\text{sunflower oil/buffer}} = 0.091 \pm 0.015$ 1/h and when WCO was used, the enzyme operational stability decay rate was $k_{d\text{WCO/buffer}} = 0.044 \pm 0.007$ 1/h.

Unlike a buffer, DES provided greater stability to lipase (Figure 1). After 48 h of the experiment, lipase activity remained almost the same. The deactivation constants were determined to be $k_{d\text{sunflower oil/DES}} = 0.009 \pm 0.006$ 1/h for sunflower oil and $k_{d\text{WCO/DES}} = 0.013 \pm 0.003$ 1/h for WCO,

respectively. According to literature [27], DES probably "protects" the enzyme by forming hydrogen bonds with methanol. This limits the penetration of the methanol in the enzyme active sight. In addition, choline and chloride DES ions can form hydrogen bonds with the surface residues of the enzyme, leading to better enzyme stability.

3.2. Biodiesel Production in A Microreactor

Prior to the microreactor experiment, enzyme operational stability was determined. While the batch processes were carried out in the period of 48 h, continuous processes in microreactors were conducted for a longer period of time. Based on the setup up of the different overall flow rate, 4 days are needed for one experiment to be conducted in a microreactor.

According to literature [11], an enzyme should be stable in buffer for more than five days. For the first four days it remained above 80%, after which the activity dropped significantly, with the measured activity of 30% of the initial activity in the sixth day. The reason for this is probably also methanol which penetrated into the syringe during the microreactor experiments due to back flow. This usually occurs when pumps are stopped, and high pressure is still present in the microchannel.

As mentioned above, in order to find the "ideal" system for biodiesel production by enzymatic transesterification, several microreactor experiments have been set up. The main goal was the intensification of the transesterification process in terms of obtaining similar biodiesel yields for shorter (residence) time in comparison to the batch system. This can be achieved due to the small microchannel size which assures a high surface to volume ratio, which leads to enhanced heat transfer and thus reduced energy demands, faster diffusion as a dominant transport, good process control and high throughput [28].

Based on the chronological order of performed experiments, different comparisons were made, according to the oil source, the reaction medium and enzyme origin. All of the experiments (5–12) were made for same range of residence times (τ = 0–30.62 min), so the comparison could be made easier. As main process parameters, biodiesel yield and volumetric productivity were compared for different residence times.

3.2.1. Influence of Oil Origin on Biodiesel Yield and Volumetric Productivity

First two experiments that were performed in a microreactor were Experiments 5 and 6 (Table 1). Those experiments were performed under the same conditions as batch experiments 1 and 2. In Experiment 5, sunflower oil was used, and in Experiment 6, WCO were used as substrates. The comparison of biodiesel yield for these two experiments is presented in Figure 2. As it can be seen, biodiesel produced from WCO results in higher yield for the same residence time, when compared to biodiesel produced form edible oil. The highest yield of 33% was obtained in the experiment performed with WCO for the longest residence time of 30 min. When it comes to productivity, the comparison between Experiment 5 and Experiment 6 shows that the setup of Experiment 6 (Q_{p6} = 20.88 kg/ (L·d)) results in double the volumetric productivity than Experiment 5 (Q_{p5} = 9.87 kg/ (L·d)), respectively.

One possible explanation for the highest yield in the experiment with WCO is the cracking of long chains in oil during frying and the resulting shorter chain fatty acids present in the reaction medium during transesterification. The WCO used in this research was analyzed and its composition was compared with fresh sunflower oil. Analysis revealed that WCO had a 4% higher concentration of palmitic acid and a 6% lower concentration of linoleic acid in comparison to fresh sunflower oil. Lipases can then process them faster as a consequence, resulting in higher production yields of biodiesel in the experiment with WCO.

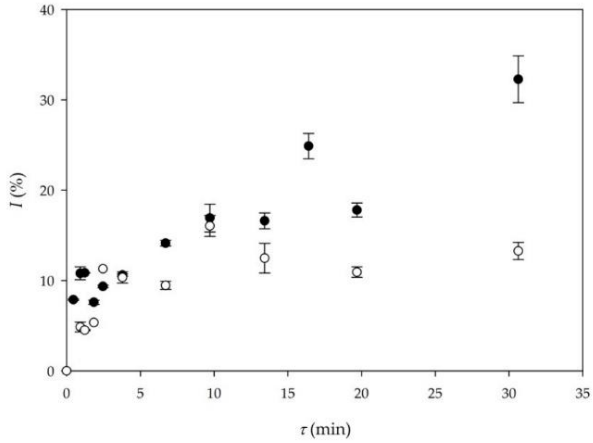


Figure 2. Comparison of biodiesel yield depending on oil origin (○—edible oil (Experiment 5), •—waste cooking oil (WCO) (Experiment 6)).

3.2.2. Influence of the Reaction Medium on Biodiesel Yield and Volumetric Productivity

Although the results obtained from batch experiments (Experiments 3 and 4) indicated that when DES is used as a medium for the transesterification process, additional water should be added to the systems to obtain a higher yield reaction. Nevertheless, transesterification in a microreactor with DES as a reaction medium was performed under the same conditions as in batch experiments (Experiments 3 and 4). The first reason was to gain more information about reaction performance in a microreactor and the second one was to test the possibility of simultaneous glycerol removal by using the DES which was present in the system. This possibility of process duality could prove to be a valuable process optimization procedure for biodiesel production in microreactors. In one of our previous papers [29], ChCl:Gly DES was efficiently used for glycerol removal from biodiesel. By using ChCl:Gly (in ratio 1:3.0) based DES, almost all free glycerol was removed from biodiesel. Based on these results, a DES with the same composition was used for biodiesel production. In order to check glycerol removal efficiency in these experiments, the concentration of glycerol was calculated based on the stoichiometry of the reaction (calculated from the FAME concentration for the longest residence time τ = 30 min) for the value of 12.58 mg/mL. Analyzing the real sample, the concentration was determined to be 10.68 mg/mL, meaning that 1.9 mg/mL (15.11%) glycerol was removed from the process. The reason for such low extraction efficiency could be the amount of DES in the process. While the amount of DES in the previously performed experiments was 50% (v/v), in this experiment, only 10% (*v*/*v*) of DES was used. Although the obtained efficiency was not enough to satisfy the norms for biodiesel purity, it clearly indicated that with future optimization, an integrated system for biodiesel production and simultaneous glycerol removal on a single microchip could be a solution for further process intensification.

Figure 3 shows the comparison between yields obtained in Experiment 6 (buffer) and Experiment 8 (DES).

As it can be seen in Figure 3, higher biodiesel yield was again obtained in the experiment where a buffer was the reaction medium (Experiment 6). In the experiment performed with a DES as the reaction medium, approximately 15% of the biodiesel yield was obtained for the residence time of 30 min. The obtained volumetric productivity in Experiment 6 (Q_{p6} = 20.88 kg/ (L·d)) was almost three times the volumetric productivity of Experiment 8 (Q_{p8} = 7.35 kg/ (L·d)), respectively.

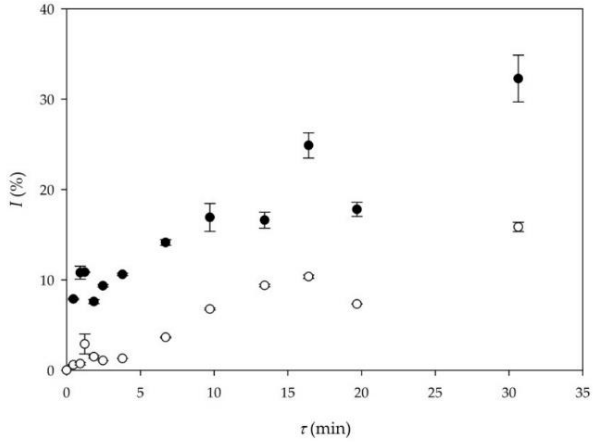


Figure 3. Comparison of biodiesel yield in different reaction mediums (•—buffer as a reaction medium (Experiment 6), ○—DES as a reaction medium (Experiment 8)).

Although the experiment performed with DES resulted in approximately 15% biodiesel yield (τ = 30 min), this is significantly higher in comparison with biodiesel yield obtained in batch experiments, where biodiesel yields were only 6% (Experiment 3) and 5% (Experiment 4), respectively. In addition, batch experiments were performed for 48 h, which also favors microreactor experiments. This indicates that using anhydrous DES in microreactor systems significantly increases productivity, which is visible in Table 3 if comparing Experiments 3 and 4 to Experiments 7 and 8. Furthermore, adding water content up to 4% (*w*/*w*) [26], could provide additional improvement in biodiesel yield for the same residence time.

Table 3. Comparison of biodiesel yield and volumetric productivity for different DES water content in the transesterification reaction catalyzed by lipase.

Water Content (% *w*/*w*)	τ (min)	I (%)	Q_p (kg/ (L·d)
0	30.62	12.38	5.74
1	30.62	13.31	6.17
2	30.62	16.81	7.79
4	30.62	31.22	14.47
6	30.62	23.03	10.68
8	30.62	10.62	4.92

3.2.3. Influence of Enzyme Origin on Biodiesel Yield and Volumetric Productivity

In order to make the overall process more economical, the application of lipase, produced by solid-state fermentation of *Thermomyces lanuginosus*, on by-products from cold-pressing oil production and partially purified, was used for biodiesel production. Results were compared with the results obtained for biodiesel production by transesterification catalyzed by commercial lipase. As it can be seen form Figure 4, there is a significant difference in biodiesel yield when commercial lipase and produced and purified lipase [22] were used. The main reason behind this is the much larger initial activity of the commercial enzyme (around 200 times higher) in comparison to the purified enzyme. At this point, without additional optimization of purification steps, purified enzyme is not suitable for biodiesel production. A new approach in making the process more economical would be the immobilization or the recirculation of commercial lipase. Furthermore, a productivity-based comparison between Experiment 5 and Experiment 9 shows that the setup of Experiment 5 (Q_{p5} = 9.87 kg/ (L·d)) provides

much higher volumetric productivity than Experiment 9 (Q_{p9} = 1.63 kg/ (L·d)), respectively, mostly due to the much higher initial lipase activity.

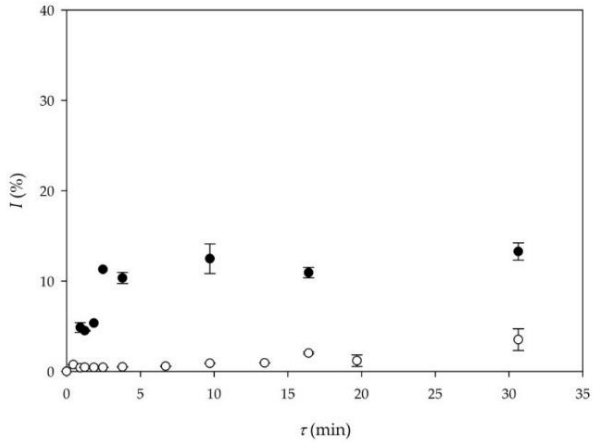


Figure 4. Comparison of biodiesel yield according to lipase origin (•—commercial lipase Lipolase 100L (Experiment 5), ○—purified lipase produced by solid-state fermentation (Experiment 9)).

3.3. Influence of DES Water Content on Biodiesel Yield and Volumetric Productivity

Additional experiments were performed to explore the possibility of using DES as adequate replacement for a buffer in terms of higher biodiesel yields. Experiments were performed with different water content ranging from 1 to 8% (*w*/*w*). Since all the previously conducted experiments have shown the highest biodiesel yield for residence time of 30 min, all experiments with different DES water content were performed for that residence time. To confirm the repeatability of experiments, the experiment with 0% of water (*w*/*w*) was compared to Experiment 7, performed at same initial conditions and the results have shown strong agreement. If water was added in the reaction medium biodiesel yield and consequently volumetric productivity, rises all the way up to 4% (*w*/*w*) of added water (Table 3). This agrees with the study conducted by Merza et al. [25], since the water content of 4% (*w*/*w*) was also optimal for enzymatic production of biodiesel by transesterification. If the water content was above 4% (*w*/*w*), biodiesel yield and volumetric productivity start to decrease and for 8% (*w*/*w*) of water, both biodiesel yield and volumetric productivity where lower than in the experiment without the addition of water. This is probably due to hydrolysis being more pronounced in the reaction system with higher water content.

3.4. Comparison of Different Systems for Biodiesel Production

The comparison of biodiesel production using different systems has clearly shown the guidelines for the forthcoming research. In Table 4 biodiesel yield and volumetric productivity obtained in a batch and in a continuous microreactor have been shown. The highest volumetric productivity achieved in a batch process (Experiment 1) has significantly lower productivity in comparison with the highest volumetric productivity obtained in a microreactor experiment (Experiment 6). These results show that enzymatic transesterification in microreactors is a viable option, even WCO was used as a substrate. Unfortunately, biodiesel yield and productivity obtained in this research are significantly lower if compared with earlier research of Šalić et al. [11] (Experiment 13, Table 4). The main reason for it is the high methanol excess (28-fold higher than stoichiometric one) used in Experiment 13. Namely, the methanol concentration in Experiment 6 was 30.60 mg/mL, while in the Experiment 13, methanol concentration was 836.97 mg/mL. Microchannel width in Experiment 13 was 1 mm, which is unfavorable in comparison with the microchannel width in Experiment 6 in terms of mass transfer. Still,

the high methanol excess was the dominant reason for better results in a bigger microchannel. If the results of this research were compared with experiments performed by Gojun et al. [30] (Experiment 14), the results go in the favor of the smaller microchannel width. Namely, in Experiment 14, the microchannel width was 250 μm, which leads to four-fold faster diffusion in this microreactor system. In addition, in Experiment 14, the microreactor was fed with three inlets. Oil, enzyme suspended in buffer and methanol were fed separately. In Experiments 5–12, oil and enzyme with the addition of emulsifier were fed as one process stream and methanol was the second inlet stream. Faster diffusion and absence of emulsifier could be a reason for better results obtained in Experiment 14.

Table 4. Comparison of the oil transesterification process performed in a batch and in different types of microreactors.

Experiment	t (h)	I (%)	Q_p (kg/ (L·d)	Reference
1	48	91.34	0.45	
2	48	70.22	0.35	
3	48	6.44	0.03	
4	48	5.23	0.02	
	τ (min)			
5	30.62	13.26	9.87	
6	30.62	32.28	20.88	This research
7	30.62	12.42	5.76	
8	30.62	15.85	7.35	
9	30.62	3.51	1.63	
10	30.62	20.08	9.31	
11	30.62	1.76	0.81	
12	30.62	5.45	2.53	
13	30	97.81	69.88	[11]
14	19.8	32.72	35.42	[30]
	t (h)			
15	8	40	1.78	[26]

Lastly, the comparison of the experiments involving a DES as the reaction medium shows some interesting insights on whether these systems have good future applications. Experiments 7 and 8 were conducted with the commercial enzyme in a DES as the reaction medium. Those experiments provided biodiesel yield of 12–15% (Table 4), which is less when compared to Experiment 15, performed by Merza et al. [25], where biodiesel yield was up to 40%. However, with calculated productivity of these systems (shown in Table 4), Experiments 7 and 8 conducted in microreactor systems provide 3–4 times higher productivity than Experiment 15. These results justify the usage of a DES as the reaction medium, with increasing the water as a possible enhancement.

All in all, the direction for future experiments should definitely be the usage of waste oil as substrate, methanol in higher surplus than conducted in this research and smaller channel width. All the data shown indicates that channel width smaller than 1 mm is suitable for process intensification.

4. Conclusions

Biodiesel production by enzymatic transesterification has been conducted successfully. This study represented a total of twelve different experiments, eight of which were conducted in microreactors. The importance has been put on developing the "ideal" microsystem, the one which will provide the highest biodiesel yield for the shortest residence time. Results show that enzymatic transesterification in microreactors is a viable option for using waste oil as a primary substrate. The highest yield of 32% for residence time τ = 30 min was obtained in the microreactor system with an emulsion of waste oil and commercial enzyme suspended in a water buffer. This knowledge may prove rather important in future experiments and in process optimization, where the next steps are kinetic studies and mathematical modeling. Furthermore, even though results obtained with DES were not as good as

those obtained in a buffer medium, this opened up another research area. While DESs have already been confirmed as great extraction mediums, their role in synthesis still needs further investigation. Future experiments will provide momentum for developing the multi-purposed significance of DES.

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Paper 2

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Biodiesel purification in microextractors: Choline chloride based deep eutectic solvents vs water

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ABSTRACT

Nowadays, the production of biodiesel by transesterification is a common process. On the other hand, once the biodiesel is produced, the purification of crude biodiesel to the extent that satisfies international standard norms still presents a significant challenge. One of the biggest challenges is the removal of glycerol. In this paper, the total of seven different deep eutectic solvents (DESs) based on a mixture of choline chloride and ethylene glycol or a mixture of choline chloride and glycerol were prepared and applied for biodiesel purification, using three microextractors of different sizes. The obtained results were compared with the results of biodiesel purification by wet washing in the same microextractor type. For the residence time of only 13.61 s, by using a ChCl:$Gly_{1:2.5}$ DES, glycerol was almost completely removed from the biodiesel. So, the obtained result clearly justifies the application of DESs in microextractors for glycerol removal during biodiesel purification.

1. Introduction

Today's modern society is continuously facing the repercussions of long-term negative influences on the environment. With the increase of the human population, there is also a constantly growing energy demand. One of the main energy sources is fossil fuel (88% of the total energy consumption in the world) which is mainly produced from non-renewable energy sources and presents a great environmental burden [1]. Due to that, there is a constant search for a good and sustainable alternative. Biofuels produced from renewable energy sources appear to be a solution. Biodiesel has found the broadest usage compared to the other biofuels (bioalcohols and biogas). It is characterized as renewable, non-toxic, environmentally friendly and biodegradable, with a favorable combustion emission profile [1,2]. The most commonly used method for biodiesel production is transesterification of abundant vegetable or animal fat catalyzed by alkali [3]. Although chemical methods have been proved effective, there are many advantages in using enzymatic methods for biodiesel production [4,5]. Mild reaction conditions, low amount of waste, low energy demand, flexibility in choosing or even constructing different enzymes that could be substrate-specific, usage of small amounts of water, etc. [6].

On the other hand, no matter which catalyst is used, there are problems with the side effects of the catalysts, like soap and glycerol formation, during the transesterification process. Complicated and costly downstream processes are still one of the main reasons why biodiesel is still not competitive with diesel fuel. According to Atadashi et al. [7], 60–80% of the overall process costs in biodiesel production are connected to purification steps. Those downstream processes include the separation of glycerol and crude biodiesel in a decanter, followed by alcohol removal using an evaporator. After these steps, crude biodiesel is still not suitable for application in engines since it contains free glycerol molecules, soap (if biodiesel is produced by chemical catalysis), traces of the catalyst, methanol, metals, water, oil, and glycerides. All those impurities have to be removed in order to reach the standards (e.g., ASTM D6751 [8] and EN 14214 [9]) for biodiesel quality.

Different methods can be applied for biodiesel purification, like wet washing, dry washing, and membrane separation processes. Liquid extraction, which also includes water washing processes, is the most commonly applied method. The main problem with water usage is the generation of large amounts of polluting wastewater, which causes significant disposal problems [7]. According to Karaosmanoğlu et al. [10], 10 dm^3 of water is necessary to purify 1 dm^3 of biodiesel to meet the requirements of the norms. As an alternative, the application of green solvents, like ionic liquids (ILs) and especially low-cost IL alternatives called deep eutectic solvents (DESs), is proposed [11,12]. DESs are formed by mixing solid organic salts like choline chloride and a hydrogen bond donor molecule such as ethylene glycol or glycerol

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[13–15]. They are characterized as green due to their high biodegradability and low toxicity. Up to now, DESs have been successfully applied for biodiesel purification [11,12,14–18], but mostly in discontinuous extractors/separators with an application of shakers to enhance mass transfer. In order to make the process more economical, the replacement of discontinuous processes with continuous process would be the next step. Additionally, since the extraction/separation processes are highly dependent on the mass transfer rate, minimizing the diffusion path from one phase to another would make the process even more efficient. Miniaturized extraction/separator systems called microextractors and microseparators offer many exceptional technical advantages (large surface to volume ratio, small dimensions, etc.), based on which the impact of diffusion on the extraction/separation becomes negligible [19,20]. They have been identified as a promising technique for the separation of different chemical and biochemical products [21–25], including biodiesel [26].

In this paper, biodiesel was synthetized by transesterification of fresh sunflower oil and waste cooking oil with the commercial enzyme lipase. After biodiesel production, purification (glycerol removal) with different DESs (choline chloride:ethylene glycol and choline chloride:glycerol) was carried out in different types of microextractors (the same configuration but with different width and volume of the microchannels). The obtained results were compared with biodiesel purification performed by wet washing in a microextractor of equal geometry.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Edible sunflower oil (Zvijezda, Croatia) was purchased from a nearby supermarket. Waste cooking oil (WCO) used in this research was collected after deep-frying of potatoes. The lipase from *Thermomyces lanuginosus* (Lipolase 100L), F.A.M.E. mix GLC-10 and ethylene glycol were purchased from Sigma-Aldrich Handels GmbH (Austria). Tris (hydroxymethyl)aminomethane (TRIS), methanol, HCl and *n*-heptane were purchased from BDH Prolabo (VWR, United Kingdom). Acetonitrile was purchased from Fisher Chemicals (United Kingdom). Choline chloride, glycerol and 4-nitrophenyil-acetate were purchased from Acros Organics (Fischer Scientific, Belgium). Potassium dihydrogen phosphate (KH_2PO_4) and KOH were purchased from Lach:ner (Czech Republic). Dipotassium hydrogen phosphate (K_2HPO_4) was purchased from Merck (Germany). Sodium hydrogen carbonate was purchased from Kemika (Croatia).

2.2. Methods

2.2.1. Preparation of DESs

Choline chloride:ethylene glycol (ChCl:Etgl) DES and choline chloride:glycerol:water (ChCl:Gly:H_2O) DES were prepared by heating cholinium-based salt (choline chloride) and a hydrogen bond donor (ethylene glycol or glycerol). ChCl:Etgl was prepared in molar ratio of 1:2.5 while ChCl:Gly:H_2O was prepared in different molar ratios without (1:1; 1:2; 1:2.5 and 1:3) and with addition of water (1:1:0.5; 1:1:1 and 1:1:2) (Table 1). Waterless DESs were prepared with different glycerol molar ratio, while in DESs with addition of water, molar ratio of the choline chloride and glycerol was kept constant while the amount of water was altered. These combinations were chosen in order to investigate the effect of glycerol and water amount on DESs properties and extraction efficiency. All DESs were prepared at magnetic laboratory stirrer (Rotamix S-10, Tehtnica, Slovenia) at 200 rpm and 50 °C until a homogeneous liquid has formed.

2.2.2. Biodiesel synthesis in a batch reactor

The production of biodiesel was carried out in a batch reactor (V = 250 cm^3) using the procedure described elsewhere [6]. Briefly, the reaction was performed in a single step at temperature of a 40 °C with a constant stirring of 600 rpm. The molar ratio of oil to methanol was 1:3.4. The reaction mixture was composed of 163.63 g of edible sunflower oil or waste cooking oil, 20.35 g of methanol, and 16.36 g of Lipolase 100L stock solution diluted with 0.01 mol dm^{-3} phosphate buffer at pH 7.4 in molar ratio 1:10. The reaction started with the addition of the enzyme. A 1 cm^3 samples were withdrawn from the reaction mixture in different period of time until the end of the reaction was detected. FAME and glycerol concentrations measurements were performed using GC.

2.2.3. Biodiesel purification in a separation funnel

After biodiesel synthesis in a batch reactor, the complete reaction mixture was transferred into a separation funnel and let to settle down during 24 h. Upper layer was then transferred in the new funnel and the procedure was repeated for the additional 24 h. During each step concentration of glycerol was monitored.

2.2.4. Biodiesel purification in microextractors

Purification of biodiesel by wet washing was performed in a glass microextractor (length:width:depth = 332 mm:250 μm:50 μm with an internal volume of 4.3 mm^3; Micronit Microfluidics B.V., Netherlands) with two "Y" shaped inlets and two "Y" shaped outlets. For purification of biodiesel using DESs three different glass microextractors of same depth (50 μm) and different width (250 μm, 350 μm and 500 μm, with an internal volume of 4.15 mm^3, 5.81 mm^3 and 8.30 mm^3, respectively, Micronit Microfluidics B.V., Netherlands) equipped with two "Y" shaped inlets and two "Y" shaped outlets were used. In all experiments, biodiesel was placed into one syringe and fed from one inlet into the microextractor while solvent (DES or water) was fed from another inlet (Fig. 1) using two syringe pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, USA) equipped with high-pressure stainless steel syringes (8 cm^3, Harvard Apparatus, USA). All experiments were performed at 25 °C. The total flow was altered from 0.7 to 150 mm^3 min^{-1} (depending on the experiment) and the influence of solvents on glycerol extraction was monitored.

2.2.5. Measurement of fatty acid methyl esters (FAME) and glycerol concentrations

FAME and glycerol concentration were determined according to the method described elsewhere [6] using GC (Shimadzu GC-2014, Japan) equipped with FID detector and Zebron ZB-wax GC capillary column (length 30 m, I.D. 0.53 mm and film thickness 1.00 μm, Phenomenex, USA). Carrier gas in this method was nitrogen, at a rate of 1.97 cm^3 min^{-1}. In the method's total determination time of 15 min, measurement starts at the temperature of 180 °C for 1 min, after which at a rate of 5 °C min^{-1}, a column is heated up to 230 °C. In order to identify peaks for corresponding esters of fatty acids, standard F.A.M.E. mix GLC-10 was used. Retention times of fatty acids esters are as follows: 7.74 min for palmitic, 10.590 min for stearic, 10.867 min for oleic, 11.575 min for linoleic and 12.615 min for linoleic. Glycerol determination was made with the same method and its retention time was 9.02 min. To confirm repeatability, all measurements were performed in triplicate. On a 95% confidence interval, the results showed no significant difference.

2.2.6. Lipase assay

Enzyme activity was determined by a test based on the hydrolysis of 1.5 mol dm^{-3} 4-nitrophenyl acetate described elsewhere [26]. 100 mm^3 of the sample was added to 3900 mm^3 of TRIS-HCl buffer and homogenized. 950 mm^3 of this mixture was added to UV-cuvette. The test started by adding 50 mm^3 of 1.5 mol dm^{-3} 4-nitrophenyl acetate (dissolved in acetonitrile). To determine the enzyme activity, a spectrophotometer (Shimadzu UV – 1601, Kyoto, Japan) was used. The determination time was 60 s, while the change of absorbance was

Table 1
Composition and characteristics of synthetized DESs, and volumetric flow ratios used in a microextractor experiments.

ChCl:Gly:H_2O	ρ (g cm^{-3})		η (Pa s)		Microchannel width (μm)		
	25 °C	40 °C	25 °C	40 °C	250	350	500
					volumetric flow ratios (BD:DES)		
1:1.0:0	Solid at room temperature						
1:2.0:0	1.225	1.210	0.267	0.096	0.036	0.036	0.036
1:2.5:0	1.220	1.200	0.277	0.097	0.030	0.030	0.030
1:3.0:0	1.225	1.215	0.283	0.098	0.029	0.029	0.029
1:1:0.5	1.200	1.160	0.169	0.053	0.046	0.046	0.046
1:1:1.0	1.225	1.190	0.070	0.026	0.055	0.055	0.055
1:1:2.0	1.170	1.150	0.043	0.018	0.126	0.126	0.126

measured at 400 nm. To confirm repeatability, all measurements were performed in triplicate. On 95% confidence interval, the results showed no significant difference.

2.2.7. Mathematical model of biodiesel purification in a microextractor

The glycerol separation in a microextractor was described with 2D model including convection in the flow direction (x) and diffusion in two directions (x and y). The mathematical model for steady-state conditions in a microextractor was composed of dimensionless partial differential equations for glycerol concentrations in biodiesel and DES (water) phase and corresponding boundary and initial conditions (Eqs. (1) and (2)).

- glycerol concentration in biodiesel phase:

$$v\cdot\frac{\partial\gamma_{glycerol,biodiesel}}{\partial\xi}=\frac{D_{glycerol,biodiesel}}{W}\cdot\left(\frac{\partial^2\gamma_{glycerol,biodiesel}}{\partial\xi^2}+\frac{\partial^2\gamma_{glycerol,biodiesel}}{\partial\psi^2}\right) \quad (1)$$

$$\gamma_{glycerol,biodiesel}(0,\psi)=\gamma_{glycerol,biodiesel,i}\quad 0\le\psi\le 1$$

$$\frac{\partial\gamma_{glycerol,biodiesel}\left(\frac{L}{W},\psi\right)}{\partial\xi}=0\quad 0\le\psi\le 1$$

$$\gamma_{glycerol,biodiesel}(\xi,0)=K_P\cdot\gamma_{glycerol,DES}(\xi,0)\quad 0<\xi<\frac{L}{W}$$

$$\frac{\partial\gamma_{glycerol,biodiesel}(\xi,1)}{\partial\psi}=0\quad 0<\xi<\frac{L}{W}$$

- glycerol concentration in DES (water) phase:

$$v\cdot\frac{\partial\gamma_{glycerol,DES}}{\partial\xi}=\frac{D_{glycerol,DES}}{W}\cdot\left(\frac{\partial^2\gamma_{glycerol,DES}}{\partial\xi^2}+\frac{\partial^2\gamma_{glycerol,DES}}{\partial\psi^2}\right) \quad (2)$$

$$\gamma_{glycerol,DES}(0,\psi)=0\quad -1\le\psi\le 0$$

$$\frac{\partial\gamma_{glycerol,DES}\left(\frac{L}{W},\psi\right)}{\partial\xi}=0\quad -1\le\psi\le 0$$

$$\frac{\partial\gamma_{glycerol,DES}(\xi,-1)}{\partial\psi}=0\quad 0<\xi<\frac{L}{W}$$

where ν represents the linear velocity, ξ and ψ represent independent dimensionless variables $\xi = x/W$, $\psi = y/W$, x and y are coordinates in the length (L) and microchannel width ($2\ W$). $D_{glycerol/biodiesel}$ and $D_{glycerol/DES(water)}$ are diffusion coefficients for glycerol in biodiesel and DES (water) phases and K_p is partition coefficient. The molecular diffusion coefficients for glycerol and biodiesel were calculated using the Hyduk-Laudie empirical correlation (Eq. (3)):

$$D_{glycerol,biodiesel(DES)}=\frac{13.26\cdot10^{-9}}{\eta_{biodiesel(DES)}\cdot V_{m,glycerol}^{0.589}} \quad (3)$$

where V_m, glycerol is the molar volume of glycerol, and η is the dynamic viscosity of both biodiesel phase and DES (water) phase. System of partial differential equations was solved using the 2D finite differences method by discretization on the static equidistant grid in Mathematica 10.0 (Wolfram Research, USA) software.

Additionally, the diffusion time was calculated according to Eq. (4).

$$\tau_D=\frac{W^2}{D_{glycerol,biodiesel}} \quad (4)$$

3. Results and discussion

3.1. Biodiesel synthesis in a batch reactor

First, biodiesel was synthetized with free Lipolase 100L in a batch

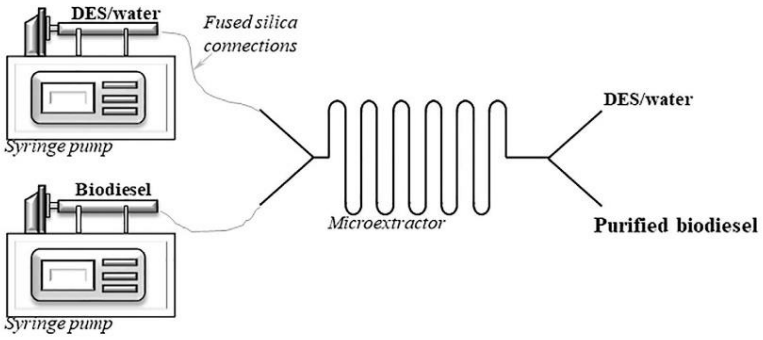

Fig. 1. Schematic diagram of the microextractor system used for biodiesel purification.

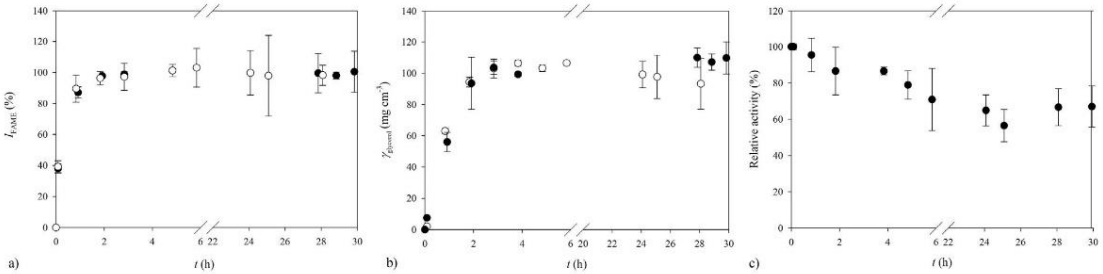

Fig. 2. Biodiesel synthesis from ○ sunflower oil and ●WCO in a batch reactor: (a) FAME yield, (b) glycerol concentration) and (c) enzyme deactivation during the process.

reactor (V = 250 cm^3), according to the procedure described elsewhere [6], using edible sunflower oil and waste cooking oil (WCO). In both experiments, methanol was used in excess as a second substrate. The maximum fatty acids methyl esters (FAME) yield for both substrates after 6 h of the experiment was around 96% (γ_{FAME} = 1049.05 ± 10.30 mg cm^{-3}, Fig. 2a). During that time, the enzyme activity was monitored for the process in which edible sunflower oil was used and it was noticed that the activity decreased in the first 6 h (Fig. 2c), for about 25% when compared to the initial activity. The deactivation constant, k_d, was calculated to be 0.053 ± 0.005 h^{-1}. According to literature, when lipase is exposed to water-miscible solvents like methanol, those solvents have a high tendency to strip off tightly bound water from the enzyme. Consequently, those molecules penetrate deeper into the enzyme active site, which in turn causes a loss of both structure and activity [27]. After the reaction ended (around 6 h) and most of the methanol was converted, no change in enzyme activity was noticed. Biodiesel synthesis from WCO deactivation was not monitored, but the same trend could be assumed.

The formation of glycerol was also monitored and the maximum concentration obtained at the end of booth processes was around 100.14 ± 4.86 mg cm^{-3} (Fig. 2b), which corresponds to the calculated glycerol concentration value of 107 mg cm^{-3} (based on the stoichiometry of the reaction calculated from the FAME concentration). Also, the ratio of the obtained glycerol in comparison to biodiesel follows the general rule, where for every 100 kg of biodiesel produced, approximately 10 kg of crude glycerol is formed.

3.2. Biodiesel purification on a macroscale

After crude biodiesel was produced, it was placed in a separation funnel to separate glycerol from the biodiesel (the most common method). Samples were taken and analyzed for glycerol concentrations before and after separation. After 24 h, 97% of the glycerol was removed and the procedure was repeated once again (Fig. 3). After the second separation, an additional 2.7% of glycerol was removed. After these steps, the biodiesel phase still contained free glycerol particles (0.3%). According to the quality parameters prescribed by the American standard ASTM D 6751 [8] and the European standard EN 14214 [9], the percentage of glycerol in biodiesel has to be less than 0.02%, therefore, additional purification techniques had to be applied. According to literature [28,29], other different separation methods can be applied for biodiesel purification. One of them is filtration. So, as the next step, simple filtration trough a 0.20 μm pore size filter (Filter Chromafil® AO-20/3; 0.45 μm, Macherey-Nagel GmbH, Dueren, Germany) was performed. Unfortunately, the process did not have an effect on glycerol removal. As the next step, centrifugation at 14,000 rpm and 4 °C for 20 min (Universal 320R, Hettich Zentrifugen, Tuttlingen, Germany) was applied. This step also led to an insignificant improvement in glycerol content (Fig. 3). The free glycerol content after this step was around 0.09% (w/w) for biodiesel synthetized from edible

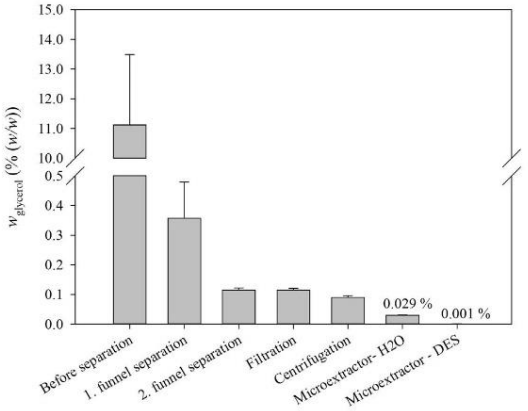

Fig. 3. Free glycerol content in different phases of biodiesel purification process.

sunflower oil and 0.106% (w/w) for biodiesel synthetized from waste cooking oil.

3.3. Biodiesel purification in a microextractor

According to Niawanti et al. [30], several factors influence the extraction processes. Extraction temperature, extraction time, characteristics of solid particles (size, shape, and condition), and the type of solvent are the most important ones.

As the next purification step, microextractors with three different microchannel widths (250 μm, 350 μm, 500 μm) and different volumes (4.15 mm^3, 5.81 mm^3, and 8.30 mm^3) were used. The assumption was that by applying the microextractors, the extraction time will be shortened. Also, as mentioned in the introduction, since the extraction/separation processes are highly dependent on the mass transfer rate, minimizing the diffusion path from one phase to another would make the process even more efficient. Choline chloride based DESs or water were used as purification media in experiments performed in a microextractor, since solvent type also has an important effect on the extraction process [30]. A ChCl:Etgl based DES was used because in one of our previous studies [26], a ChCl:Etgl based DES was successfully applied in the development of an integrated system with continuous biodiesel synthesis and purification. After the process, the absence of glycerol was observed and it was therefore concluded that the glycerol content in biodiesel was below 0.02%. In this study, a step forward was the application of a smaller channel diameter to enhance process efficiency. A ChCl:Gly:H_2O based DES was used because, according to the literature [11,12], it also has the ability to successfully remove glycerol and other residues (like KOH) from biodiesel. It is also important to

stress down that, according to the literature, ethylene glycol as solvent could be used for glycerol removal. According to Sinaga et al. [31], in a batch extractor at the ethylene glycol/glycerol volume ratio of 1:1 (v/v) with extraction time of 60 min, the highest purity produced amounted to 90.65%. On the other hand, by applying ChCl:Etgl DES in a batch extractor it was possible to achieve 97.78% efficiency for extraction time of 20 min [32]. Since the obtained results are in favor of DESs, they were chosen as extraction solvents in this research.

Prior to the separation experiments, it was necessary to define the volumetric flow ratio of phases, which will enable the formation of a stable and parallel flow with the interface placed exactly in the middle of the microextractor channel. This was necessary in order to enable the separation of phases at the "Y" shaped exit of the system and to obtain the same volumes of each of the phases in the extractor. In our previous paper [33], a parallel flow was formed if a glass microchannel with a smooth channel (relative roughness around 1%) was used. Additionally, it was noticed that when two phases with different relative viscosity enter the microchannel with the same flow rate, the less viscose phase will occupy a much smaller part of the channel. In order to place the interphase area exactly in the middle of the microchannel, the volumetric flow ratios (biodiesel/solution) were altered in terms of the viscosity ratio of phases and determined to be 0.18 when water was used as a solvent, and 1.53 if a ChCl:Etgl DES was applied as a solvent.

As for ChCl:Gly:H_2O DESs, the volumetric flow ratio also varied depending on the DES viscosity. Values are presented in Table 1.

The parallel flow was observed using a microscope (Motic B1-220A, binocular, Weltzar, Germany) at magnifications of 40× and 100× (eyepiece magnification = 10×; objective magnification = 4× and 10×). The obtained flow profiles are presented in Fig. 4. As can be seen, two distinctive layers are formed and no emulsion formation was noticed.

The flow was completely stable from the entrance to the end of the microchannel for all tested flow rates (Fig. 4c). Additionally, the Reynolds number was also calculated for all flow rates, and all values were below 100, indicating that all experiments were performed in a laminar flow regime [34].

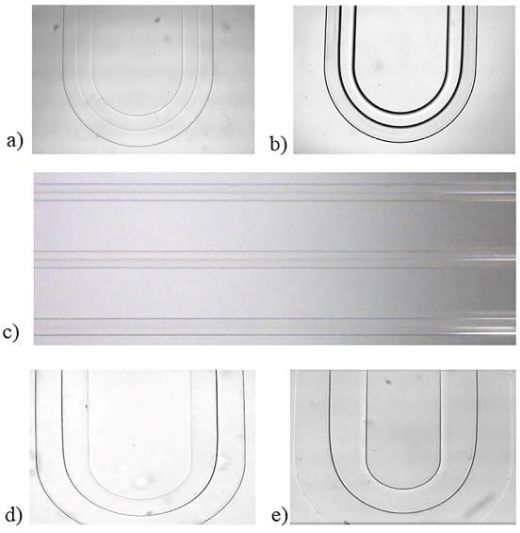

Fig. 4. Microscopic observation of the flow pattern of a biodiesel (BD) and (a) ChCl:Etgl DES at flow ratio BD: ChCl:Etgl = 1:0.64 and (b) water (W) solution at flow ratio BD:W = 1:5.5, (c) stability of flow pattern along microchannel length, (d) ChCl:Gly DES at flow ratio BD:ChCl:$Gly_{1:2.5}$ = 1:0.65 in a 250 mm width microextractor and (e) ChCl:Gly DES at flow ratio BD: ChCl:$Gly_{1:2.5}$ = 1:0.07 in a 500 mm width microextractor.

3.3.1. Biodiesel purification by wet washing

As mentioned in the introduction, the biodiesel wet washing process is the most commonly applied method for biodiesel purification [7], so water was used as a solvent in a microextractor. Separation was performed for different flow rates at the flow ratio biodiesel:water = 1:5.5. The results are presented in Fig. 5a–c.

The separation efficiency was determined to be 75.19% for a residence time of only 8.3 s. This led to insufficient glycerol removal, and at the end of the process, the amount of remaining glycerol was higher than the recommended values according to the American standard ASTM D 6751 [8] and the European standard EN 14214 [9]. The reason why separation efficiency wasn't higher than 75% is probably because the water extraction capacity was too low to perform a complete extraction in one step. In order to estimate how many microextractors are necessary for complete glycerol removal, a mathematical model was developed (Eqs. (1) and (2)). The proposed model was validated on all performed experiments in this paper and a good agreement between the model and the experimental results indicated that the model could be used for process predictions. According to the mathematical model (Fig. 5d), two microextractors have to be connected into a series to lower the free glycerol concentration sufficiently to fulfill the standards, and as many as three extractors are needed to achieve the same results obtained by using DES. In comparison, when the same process was performed in a batch reactor, three washing processes were necessary to reduce the glycerol content in biodiesel. The first two washings include the extraction time and the settling time, which is around 1 h, while in the final washing, the sedimentation time was performed for 3 h to provide the needed separation efficiency. As for the amounts of used water, as already mentioned, according to Karaosmanoğlu et al. [10], in order to purify 1 dm^3 of biodiesel to meet the requirements of the norms, 10 dm^3 of water is necessary. After that, additional purification steps are needed to remove the existing water in biodiesel (usually drying at 110 °C) [35]. If microextractors are used for biodiesel purification the extraction time is reduced and settling time is avoided, since phase separation was possible at the exit of the microchannel, because a stable and parallel flow with the interface placed exactly in the middle of the microextractor channel was formed.

3.3.2. Biodiesel purification by ChCl:Etgl based DES

After the water was used as the solvent for biodiesel purification in a microextractor experiments, the new experiments with unpurified biodiesel and ChCl:Etgl based DES (T = 25 °C, ρ = 1.123 g cm^{-3}, η = 0.036 Pa s) as solvent were performed. Experimental results are shown in Fig. 6a-c. As can be observed, the DES provided a much more efficient transfer over water and almost all glycerol was extracted from the biodiesel. As mentioned, the separation efficiencies were determined to be 75.19% for water in the wet washing process, while 98.35% efficiency was achieved if a DES was used as a solvent. On the other hand, maximum efficiency was achieved for a much higher residence time in comparison with the experiment performed with water as the solvent (τ = 174 s for DES and τ = 8.2 s for water as the solvent).

Comparing the overall process, when glycerol separation was performed within a microextractor using a DES in combination with a separation funnel as the first step, 99.97% of glycerol was removed overall, which corresponds to ASTM D 6751 and EN 14214 standards (Fig. 3). In the second experiment performed in a microextractor and with water as the purification agent, 99.19% of glycerol was removed overall. According to the biodiesel quality standards for purification experiments performed with water, additional steps are needed to achieve the quality standards. Therefore, all further biodiesel purification experiments were performed using a DES as the solvent. Also, by comparing the flow ratios of biodiesel and water and biodiesel and DES, respectively, it can be observed that much smaller amounts of DES (1:0.65) are needed to achieve higher efficiency than in the experiments

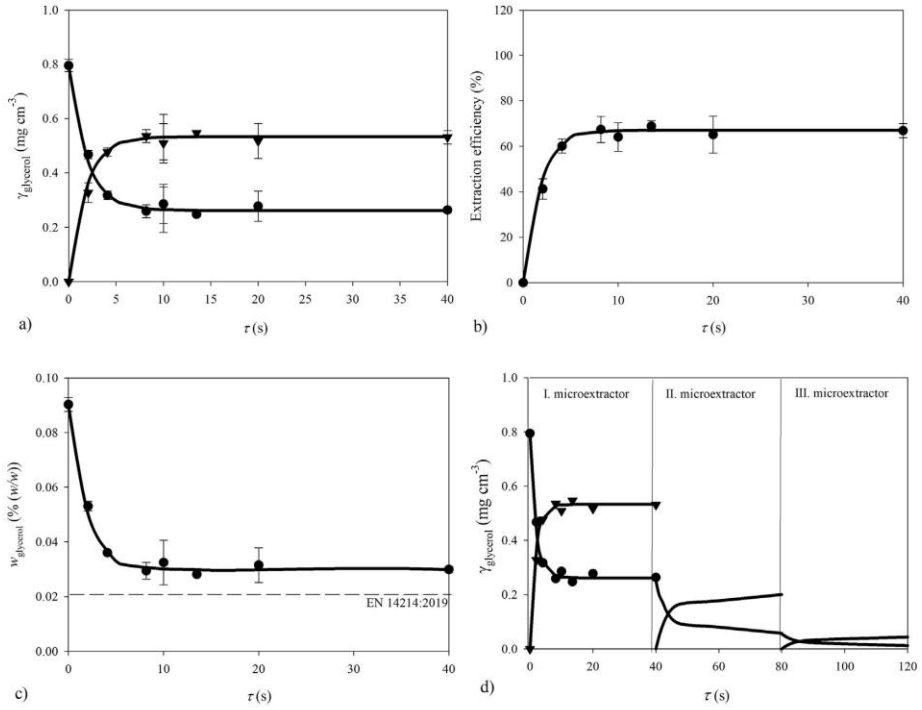

Fig. 5. Influence of the residence time on the extraction of glycerol from the biodiesel using water as a solvent (wet washing): (a) glycerol concentration, (b) extraction efficiency, (c) free glycerol content and (d) extraction of glycerol from the biodiesel by wet washing process in system composed of three microextractor connected into series (▬ mathematical model,● biodiesel phase, ▼ water phase).

where water was used as the solvent (1:55). Another advantage of the use of DESs is their ability to reduce the biodiesel water content to the value below international standards [17]. By this, the additional step in the removal of existing water in biodiesel (usually drying at 110 °C) [35] after purification with water (wet washing), is avoided.

The same results were obtained for biodiesel synthetized from WCO when purified with a ChCl:Etgl based DES. Even the residual concentration of glycerol after the separation funnel was higher than the one obtained after biodiesel was synthetized from edible sunflower oil, efficiency was the same, resulting in purified biodiesel that satisfies the quality standards (Figs. 7 and 8).

3.3.3. Biodiesel purification by ChCl:Gly:H_2O based DES

As mentioned, aside from a ChCl:Etgl based DES, a ChCl:Gly:H_2O based DES was also tested in this research. According to the literature [11,12], a ChCl:Gly:H_2O based DES also has the ability to successfully remove glycerol and other residues (like KOH) from biodiesel. First, the purification of biodiesel was performed by using ChCl:$Gly_{1:2.5}$, and results are presented in Fig. 9. As can be seen, for a residence time of only 13.61 s, glycerol was almost completely removed from the biodiesel. This was a significant improvement in comparison to the ChCl:Etgl based DES, where the same efficiency was obtained after 180 s, and especially in comparison to batch processes [11,12], where the same extraction process takes a few hours (two hours of shaking on an orbital shaker followed by settling to separate the biodiesel from the DES, which can last from two hours to overnight). Also, due to the fact that the phase's flows were set in the way that both phases occupy the same volume in the microextractor channel, and that the interphase area was exactly in the middle of the microchannel, it was possible to separate both phases at the exit of the microchannel. In this way, as for wet

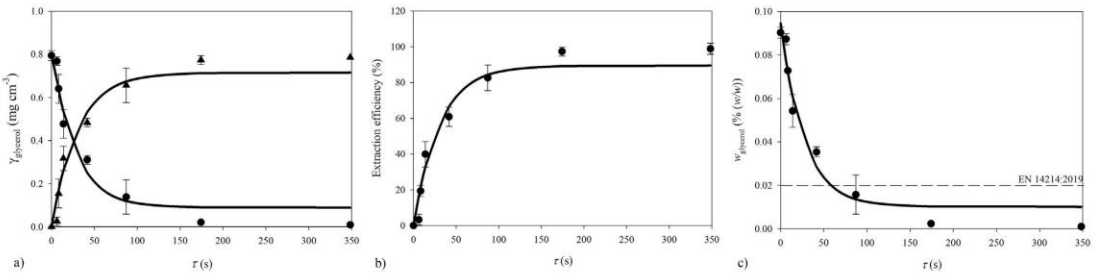

Fig. 6. Influence of the residence time on the extraction of glycerol from the biodiesel using ChCl:Etgl based DES (a) glycerol concentration, (b) extraction efficiency, (c) free glycerol content (▬ mathematical model, ● biodiesel phase, ▼ DES phase).

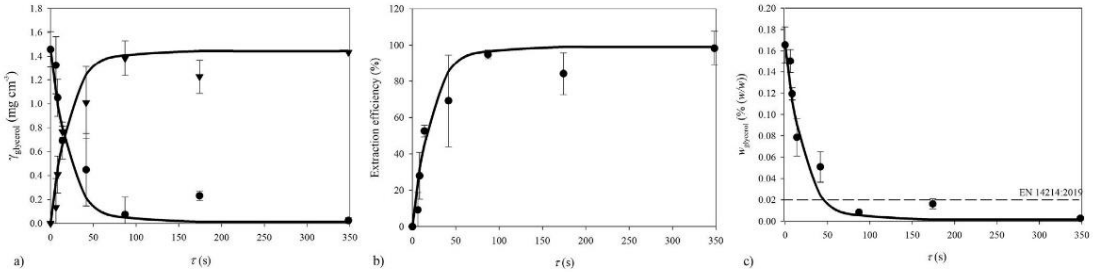

Fig. 7. Influence of the residence time on the extraction of glycerol from the biodiesel synthetized from WCO using ChCl:Etgl based DES (a) glycerol concentration, (b) extraction efficiency, (c) free glycerol content (▬ mathematical model, ● biodiesel phase, ▼ DES phase).

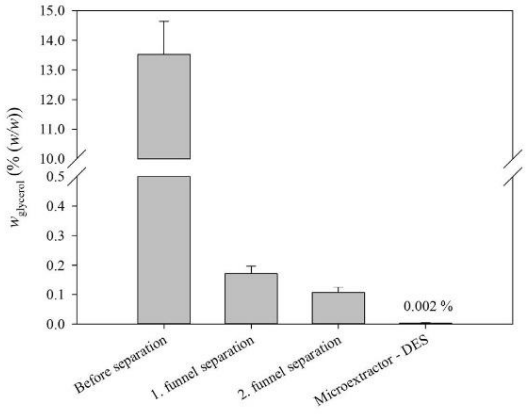

Fig. 8. Free glycerol content in different phases of biodiesel (from WCO) purification.

washing in a microextractor, the settling time that is necessary for phase separation after batch separation is completely avoided. An additional improvement was in the amount of ChCl:Gly (flow ratio biodiesel:ChCl:Gly = 1:0.07) used, compared to the experiment performed with ChCl:Etgl (flow ratio biodiesel:ChCl:Etgl = 1:0.65).

Based on the promising results with a $ChCl:Gly_{1:2.5}$ based DES, six additional choline chloride:glycerol-based DESs (Table 1) were prepared, and their density and viscosity were determined for the temperature of 25 °C and 40 °C. Three of them were formed with different choline chloride and glycerol molar ratio (1:1; 1:2, 1:2.5, and 1:3), while additional three were prepared with the same choline chloride and glycerol molar ratio (1:1), but with the addition of water (0.5, 1, and 2 molar ratio). All prepared DESs were in the form of homogeneous colorless liquids at room temperature, except for $ChCl:Gly:H_2O_{1:1.0:0}$, which appeared solid. Due to that, this DES was excluded from further research. Additionally, in order to place the interphase area exactly in the middle of the microchannel, the volumetric flow ratios (biodiesel/DES) were altered. The obtained results are presented in Fig. 4d–e and Table 1. As can be observed, by increasing the amount of glycerol in the DES the viscosity increases and the volumetric flow ratio decreases. This means that the flow velocity of a DES has to be increased in order to maintain a stable parallel flow with the interface area exactly in the middle of the microchannel. The addition of water in the DES decreased viscosity and led to an increase of volumetric flow ratios (Table 1).

Also, two additional microextractors with different widths (350 and 500 μm) and volumes (5.81 mm^3, and 8.30 mm^3) were tested. As can be seen from the results shown in Table 1, microchannel width does not have an impact on the volumetric flow ratios of phases.

After the extraction was performed in a microchannel with the width of 250 μm, the biodiesel purification process was performed in two additional microextractors (microchannel width 350 μm and microchannel width 500 μm). Results are presented in Table 2. Since diffusion is the controlling mechanism for the extraction process, as can be observed, by increasing the diffusion distance within the channel, the residence time necessary to achieve the same efficiency increases [36]. In order to calculate the theoretical diffusion time (τ_D; Eq. (4)) necessary to complete the glycerol diffusion from biodiesel to DES, diffusion coefficients were estimated using the empirical correlations (Eq. (3)). As can be observed, glycerol can bridge the distance from one phase to the other in only a few seconds.

Comparing the performance of all types of microextractors, even though the extraction was fastest (13.61 s for $ChCl:Gly_{1:2.5.0}$ and 15.81 s for $ChCl:Gly:H_2O_{1:1.0:0.5}$) in the smallest channel diameter (250 μm), further research was conducted in the largest microextractor (500 μm), due to the fact that it has a larger capacity and larger amounts of crude biodiesel could be processed at same time.

All prepared choline chloride:glycerol based DESs were able to remove free glycerol almost completely. According to the literature [12], the molar ratio of a DES is one of the factors that affects the extraction

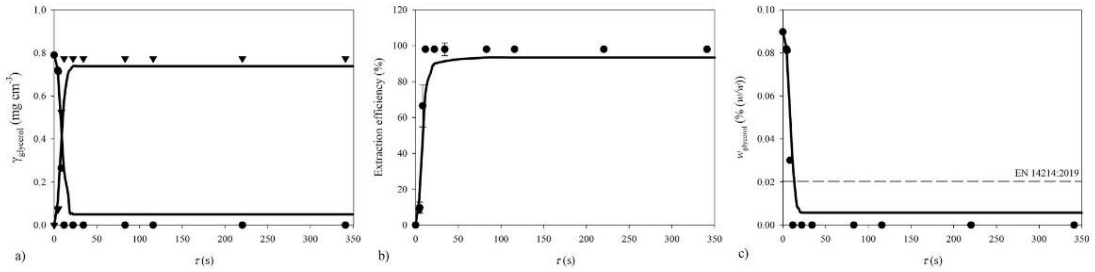

Fig. 9. Influence of the residence time on the extraction of glycerol from the biodiesel synthetized from WCO using $ChCl:Gly_{1:2.5}$ based DES (a) glycerol concentration, (b) extraction efficiency, (c) free glycerol content (▬ mathematical model, ● biodiesel phase, ▼ DES phase).

Table 2
Comparison of extraction efficiency achieved for experiments performed with different DESs and different microextractors.

ChCl: Gly:H_2O	Microextractor width (μm)											
	250				350				500			
	τ (s)	E (%)	D (m^2 s^{-1})	τ_D (s)	τ (s)	E (%)	D (m^2 s^{-1})	τ_D (s)	τ (s)	E (%)	D (m^2 s^{-1})	τ_D (s)
1:2.0:0	–	–	–	–	–	–	–	–	55.03	98.01	$1.813 \cdot 10^{-8}$	3.45
1:2.5:0	13.61	98.37	$1.743 \cdot 10^{-8}$	0.89	21.39	98.28	$1.743 \cdot 10^{-8}$	1.75	49.31	98.18	$1.743 \cdot 10^{-8}$	3.58
1:3.0:0	–	–	–	–	–	–	–	–	44.46	98.40	$1.701 \cdot 10^{-8}$	3.67
1:1:0.5	15.81	98.92	$3.056 \cdot 10^{-8}$	0.51	26.21	98.81	$3.056 \cdot 10^{-8}$	1.01	39.21	98.21	$3.056 \cdot 10^{-8}$	2.05
1:1:1.0	–	–	–	–	–	–	–	–	24.90	98.79	$8.361 \cdot 10^{-8}$	0.75
1:1:2.0	–	–	–	–	–	–	–	–	11.32	98.78	$1.454 \cdot 10^{-7}$	0.43

efficiency. By increasing the mole fraction of ethylene glycol in a DES, efficiency also increases. On the other hand, the increase of the glycerol mole fraction has a negative effect on free glycerol removal. According to Hayyan et al. [11], lower glycerol content in a DES has a greater tendency to attract more glycerol into the DES and to form a higher glycerol ratio DES. By increasing the glycerol content in a DES, this effect decreases. On the other hand, the same authors claim that this is not the only factor that affects the extraction efficiency. The molar ratio of biodiesel and the DES also has a significant effect, so in order to find the optimal DES, both effects have to be monitored. The negative effect of the glycerol mole fraction increase was not expressed that much in the microextractor experiments (Table 2), where the same efficiency was noticed (99%) for almost the same residence time ($\tau \approx 50$ s). According to this, it can be concluded that mass transfer is the main effect in the process.

Also, despite the fact that only free glycerol content was monitored in these experiments, according to the literature [30,37], mono and diglyceride can successfully be removed from crude biodiesel by applying DESs. The reason for this is hydrogen binding between glycerides and the DES. Since free fatty acids, residual methanol, and water also have hydrogen groups, it can be assumed that they will also be removed. The extraction of FAME, the main component of biodiesel, will not occur, since FAME does not have hydroxyl groups, and as a consequence, biodiesel is immiscible with the DES. So, it can be concluded that the application of proposed DESs and extraction systems could lead to an overall increase in the efficiency of the biodiesel purification process.

3.3.4. *Proposed microextractor systems for biodiesel purification*

In the end, two different approaches were proposed for biodiesel purification based on the shown experimental data and mathematical model simulations. The first one is a single microextractor unit with DES recirculation (Fig. 10a). Recirculation was proposed to minimize the waste stream and to justify the application of DESs, since DESs have great affinity and capacity towards glycerol, and since it is possible to separate the DES phase and biodiesel at the end of the microchannel. Once DESs become too saturated by glycerol (and other impurities) they can be recovered and reused. Several methods for successful DES regeneration are mentioned in the literature, such as distillation (especially vacuum distillation) and re-crystallization [11]. According to Hayyan et al. [11], lower glycerol content in a DES has a greater tendency to attract more glycerol into the DES; therefore, the application of a lower glycrol content DES is proposed. This effect was not so expressed in a microchannel. Lower glycerol content in a DES will probably prolong the time necessary for the DES to get saturated by all impurities from biodiesel and prolong the usage/recirculation time of the DES.

The second proposed system is for wet washing, where two or three microextractors (depending on the purity needed) are connected into a series (Fig. 10b). Multiple microextractors for biodiesel purification are necessary, since water has a low capacity towards glycerol and additional fresh water has to be fed into a microextraction system to achieve biodiesel purity in accordance with the standards.

Beside these systems, which were proposed upon the experimental results, another theoretical system can be proposed. When working with immiscible fluids like biodiesel and DES it is possible to disperse one phase in form of droplets or slugs in to another phase. According to Nightingale and deMello [38] the biggest advantage of this flow type is the elimination of velocity dispersion and microchannel fouling is greatly reduced. Microchannel fouling is one of the major problems when working with laminar phase flow. Additional problem is that the size of the interface area is limited in parallel flow microsystems. It depends on microchannel length and depth. In slug flow microsystems, size depends on slug size and it can be significantly increased by reducing the slug size. Increasing the interface area, mass transfer is increased and consequently productivity is increased. Therefore, the third system that could be used is the combination of slug flow microextractor followed by separator that will allow successful phase separation and DES recirculation.

4. Conclusion

Glycerol removal during biodiesel purification by extraction was proved to be very efficient with a ChCl:Etgl based DES and a ChCl:Gly:H_2O based DES as solvents. All investigated DESs have the overall advantage when compared to the biodiesel wet washing process using water. Namely, the higher extraction efficiency and a significantly lower amount of solvents used are the main advantages of the DES application for biodiesel purification. Also, there is less waste to be treated at the end of the process. Additionally, since it is possible to separate the DES and biodiesel phases at the end of the microchannel, the recirculation of the DES is possible, which, in combination with its

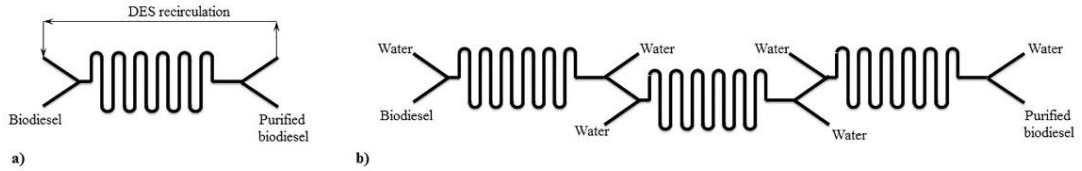

Fig. 10. Scheme of proposed microextraction system for glycerol removal using (a) DES and (b) water.

regeneration, justifies the application of DESs for biodiesel purification

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CRediT authorship contribution statement

Anita Šalić: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization, Writing - review & editing. **Ana Jurinjak Tušek:** Software, Data curation, Writing - review & editing. **Martin Gojun:** Investigation, Writing - review & editing. **Bruno Zelić:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Paper 3

T. Sokač, **M. Gojun**, A. Jurinjak Tušek, A. Šalić, B. Zelić, Purification of biodiesel produced by lipase catalysed transesterification by ultrafiltration: selection of membranes and analysis of membrane blocking mechanisms, Renewable Energy 159 (2020) 642-651, doi:10.1016/j.renene.2020.05.132

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Purification of biodiesel produced by lipase catalysed transesterification by ultrafiltration: Selection of membranes and analysis of membrane blocking mechanisms

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ABSTRACT

The most common reaction for biodiesel production is catalysed by acid, base or enzyme catalysts. When transesterification is completed, the reaction mixture contains various impurities such as soap, catalyst, free glycerol and alcohol that have to be removed to meet the biodiesel international standard specifications. In this work, biodiesel was produced by lipase catalysed transesterification from edible sunflower oil and methanol as substrates. The purification of crude biodiesel was carried out by decantation followed by ultrafiltration membrane technology. Four different membranes, polyethersulfone, polyacrylonitrile, polypropylene and regenerated cellulose were selected and used for biodiesel ultrafiltration. Based on permeate flux and glycerol content in the permeate membrane performance was evaluated. The obtained results showed that two out of four tested membranes have potential for biodiesel purification. Polyacrylonitrile membrane showed the best performance resulting in lowest glycerol content in permeate (0.006% (w/w)). Additionally, polyacrylonitrile membrane was successfully reused six times without significant loss of performance. Furthermore, the membrane blocking mechanisms were analysed for all membranes by Hermina's model. Consequently, complete cake layer formation was identified as the most dominant blocking effect.

1. Introduction

Conventional energy sources like fossil fuel, petroleum, coal and methane are non-renewable sources and rapid industrializations leads to decrease of its reserves [1,2]. Also, their application presents a great ecological burden. Combinations of both negative effects lead to a constant search for a good and sustainable alternative. Less pollution potential and less contribution in global warming are one of the key demands that this new alternate solution should meet. Biodiesel, as an alternative of conventional fuel [3,4] could meet energy and environmental requirements. In the last decade, the interest towards biodiesel significantly increased in comparison to other biofuels. Reason for this are similar physicochemical properties of biodiesel to those of petroleum diesel and possible direct use in vehicles without any engine modification [5,6]. Furthermore, biodiesel is characterised as a non-toxic, renewable and biodegradable fuel [7,8]. Biodiesel combustion has less harmful effects on human health compared to petroleum diesel, with lower emissions of pollutants such as carbon monoxide and fine particular matter and there is no sulphur in its composition [9]. However, biodiesel exhibits some disadvantages such as higher viscosity than conventional diesel, which can lead to problems with fuel pumping, combustion and atomization [10].

The most common method for biodiesel production is the transesterification, a reaction between triglycerides (from vegetable oils and animal fats) and short chain alcohols in the presence of acid or basic catalysts. As a product of the reaction, alkyl esters of fatty acids (biodiesel), glycerol and soap as the by-products, are formed. Other, less widely used methods of biodiesel production are: blending with oils, micro-emulsifications and pyrolysis [11,12].

As mentioned, produced biodiesel contains various impurities such as soap, catalyst, free glycerol and alcohol that must be removed from the resultant biodiesel product to meet international standard specifications [13]. Free glycerol removal is important due to its negative effect on diesel engines and on the quality of biodiesel fuel. Higher amount of soap in biodiesel could damage

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injectors, posing corrosion problem in diesel engines, plugging of filters and weakening of engines so it also needs to be removed [14,15].

After transesterification, the most common separation process for the separation of glycerol from crude biodiesel is a decanter [16]. Since the glycerol, known as free glycerol, is still present in the biodiesel after this step, additional downstream purification processes are needed. They can be generally divided to wet and dry washing processes [17–19]. Both methods are generally accepted and applied at commercial scale. Wet washing is the most frequently used method for biodiesel purification. It is performed by distilled water or acidulated water (aqueous mineral solution) [20]. This method is successful in removing impurities, but the main disadvantage is generation of huge amounts of wastewater that has to be treated before discharging in sewage systems. The amount of wastewater can range from 0.2 to 10 L of water per L of purified biodiesel. Beside environmental inconvenience, wet washing is also known for being energy and time-consuming and economically inefficient [21,22]. The dry washing technique commonly employed to purify crude biodiesel is usually achieved through the use of silicates, ion exchange resins, cellulosic, activated carbon and activated fibre. These adsorbents have acidic and basics adsorption sites and have strongly affinity for polar compounds such as methanol, glycerol, metals and soaps [19,23]. The dry washing procedure is waterless, improves fuel quality and reduces washing time. Thus, problems associated with both conventional biodiesel purification methods have resulted to the exploration and exploitation of membrane technology to separate biodiesel from impurities [24,25].

Membrane technology for biodiesel purification has received attention from the scientific community over the last decade. It has been verified that membrane technology can reduce water consumption during the biodiesel purification step, leading to a significant impact on process costs [26,27]. Membrane technology provides high-quality fuel. It has been reported that other positive points, such as moderate temperature and pressure conditions of membrane separation process, safety, low usage of energy, elimination of waste water treatment, higher mechanical, thermal and chemical stability, have led to widely use of membrane technology for biodiesel production [28].

Basically, membranes are semi-permeable barriers that separate different species of solution by allowing restricted passage of some component of mixture in a selective manner [23]. The most widely used are membrane for microfiltration (MF) and ultrafiltration (UF). MF is a pressure-driven membrane process for the separation of fine particles, microorganisms and emulsion droplets. The membranes used have a microporous structure which separates fine particles in the size range 0.1–10 μm. UF is a separation process using membranes with pore sizes in the range of 1–100 nm. Typically, UF will remove high molecular weight substances, colloidal materials and organic and inorganic molecules. UF processes have several applications such as rejection of viruses, bacteria wastewater reuse and sewage reuse [29].

Although membranes are effective in oil separations, membrane fouling remains one of the most technical challenges. Fouling can be minimized through the choice of suitable membrane materials [29]. Membranes are typically classified into organic membranes (they are made of polymeric material such as polysulfone, polyethersulfone, polyacrylonitrile and cellulose) and inorganic membranes. In organic solvents, polymeric membranes may swell, resulting to instant and/or long-term pore size-changes. Consequently, polymeric membranes in solvent applications have shorter operating lifetimes [11,23]. Inorganic membranes especially ceramic ones are more expensive but they have some advantages such as narrow and well-defined pore size distribution, high thermal and chemical stabilities and resistance to microbial degradation [30].

In this paper, biodiesel was produced by transesterification of edible oil using enzyme lipase from *Thermomyces lanuginosus*. After the production, biodiesel was purified using decanter followed by ultrafiltration membranes. Total of four different membranes were tested and compared: polypropylene, polyethersulfone, polyacrilonitrile and regenerated cellulose mebrane. To get better insight into mechanism leading to flux decline in process of dead-end filtration, Hermina's model (covering four basic types of fouling: complete blocking model, intermediate blocking model, standard blocking model and cake layer model) was used to analyse the predominate fouling mechanism for each membrane.

2. Materials and methods

2.1. Materials

Edible oil (Zvijezda, Croatia) was purchased in a nearby supermarket. The lipase from *Thermomyces lanuginosus* (Lipolase 100 L) was purchased from Sigma-Aldrich Handels GmbH (Austria). Methanol was purchased from Lach:ner (Czech Republic). Acetylaceton and sodium (meta)preiodate were purchased from BDH Prolabo (VWR, United Kingdom). Ammonium acetate, ethanol and glycerol were purchased from Gram-mol (Croatia). Acetic acid was purchased from Kemika (Croatia).

The ultrafiltration module (model number UFSC05001) was purchased from Amicon (USA).

2.2. Methods

2.2.1. Biodiesel production in a batch reactor

Production of biodiesel from edible sunflower oil using the Lipolase 100 L was performed in a laboratory batch reactor (V = 250 mL) at constant stirring at 40 °C (Fig. 1). The reaction mixture was composed of 225 g of sunflower oil, 27.97 g of methanol and 22.5 g of Lipolase 100 L stock solution diluted with 0.01 M phosphate buffer at pH 7.4 in molar ratio 1:10. The reaction started by the addition of the enzyme into the reaction mixture and the process of transesterification was carried out for 48 h [31].

2.2.2. Biodiesel purification in a separation funnel

After the reaction of transesterification, the mixture was transferred into a decanter for 24 h to separate the ester (biodiesel) and glycerol phase. The concentration of glycerol in biodiesel was monitored priori and after separation.

2.2.3. Purification of biodiesel by ultrafiltration membrane

The purification of biodiesel was carried out in a semi-batch module presented in Fig. 1 using membranes (45 mm in diameter). The overpressure in the module was adjusted with nitrogen gas at 4 bars (pressure monitored by manometer). Overall four membranes were tested and compared: polypropylene (PP, 13.4 cm^2, 0.2 μm, Pall, USA), polyethersulfone (PES, 13.4 cm^2, 10 kDa, Millipore, Germany), polyacrilonitrile (PAN, 13.4 cm^2, 0.2 μm, Millipore, Germany) and regenerated cellulose (RC, 13.4 cm^2, 1 kDa, Millipore, Germany). The initial volume of biodiesel in a membrane module was 35 mL in each experiment. The process was carried out until the remaining biodiesel volume in the membrane module was 5 mL. The permeate flux was collected in a 1 mL vial and the filtration time necessary for the mentioned volume to be collected was measured. In selected samples content of free glycerol was measured according to procedure described below. In order to test the reusability of membranes, semi-continuous and discontinuous processes were performed. Both

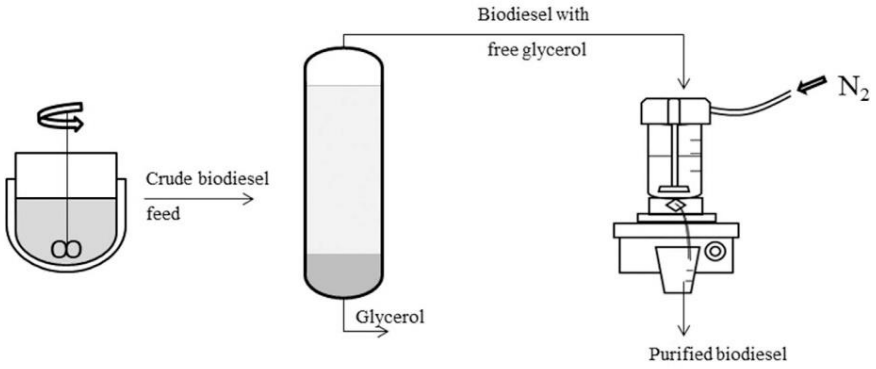

Fig. 1. Schematic diagram of biodiesel production (batch reactor) and purification (decanter and ultrafiltration membrane) process.

processes started by feeding 35 mL of the crude biodiesel in a module. During the discontinuous process, after first filtration cycle ended, membrane was removed from the module, washed with ethanol and reused again. During the semi-continuous process, the amount of 5 mL of biodiesel which remained in a module after first cycle was removed and 35 mL of new crude biodiesel was fed in the system without membrane washing. Cycles were repeated until the permeate flux decreased significantly.

2.2.4. Measurement of fatty acid methyl esters (FAME) in a biodiesel

FAME concentrations were determined according to the method described by Budžaki et al. [31] using a gas chromatograph (Shimadzu GC-2014, Japan) equipped with FID and Zebron ZB-wax GC capillary column (length 30 m, I.D. 0.53 mm and film thickness 1.00 μm, Phenomenex, USA). Carrier gas in this method was nitrogen, at rate of 1.97 mL/min. In the method's total determination time of 15 min, measurement starts at the temperature of 180 °C for 1 min, after which at a rate of 5 °C/min, column was heated up to 230 °C. In order to identify peaks for corresponding esters of fatty acids, standard F.A.M.E. mix GLC-10 was used. Retention times of fatty acids esters are as follows: 7.74 min for palmitic, 10.590 min for stearic, 10.867 min for oleic, 11.575 min for linoleic and 12.615 min for linoleic. To confirm repeatability, all measurements were performed in triplicate. On 95% confidence interval, the results showed no significant difference.

2.2.5. Measurement of glycerol in a biodiesel

Gas chromatography method (for higher glycerol concentrations): Glycerol determination was performed on the same way already described for measurements of FAME concentrations (2.2.4.) while glycerol retention time was 9.02 min. To confirm repeatability, all measurements were performed in triplicate. On 95% confidence interval, the results showed no significant difference.

Spectrophotometric method (for lower glycerol concentrations): Glycerol concentration in a biodiesel was determined by spectrophotometric method described by Bondioli et al. [32]. Briefly, 0.5 g of biodiesel sample was weighed into a 10-mL test tube, dissolved in 2 mL of hexane with addition of ethanol (2 mL). The tube with a sample was homogenised using a Vortex mixer (DLab MX-S, China) for 5 min and centrifuged on a centrifuge (Hettich Zentrifugen, Universal 320R, Germany) for 15 min at 2000 rpm. After that, exactly 0.5 mL of the lower layer was transferred into a new 10-mL test tube. 1.5 mL of working solvent (mix of equal volumes of distilled water and 95% ethanol) was added in to the sample, followed by 1.2 mL of a 10 mM sodium periodate solution. Mixture was homogenised for 30 s on a laboratory mixer. After that, 1.2 mL of a 0.2 M acetylacetone solution was added. Sample was mixed and emerged in to a water bath thermostated at 70 °C for 1 min, stirring manually. After the reaction time, the sample was immediately cooled by immersing the tube in a baker containing tap water for 2 min. After cooling, the sample was centrifuged for 1 min and the samples were read in a spectrophotometer set in a double beam mode at 410 nm. Glycerol concentration was determined from calibration curve ($A_{410\ nm} = 34.953 \cdot \gamma_{glycerol} + 0.1887$) prepared using the same procedure but with the know glycerol concentrations. To confirm repeatability, all measurements were performed in triplicate. On 95% confidence interval, the results showed no significant difference.

2.2.6. Analysis of blocking mechanism

During the process of membrane filtration the reduction of permeate flux was determinate by different mechanisms; the pore blocking, the polarization of the concentration and formation of the precipitation layer [33]. To get better insight into mechanism leading to flux decline in process of dead-end filtration at constant pressure Hermia, 1982 developed fouling model that includes four basic types of fouling: complete blocking model, intermediate blocking model, standard blocking model and cake layer model. Mathematical representation of basic process is given by Eqs. (1) and (2) [33–36].

$$\frac{d^2t}{dV^2} = k \cdot \left(\frac{dt}{dV}\right)^n \quad [1]$$

$$\frac{dV}{dt} = F \quad [2]$$

where t is filtration time, V is permeate volume, k proportionality constant, n constant for different types of fouling and F flux though the membrane; $n = 2$ represents complete blocking model, $n = 1.5$ represents the standard blocking model, $n = 1$ represents the intermediate blocking model and $n = 0$ represents a cake layer model. There have been studies indicating the change of fouling mechanism over the filtration process [33].

According to complete blocking model, every molecule that reaches the membrane surface completely blocks the pore entrance of the membrane. For $n = 2$, linearized form of Eq. (1), is given by Eq. (3) [33–36]:

$$\ln F = \ln F_0 - k_c \cdot t \quad [3]$$

In the case of the standard blocking model for $n = 1.5$, molecule size is smaller than membrane pores and molecules can enter the membrane pore and block the pores. For $n = 1.5$, linearized form of Eq. (1), is given by Eq. (4) [33–36]:

$$\frac{1}{F^{0.5}} = \frac{1}{F_0^{0.5}} + k_s \cdot t \quad [4]$$

Intermediate blocking model ($n = 1$) assumes that not every molecule that arrives on the membrane surface blocks a membrane. It also includes the assumption that some molecules' may deposit on previously settled molecules. For $n = 1$, linearized form of Eq. (1), is given by Eq. (5) [33–36]:

$$\frac{1}{F} = \frac{1}{F_0} + k_i \cdot t \quad [5]$$

The fourth mechanism describes complete cake layer formation on the surface of the membrane due to the fact that molecules have larger diameter that membrane pores. For $n = 0$ linearized form of Eq. (1), is given by Eq. (6) [33–36]:

$$\frac{1}{F^2} = \frac{1}{F_0^2} + k_g \cdot t \quad [6]$$

In all equations F represents the permeate flux, t represents filtration time, F_0 represent flux at $t = 0$ and k_c, k_s, k_i, k_g are model constants.

Model constants were estimated from Eqs. (3)–(6) using simple regression analysis of TRIBCO Statistica™ Trial.

3. Results and discussion

3.1. Biodiesel synthesis in a batch reactor

In order to produce biodiesel, edible sunflower oil and methanol were used as substrates and free enzyme Lipolase 100L as biocatalyst. There are many advantages in using enzymes over chemical catalysts (like KOH and NaOH). Besides mild reaction conditions and lower energy consumption, big advantage is the absence of unwanted soap that is common by-product of chemically catalysed biodiesel production [37]. Additionally, glycerol produced by enzymatic process is characterised as bio- and can be used in food and pharmaceutical industry. Another big advantage is that raw materials used in the enzymatic catalysis can/must contain higher amounts of water (usually between 2 and 20%) since water is necessary for enzyme activation. For chemical processes water content has to be lower than 0.6% (w/w) otherwise it can significantly affect soap formation [38]. Soap affects catalyst, lowers it catalytic performance and makes biodiesel purification more difficult [39,40].

In this investigation the biodiesel yield was around 94% after the transesterification process. The disadvantage of the produced biodiesel was the percentage of glycerol that was around 11%. The amount of obtained glycerol was expected since 1 ton of glycerol is formed for every 9 tons of biodiesel produced [41]. As already mentioned advantage in enzymatically catalysed transesterification was absence of soap.

Since glycerol is insoluble in biodiesel, most of it can be removed by settling. In order to remove majority of glycerol, a decanter was used. At the end of the process, there was around 0.04–0.08% (w/w) glycerol remaining in the biodiesel (free glycerol) that needed to be removed. To do so, membrane technology was used.

3.2. Biodiesel purification in an ultrafiltration module

According to literature, cellulose membranes are considered as the state of the art for low fouling membranes [42]. On the other hand they have significant limitations like low chemical stability and relatively low porosity. The alternative is the application of surface modifications on commercially available membranes. Some of them are polypropylene, polyethersulfones, and poly(vinyldiene fluorine) membranes [43]. In this paper, total of four different membranes: polypropylene, polyethersulfone, polyacrilonitrile and regenerated cellulose were applied for free glycerol removal from biodiesel.

Based on literature data [42], first membrane tested in this research was polyethersulfone membrane. Polyethersulfone membrane was chosen according to research of Alves et al. [16] where several different membranes were compared for glycerol removal (mixed cellulose ester microfiltration membranes of 0.22 and 0.3 μm pore size and polyethersufone membranes of 10 and 30 kDa). Among all tested membranes only 10 kDa polyethersulfone membrane was able to reduce glycerol content below 0.02% (w/w) [16]. Although the obtained data demonstrated that the 10 kDa polyethersulfone membrane can be used as a good alternative for biodiesel purification no additional data about reusability of the membrane were given.

Besides membrane type, several different factors can have significant impact on purification process like temperature, pH, water content and transmembrane pressure. In their work, Alves et al. [16] concluded that the addition of water has a significant effect on glycerol removal process. Reason for this is the formation of complex between glycerol and water resulting in larger molecules that were not able to pass through a membrane. In their paper addition of 0.1 and 0.2% (w/w) of acidified water to crude biodiesel samples before ultrafiltration was investigated. According to obtained results the addition of acidified water at higher concentration improved the glycerol removal. In research performed by Rodriguez et al. [44] addition of 5% (v/v) of water increases the flux four times, 10% (v/v) six times and 20% (v/v) twenty times.

In this research no additional water was added in to the crude biodiesel before the ultrafiltration because water was already present in the crude biodiesel after transesterification process. Namely, water was component of reaction mixture during enzymatically catalysed transesterification while lipase activity enhances the productivity of biodiesel production [45].

Transmembrane pressure (TMP) is another significant parameter that affects the process. It is the main driving force of the ultrafiltration process and by increasing TMP the flux increases [46]. According to the literature [47] in the processes with high glycerol concentrations, the pressure in the range of 2–2.5 bars has the positive effect on the flux and the pressure in the range of 3–4 bar, does not prove positive effect on flux reduction in comparison to 2–2.5 bar. On the other hand, increasing the TMP, a less pronounced flux cut-off is observed [15]. Also, in their paper Alves et al. [16] concluded that TMP in the range 1–4 bars does not have any effect on biodiesel quality. Based on this data, the pressure of 4 bars was chosen as the working pressure in this investigation.

Temperature can also affect filtration process. According to literature, by increasing the temperature, the mobility, of the polymer binding is increasing. This leads to reduction of membrane resistance to fluid [48]. Additionally, by increasing the temperature, viscosity is decreasing. This directly affects the flux by increasing it and also impurities can penetrate deeply in to the membrane. On the other hand, heating is affecting the economic balance of the process making it more expensive. Due to this reason, all ultrafiltrations in this research were performed at 25 °C.

Acidity and/or alkalinity is also important for flux declining and

fouling of membranes due to the nature of fatty acids. In acidic media fatty acids are in undissociated state. This leads to formation of larger agglomerates and faster flux decrease [49]. Based on this it would be expected that alkaline medium would have positive impact on flux increase. On the other hand, in paper of Harivram et al. [47] the fastest flux decrease was observed at neutral pH.

Having all this previously published research in a mind, the first membrane that was tested in this research was polyethersulfone membrane at 4 bars and 25 °C, without water addition in semi-continuous and discontinuous process. As mentioned before, all the processes were carried out until the remaining biodiesel volume in the membrane module was 5 mL. If the volume drops below 5 mL the concentration of glycerol in permeate flux increases. According to Alves et al. [16] reason for this is the accumulation of glycerol particles near the membrane surface. That caused their permeation for longer filtration times that occurred at the end of the process.

Obtained results are presented in Fig. 2. During the discontinuous biodiesel ultrafiltration process (three cycles), different permeate fluxes were generated (Fig. 2a–c). The initial fluxes were slightly different depending on initial free glycerol content in biodiesel (Table 1). As already mentioned, the amount of glycerol in different samples varied from 0.04 to 0.08% (w/w) depending on performed enzymatic biodiesel synthesis. In all ultrafiltration processes the flux decreases slowly in time. It can be explained by formation of a layer on membrane surface and membrane pore blocking which increases the membrane resistance. Also in the last ultrafiltration process, the permeate flux is higher at the beginning of the process and at the end flux is negligible. Observed fluxes indicated that selected membrane could be used for biodiesel ultrafiltration.

Glycerol content in permeate during the first ultrafiltration on polyethersulfone membrane is presented in Fig. 2d. As it can be seen, polyethersulfone membrane was efficient in glycerol removal and in all samples glycerol content was below 0.02% (w/w). The same trend was observed for all further cycles and average glycerol content as well as glycerol content in each cycle are presented in Table 1. As it can be observed, polyethersulfone membrane was able to successfully remove glycerol in all cycles with average efficiency of 83.83%. Unfortunately, in the third cycle flux decline was very significant and flux was low. After that cycle membrane was not used any more.

As mentioned, in discontinuous process, after every cycle membrane was taken out and washed with 70% ethanol. In order to investigate the behaviour of the system without membrane removal, a semi-continuous process was performed. During the process, 5 mL of biodiesel which remained in a module after ultrafiltration cycle was removed and 35 mL of new crude biodiesel was fed in the system. Total of four cycles were performed until the flux drastically declined. The results are showed in Fig. 3a. As it can be observed the flux was higher in comparison to discontinuous process because the amount of glycerol present in the crude biodiesel (Table 1) was 2.3 fold lower than in previous discontinuous experiment.

This process was also efficient for glycerol removal since in all cycles, the glycerol content was below international legislation limit (Table 1, Fig. 3b). If the contents of free glycerol in each individual cycle were compared it can be seen that ultrafiltration efficiency is increasing during ultrafiltration if experiment is performed at the low initial glycerol content. Low glycerol concentration probably resulted in less complex formation between glycerol and water and glycerol molecules could pass through membrane. Prolonging the filtration time, membrane surface becomes more saturated and ultrafiltration efficiency increases. With time membrane will probably become oversaturated and the efficiency will decrease. The same effect was noticed when the first

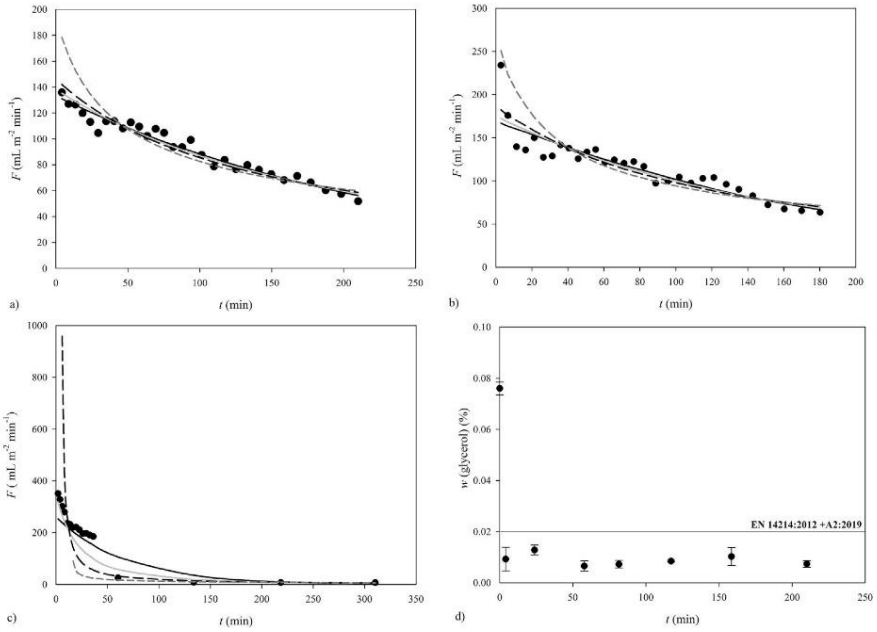

Fig. 2. Permeate flux for biodiesel ultrafiltration using polyethersulfone membrane in three discontinuous process (a–c) and glycerol content in permeate for the first ultrafiltration cycle (a) of discontinuous process (d) (• permeate flux, Hermina's model: — complete blocking, ═ standard blocking, – – – intermediate blocking, - - - complete cake).

Table 1
Glycerol content before and after ultrafiltration and ultrafiltration efficiency for different membranes.

14214:2012 + A2:2019 - free glycerol <0.02% (m/m) [13]				
Membrane type and process D – discontinuous SC - semicontinous	Cycle	The initial free glycerol content (%, w/w)	The content of free glycerol after UF (%, w/w)	Efficiency of UF, (%)
PES-D	1st	0.076 ± 0.003	0.009 ± 0.002	88.35 ± 2.11
	2nd	–	–	–
	3rd	0.032 ± 0.002	0.008 ± 0.001	74.63 ± 1.42
	average	*0.054 ± 0.020*	*0.009 ± 0.001*	*83.83 ± 1.75*
PES-SC	1st	0.032 ± 0.002	0.013 ± 0.002	57.57 ± 2.67
	2nd	0.032 ± 0.002	0.013 ± 0.003	60.92 ± 5.77
	3rd	0.032 ± 0.002	0.008 ± 0.001	75.06 ± 1.34
	4th	0.032 ± 0.002	0.014 ± 0.002	78.08 ± 2.57
	average	*0.032 ± 0.002*	*0.012 ± 0.001*	*65.27 ± 3.18*
PAN-D	1st	0.076 ± 0.003	0.007 ± 0.008	91.03 ± 0.63
	2nd	0.076 ± 0.003	0.005 ± 0.001	91.81 ± 1.91
	3rd	0.064 ± 0.001	0.011 ± 0.001	82.27 ± 1.27
	4th	0.064 ± 0.001	0.003 ± 0.001	95.24 ± 1.26
	5th	0.064 ± 0.001	0.005 ± 0.001	92.37 ± 1.21
	6th	0.064 ± 0.001	0.004 ± 0.001	93.29 ± 1.65
	average	*0.068 ± 0.002*	*0.006 ± 0.001*	*91.48 ± 1.32*
RC-D	1st	0.064 ± 0.001	0.010 ± 0.001	84.38 ± 1.89
	2nd	0.064 ± 0.001	0.012 ± 0.001	81.99 ± 2.10
	average	*0.064 ± 0.001*	*0.011 ± 0.005*	*83.18 ± 1.46*
PP	1st	0.076 ± 0.003	–	–

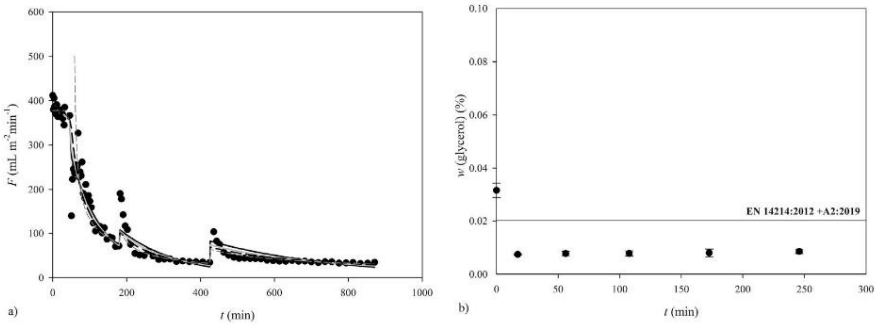

Fig. 3. Permeate flux (a) and glycerol content in permeate for first cycle (b) for semi-continuous biodiesel ultrafiltration using polyethersulfone membrane (• permeate flux, Hermina's model: ▪ complete blocking, ▪ standard blocking, – – – intermediate blocking, - - - complete cake).

cycles of semi-continuous and discontinuous ultrafiltrations were permed and compared. In the first cycle of discontinuous process the initial amount of glycerol in crude biodiesel was 0.076% (w/w) (Table 1). At the end of the cycle efficiency of glycerol removal was 88.35%. When the same, completely new membrane was used in the first cycle of semi-continuous process, under same conditions but with less free glycerol in crude biodiesel (0.036% (w/w)) the efficiency was 57.57%.

Overall, comparing the performance of discontinuous and semi-continuous ultrafiltration processes, based on UF efficiency it was concluded that both processes have same efficiency.

Besides polyethersulfone membrane, polyacrilonitrile membrane was successfully used for biodiesel purification. Saleh et al. [50] demonstrated application of polyacrilonitrile membrane in reducing glycerol content in biodiesel obtained from canola oil. According to Bansod and Rathod [51] glycerol was successfully separated by using 6 kDa and 15 kDa polyacrilonitrile membranes. 6 kDa membrane was slightly more successful leading to 0.017 mass % of glycerol after ultrafiltration in comparison to 0.02% of glycerol for 15 kDa polyacrilonitrile membrane.

In the experiments performed in this research, polyacrilonitrile membrane was tested in six repetitive discontinuous processes. Results are presented in Fig. 4. As it can be observed, there is a significant flux decrease for the first few minutes of each cycle. After that fluxes were similar to those obtained for polyacrilonitrile membrane.

On the other hand, comparing the glycerol content in permeate (Table 1) it can be seen that polyacrilonitrile membrane is more efficient for glycerol removal in comparison to polyethersulfone membrane. In each cycle UF efficiency was high and similar, leading to average efficiency of 91.48%.

Except polyethersulfone and polyacrilonitrile membranes two additional membranes were tested. First one was regenerated cellulose membrane while the second one was polypropylene membrane. Regenerated cellulose membrane was chosen while according to the literature it is state of the art for low fouling membranes [42]. On the other hand cellulose membranes have significant limitations like low chemical stability and relatively low porosity. The alternative is the application of surface modifications on commercially available membrane. Some of them are already tested polyacrilonitrile and polyethersulfone membranes. Also, polypropylene membrane can be used as well [43].

Results obtained for regenerated cellulose membrane are presented in Fig. 5a and b. As it can be seen significant flux decline was

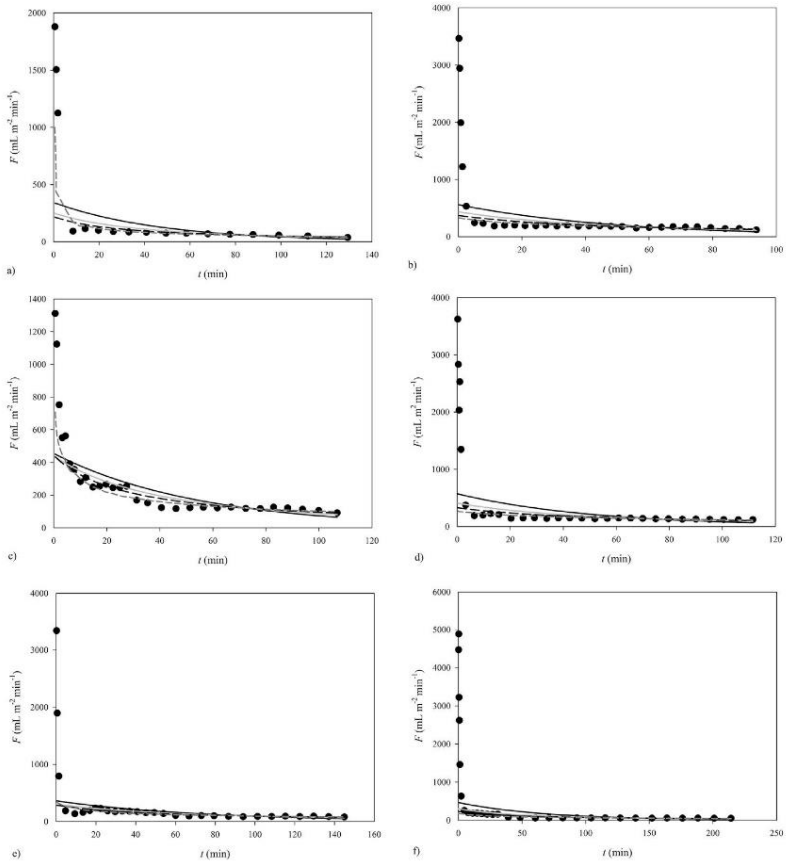

Fig. 4. Permeate flux for biodiesel ultrafiltration using polyacrylonitrile membrane in six (a–f) semi-continuous process (• permeate flux, Hermina's model: — complete blocking, ═ standard blocking, – – – intermediate blocking, - - - complete cake).

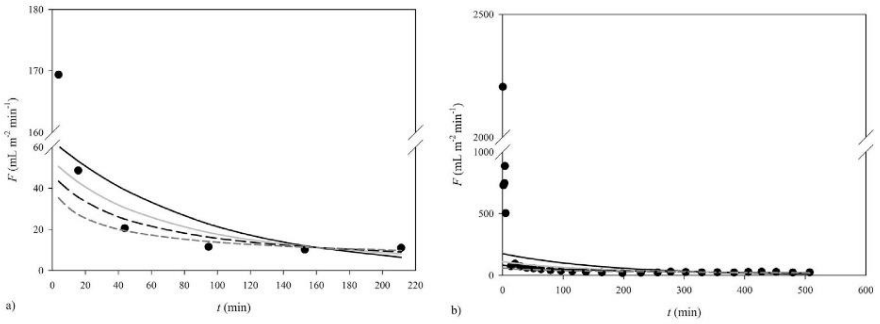

Fig. 5. Permeate flux for biodiesel ultrafiltration performed with regenerated cellulose membrane in two discontinuous processes (• permeate flux, Hermina's model: — complete blocking, ═ standard blocking, – – – intermediate blocking, - - - complete cake).

observed in both cycles immediately after the beginning of the process and only two cycles were performed. As mentioned this was due to the fact that cellulose membranes have low porosity.

If the glycerol content and UF efficiency are compared regenerated cellulose membrane is in the range of polyethersulfone membrane.

The final membrane that was tested was polypropylene membrane. When assembling the apparatus it was noted that the polypropylene membrane failed to hold crude biodiesel even without nitrogen. Due to this, it was concluded that this membrane,

Table 2
Model constants k_c, k_s, k_i and k_g and flux at $t = 0$ for different types of fouling in different UF processes.

Membrane type and process (D – discontinuous SC – semicontinous)	Filtration cycle	Complete blocking $n = 2$			Standard blocking $n = 1.5$			Intermediate blocking $n = 1$			Complete cake layer $n = 0$		
		k_c (min)	F_0 (mL m^{-2} min^{-1})	R^2	$k_s \cdot 10^3$ (min)	F_0 (mL m^{-2} min^{-1})	R^2	$k_i \cdot 10^5$ (min)	F_0 (mL m^{-2} min^{-1})	R^2	$k_g \cdot 10^6$ (min)	F_0 (mL m^{-2} min^{-1})	R^2
PES-D	1st	0.004	131.920	0.965	0.200	138.408	0.956	4.900	146.199	0.939	1.000	196.116	0.881
	2nd	0.004	170.068	0.882	61.500	176.833	0.894	4.300	188.679	0.885	1.000	288.675	0.826
	3rd	0.015	260.525	0.823	90.500	349.375	0.877	60.000	454.545	0.908	0.800	28.808	0.935
PES-SC	1st	0.002	475.516	0.387	0.053	480.917	0.385	0.500	490.196	0.383	1.000	500.000	0.378
	2nd	0.011	1268.899	0.832	0.500	21003.990	0.856	8.000	118.343	0.865	1.000	71.612	0.836
	3rd	0.006	579.868	0.716	0.400	297.265	0.789	9.700	59.523	0.850	4.000	33.539	0.928
	4th	0.003	82.545	0.665	0.200	74.316	0.765	4.500	69.444	0.836	2.000	64.550	0.916
PAN-D	1st	0.021	342.236	0.507	0.800	250.361	0.691	20.000	218.818	0.839	4.000	10000.000	0.825
	2nd	0.020	559.196	0.431	0.500	435.842	0.549	5.600	371.747	0.663	1.000	333.333	0.786
	3rd	0.018	454.456	0.704	0.600	439.504	0.811	8.300	434.783	0.871	1.000	707.107	0.899
	4th	0.020	571.126	0.443	0.500	409.942	0.549	5.900	327.761	0.652	1.000	267.261	0.799
	5th	0.014	361.405	0.476	0.500	306.388	0.682	7.000	294.117	0.810	1.000	333.333	0.879
	6th	0.015	464.193	0.494	0.500	301.408	0.681	9.300	232.558	0.785	1.000	179.605	0.862
RC-SC	1st	0.011	63.023	0.636	1.000	53.513	0.735	40.000	46.685	0.791	0.470	40.0000	0.817
	2nd	0.006	175.178	0.467	0.300	111.037	0.588	7.700	81.967	0.640	1.000	58.028	0.594

without any modifications, was not suitable for biodiesel purification.

3.3. Membrane blocking mechanism

When working with membranes, fouling, the deposition of substances on the membrane surface is one of the most serious problems. It leads to deterioration of all membranes and presents a great economic burden to the processes that use membranes. Besides pore blocking, there are two additional processes that can lead to the reduction of permeate flux, the polarization of the concentration and formation of the precipitation layer [32]. To get better insight into mechanism leading to flux decline in process of dead-end filtration at constant pressure Hermia's model [33] was used. It includes four basic types of fouling: complete blocking model, intermediate blocking model, standard blocking model and cake layer model (Eqs. (3)–(6)).

Estimated model constants (k_c, k_s, k_i and k_g) and flux at $t = 0$ min together with correlation coefficients (R^2) are given in Table 2 (additional statistical data are given in Supplementary material). The predominate fouling mechanism for each membrane was analysed by fitting the complete blocking model, intermediate blocking model, standard blocking model and cake layer model to experimental data and based on the obtained R^2, R^2_{adj} and F-vales (Supplementary material) for each individual model.

By analysing the results presented in Table 2 it can be noticed that dominant fouling mechanism changes over the filtration cycles. In case of filtration using polyethersulfone membrane in discontinuous mode, after first filtration cycle complete blocking appears to be dominant fouling mechanism based on the obtained R^2, R^2_{adj} and F-vales ($R^2 = 0.965$, $R^2_{adj} = 0.964$, $F_0 = 776.365$ mL m^{-2} min^{-1}). After second filtration cycle standard blocking was the dominant mechanism ($R^2 = 0.894$, $R^2_{adj} = 0.890$, $F_0 = 236.456$ mL m^{-2} min^{-1}) while after third filtration cycle complete cake layer was the dominant mechanism ($R^2 = 0.935$, $R^2_{adj} = 0.931$, $F_0 = 226.494$ mL m^{-2} min^{-1}). The same effect (change in the dominant fouling mechanism form complete blocking ($R^2 = 0.387$, $R^2_{adj} = 0.336$, $F_0 = 7.572$ mL m^{-2} min^{-1}) over intermediate blocking ($R^2 = 0.865$, $R^2_{adj} = 0.860$, $F_0 = 178.715$ mL m^{-2} min^{-1}) to complete cake layer ($R^2 = 0.929$, $R^2_{adj} = 0.928$, $F_0 = 233.283$ mL m^{-2} min^{-1}) during time was noticed when working with polyethersulfone membrane in semi-continuous mode. When working with polyacrilonitrile membrane in discontinuous mode it can be seen that intermediate blocking was dominant mechanism after first filtration cycle and complete cake layer for following five filtration cycles. For filtration with regenerated cellulose membrane in discontinuous mode complete cake layer was the dominant fouling mechanism. This can be probably explained with the composition of produced biodiesel that still contains fatty acids. It was composed of palmitic (C16:0) 6.73%, stearic (C18:0) 2.95%, oleic (C18:1) 32.97% and linoleic (C18:2) 57.34% acids. Since C18 acids have long carbon chains they cannot penetrate in to the membrane pores but they deposit on the membrane surface was expected [48]. Also different fouling mechanisms can take place during the same ultrafiltration run and this can be the reason for the deviation between experimental and predicted data for certain operating conditions.

4. Conclusion

Four different ultrafiltration membranes, polypropylene (PP), polyethersulfone (PES), polyacrilonitrile (PAN) and regenerated cellulose (RC) were used for glycerol removal from biodiesel produced by lipase catalysed transesterification. Membranes were evaluated based on permeate flux and glycerol content in the permeate. Based on the obtained results it was shown that the polyacrilonitrile membrane is most efficient for glycerol removal. It was efficiently reused for 6 times and in every cycle of ultrafiltration efficiency was around 91.48% with average free glycerol content in permeate of 0.006% (w/w). Hermia's model was used for the analysis of the blocking mechanism. When working with polyacrilonitrile membrane in discontinuous mode intermediate blocking was dominant mechanism after first filtration cycle while complete cake layer was dominant mechanism for following five filtration cycles.

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CRediT authorship contribution statement

Tea Sokač: Investigation, Visualization, Writing - original draft. **Martin Gojun:** Investigation. **Ana Jurinjak Tušek:** Software, Data curation, Writing - review & editing. **Anita Šalić:** Conceptualization, Methodology, Visualization, Writing - review & editing. **Bruno Zelić:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2020.05.132.

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Paper 4

M. Bačić, A. Ljubić, **M. Gojun**, A. Šalić, A. Jurinjak Tušek, B. Zelić, Continuous Integrated Process of Biodiesel Production and Purification - the end of the Conventional Two-stage Batch Process? Energies 14 (2021) 403, doi:10.3390/en14020403

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Article

Continuous Integrated Process of Biodiesel Production and Purification—The End of the Conventional Two-Stage Batch Process?

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Abstract: In this research, optimization of the integrated biodiesel production process composed of transesterification of edible sunflower oil, catalyzed by commercial lipase, with simultaneous extraction of glycerol from the reaction mixture was performed. Deep eutectic solvents (DESs) were used in this integrated process as the reaction and extraction media. For two systems, choline chloride:glycerol (ChCl:Gly) and choline chloride:ethylene glycol (ChCl:EG), respectively, the optimal water content, mass ratio of the phase containing the mixture of reactants (oil and methanol) with an enzyme and a DES phase (mass ratio of phases), and the molar ratio of deep eutectic solvent constituents were determined using response surface methodology (RSM). Experiments performed with ChCl:Gly resulted in a higher biodiesel yield and higher glycerol extraction efficiency, namely, a mass ratio of phases of 1:1, a mass fraction of water of 6.6%, and a molar ratio of the ChCl:Gly of 1:3.5 were determined to be the optimal process conditions. When the reaction was performed in a batch reactor under the optimal conditions, the process resulted in a 43.54 ± 0.2% yield and 99.54 ± 0.19% glycerol extraction efficiency (t = 2 h). Unfortunately, the free glycerol content was higher than the one defined by international standards (w_G > 0.02%); therefore, the process was performed in a microsystem to enhance the mass transfer. Gaining the same yield and free glycerol content below the standards (w_G = 0.0019 ± 0.003%), the microsystem proved to be a good direction for future process optimization.

Keywords: biodiesel; lipase; deep eutectic solvent; purification; process optimization

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1. Introduction

In order to reduce the excessive depletion of fossil fuel stocks and its negative impact on the environment, environmentally friendly alternative fuels are increasingly being explored [1]. Biodiesel, a mixture of fatty acid methyl esters, stands as a suitable replacement for fossil diesel due to its reduced greenhouse gas and impurity emissions compared to fossil diesel combustion [2–5]. The use of biodiesel in a mixture with fossil diesels such as B5, B7, and B20 blends has become common in Europe [6]. Therefore, the production of significant quantities of biodiesel for the lowest possible price is required for its competitiveness on the market [7]. Nowadays, the most common industrial process for biodiesel production is the transesterification of vegetable oils with methanol, carried out in a batch reactor with an alkaline catalyst. Besides the fact that several hours are needed to achieve a sufficient yield in the batch production process, synthetized biodiesel requires additional expensive purification methods to become suitable for application in internal combustion engines [1,8].

Other approaches in biodiesel production include heterogeneous transesterification [9,10] and transesterification under supercritical conditions [11–13], the dilution/

blending process [14], microemulsification [15–17], and pyrolysis/cracking [14]. Regardless of the production method, biodiesel must meet some quality standards related to its physical and chemical characteristics. According to the standard EN 14214:2012+A2:2019, the ester content in the biodiesel must be higher than 96.5% and the glycerol content must be lower than 0.02% [18]. Since there is an increasing trend towards continuous production instead of batch production, microreactors could be applied for that purpose [19]. Microreactors are commonly tubular reactors in which the efficiency of the reactions can be significantly increased due to their small dimensions (large surface-to-volume ratio). In that way, mass and energy transfer becomes more intense, making the reaction rates much higher in comparison to traditional batch reactors [20]. Furthermore, significant progress can be achieved by using enzymes as transesterification catalysts due to their selectivity, biodegradability, and high activity in a wide range of conditions. In comparison to chemical catalysts, the purity of the product is higher when enzymes are used as catalysts due to the lower amount of by-products formed during the reaction. Furthermore, energy consumption is lower due to the mild reaction conditions that are required during the process [21]. Furthermore, lipase from *Thermomyces lanuginosus*, an enzyme suitable for transesterification, has commercial significance due to its low price, which makes it affordable compared to other enzymes [22]. All these enzyme-related factors, together with advantages provided by microreactors, could give an environmentally friendly and economically justified biodiesel production process [8]. Production of biodiesel on a microscale is already an established practice [23], but it is mostly oriented towards the use of chemical catalysts [24–27]. Different types of microreactors such as microtube reactors, micro-structured reactors, membrane microreactors, etc., can be used for biodiesel production [1].

The next step in overcoming obstacles in biodiesel production is replacing the most common industrial purification method of wet washing, which generates up to 10 L of wastewater per 1 L of produced biodiesel [1,8]. Taking into consideration that 60–80% of the production costs belongs to the purification steps, it can be concluded that a cheaper and more efficient solution needs to be found [28]. Lately, deep eutectic solvents (DESs) are attracting more and more attention as a potential solution for biodiesel purification [29]. A DES is a binary or ternary mixture consisting of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), which forms a hydrogen bond [30]. Their advantages include cheap constitutive components such as choline chloride as an HBA representative and naturally derived HBDs such as various sugars, alcohols, and amides [31]. The production of DESs is very simple and cheap. It is possible to easily adjust their properties by changing the constitutive components of DESs and their ratios [32]. Furthermore, DESs are especially attractive because of their stability, biodegradability, non-toxicity, and low volatility, thus fitting into the concept of green chemistry as green solvents [31].

DESs composed of choline chloride as the HBA and ethylene-glycol or glycerol as HBDs are most commonly used for biodiesel purification. These DESs effectively remove not only glycerol, which is the most challenging impurity in biodiesel, but also other unwanted compounds such as unreacted products and catalysts [33]. Furthermore, DESs are increasingly being investigated in the context of their use as reaction media, especially in biocatalysis, because some studies have shown that lipases have high activity and stability in DESs in the presence of a certain percentage of water [3,31,34]. Since the simultaneous use of DESs as reaction and extraction media is still unexplored, it is necessary to optimize such an integrated process [6]. An optimization method that is very popular among researchers is response surface methodology (RSM) [35]. RSM is based on determining the functional dependence of the process response on independent variables that affect the response by mathematical and statistical techniques. In addition, based on the obtained experimental data, a mathematical model of the process is generated, which is used for numerical optimization of the process [36,37].

In this paper, the optimization of the transesterification process with integrated purification—glycerol extraction from produced biodiesel—was performed. Edible sun-

flower oil and methanol were used as substrates and the commercial enzyme lipase from *Thermomyces lanuginosus* was used as a catalyst in transesterification, while a DES was used as the reaction medium and as an extraction medium for biodiesel purification. Two DESs were used for integrated production and purification of biodiesel: choline chloride with glycerol (ChCl:Gly) as an HBD and choline chloride with ethylene glycol (ChCl:EG) as an HBD. RSM was applied to optimize the process conditions where biodiesel yield was an objective function. For the two systems that differed in the DES used, the optimal values of the mass ratio of the phase containing a mixture of reactants (oil and methanol) with an enzyme and a DES phase (mass ratio of phases), water content, as well as the molar ratio of the DES constituents were determined by numerical optimization. Finally, the integrated biodiesel production and purification process was carried out under optimal conditions in a batch reactor and in a microreactor.

2. Materials and Methods

2.1. Materials

Chemicals

The following chemicals were used to perform the experiment. Acetylacetone, acetonitrile, *n*-hexane, *n*-heptane, hydrochloric acid, methanol, sodium periodate, and tris (hydroxymethyl)aminometal (TRIS) were produced by VWR Chemicals, BDH Prolabo (Lutterworth, United Kingdom). Edible sunflower oil (Zvijezda, Zagreb, Croatia) was purchased at a local store. Enzyme lipase from *Thermomyces lanuginosus* (Lipolase 100 L) and a fatty acid methyl ester (FAME) mix GLC-10 were purchased from Sigma-Aldrich Handels GmBH (Vienna, Austria). Glycerol was purchased from Kemika (Zagreb, Croatia) and ethanol from Gram mol d.o.o. (Zagreb, Croatia). Choline chloride and 4-nitrophenyl acetate were purchased from Acros Organics (Geel, Belgium), the ethylene glycol was purchased from Lach-Ner (Prague, Czech Republic), and acetic acid from Carlo Erba Reagents (Sabadell, Spain). With the exception of sunflower oil, chemicals and reagents were of analytical grade and were used without any further purification.

2.2. Methods

2.2.1. Preparation of Deep Eutectic Solvents (DESs)

Choline chloride (ChCl) and glycerol (Gly), and choline chloride and ethylene glycol (EG) in different molar ratios were used to prepare anhydrous ChCl:Gly and ChCl:EG DESs, respectively (Table 1). After weighing each component according to the desired molar ratio, the components were placed in a Schott bottle (V = 50 mL) and mixed on a magnetic stirrer (MS-H-S, DLAB, Ontario, CA, USA) at 200 rpm and 50 °C. The process was carried out for 30–60 min until a homogeneous, colorless, transparent liquid was obtained. DESs were then cooled to 25 °C.

Table 1. The molar ratios of choline chloride:glycerol (ChCl:Gly) and choline chloride:ethylene glycol (ChCl:EG) used for the preparation of deep eutectic solvents (DESs).

Molar Ratio	Prepared DES	
1:2	$ChCl:Gly_{1:2}$	$ChCl:EG_{1:2}$
1:3	$ChCl:Gly_{1:3}$	$ChCl:EG_{1:3}$
1:3.5	$ChCl:Gly_{1:3.5}$	–
1:4	$ChCl:Gly_{1:4}$	$ChCl:EG_{1:4}$

2.2.2. Optimization of Integrated Biodiesel Production and Purification Processes

In order to optimize the integrated process composed of simultaneous biodiesel production (by transesterification) and purification (glycerol extraction), experiments were carried out according to the experimental plan (Table 2), changing the mass ratio of a reaction phase (oil, methanol, enzyme, and water) and the DES phase, water content, and molar ratios of DES constituents (Table 2). The required masses of oil and methanol were weighed into conical centrifuge tubes (V = 15 mL), with their molar ratio always being 1:3.4 [38].

The content of the centrifuge tubes was well homogenized on a homogenizer (Vibromix 10, Tehtnica, Železniki, Slovenia) and the appropriate amount of DESs was added into the centrifuge tube. The content of the centrifuge tubes was well homogenized, followed by the addition of water and lipase according to the experimental plan (Table 2). The initial concentration of lipase was equal in all performed experiments ($\gamma_{E,0}$ = 0.1 mg mL^{-1}). After homogenization, the centrifuge tubes were attached to a rack and placed in a shaker (Innova 4330 Refrigerated Incubator Shaker, New Brunswick Scientific, Enfield, CT, USA) for 2 h at 400 rpm and at a temperature of 40 °C. After incubation, the centrifuge tubes were removed and immediately centrifuged (Universal 320 R, Hettich, Buford, GA, USA) for 10 min at 4000 rpm and at 40 °C. As a result, two clearly visible layers were formed. Layer volumes were measured and the top layer containing mainly biodiesel was centrifuged again for 10 min at 14,000 rpm and at 40 °C in order to completely precipitate the DES that may have remained during the first centrifugation. Biodiesel samples obtained in this manner were analyzed by gas chromatography and on a spectrophotometer. Based on the measured concentrations of biodiesel and glycerol in the samples, the yield of biodiesel (Y), the extraction efficiency of glycerol (η), and the mass fraction of glycerol in biodiesel (w_G) were calculated.

Table 2. Experimental design and analyzed responses for investigated DESs.

Experimental Design				Analyzed Responses					
Run	w_{water}, % Mass	ChCl:Gly or ChCl:EG (1:X)	Mass Ratio of Phases	ChCl:Gly			ChCl:EG		
				Y, %	η, %	w_G, %	Y, %	η, %	w_G, %
1	0.6 (−1)	2.0 (−1)	1:1 (0)	6.96 ± 0.21	86.36 ± 0.40	0.129 ± 0.01	1.15 ± 0.11	58.65 ± 2.84	0.065 ± 0.02
2	8.6 (1)	2.0 (−1)	1:1 (0)	40.82 ± 0.66	86.92 ± 0.15	5.629 ± 1.02	12.15 ± 0.64	33.52 ± 2.54	1.098 ± 0.30
3	0.6 (−1)	4.0 (1)	1:1 (0)	14.31 ± 0.35	89.97 ± 0.18	0.195 ± 0.22	1.18 ± 0.07	40.66 ± 2.69	0.095 ± 0.04
4	8.6 (1)	4.0 (1)	1:1 (0)	44.55 ± 1.16	91.63 ± 0.16	0.507 ± 0.07	13.05 ± 0.40	95.93 ± 0.09	0.072 ± 0.03
5	0.6 (−1)	3.0 (0)	1:9 (1)	21.12 ± 0.15	98.54 ± 0.01	0.042 ± 0.02	0.35 ± 0.08	55.39 ± 0.14	0.021 ± 0.01
6	8.6 (1)	3.0 (0)	1:9 (1)	30.37 ± 0.23	54.13 ± 0.26	1.894 ± 0.36	4.04 ± 0.19	64.47 ± 6.05	0.195 ± 0.09
7	0.6 (−1)	3.0 (0)	9:1 (−1)	3.82 ± 0.54	81.06 ± 1.95	0.098 ± 0.02	1.90 ± 0.21	67.97 ± 0.01	0.083 ± 0.05
8	8.6 (1)	3.0 (0)	9:1 (−1)	33.40 ± 0.27	98.82 ± 0.01	0.054 ± 0.01	30.38 ± 0.27	95.04 ± 0.09	0.205 ± 0.10
9	4.6 (0)	2.0 (−1)	1:9 (1)	33.42 ± 1.36	99.29 ± 0.02	0.032 ± 0.05	3.55 ± 0.32	57.37 ± 1.23	0.206 ± 0.07
10	4.6 (0)	4.0 (1)	1:9 (1)	29.40 ± 0.52	99.02 ± 0.01	0.039 ± 0.01	3.56 ± 0.98	92.15 ± 0.06	0.038 ± 0.01
11	4.6 (0)	2.0 (−1)	9:1 (−1)	25.18 ± 0.32	99.80 ± 0.02	0.007 ± 0.01	9.87 ± 0.26	55.19 ± 7.68	0.601 ± 0.15
12	4.6 (0)	4.0 (1)	9:1 (−1)	28.80 ± 0.07	98.73 ± 0.00	0.050 ± 0.03	13.92 ± 2.16	96.03 ± 0.29	0.075 ± 0.01
13	4.6 (0)	3.0 (0)	1:1 (0)	45.41 ± 1.29	94.47 ± 0.74	0.341 ± 0.27	9.02 ± 0.23	41.68 ± 0.58	0.715 ± 0.43
14	4.6 (0)	3.0 (0)	1:1 (0)	40.59 ± 0.13	87.56 ± 0.29	0.687 ± 0.14	4.79 ± 0.55	39.13 ± 3.39	0.396 ± 0.21
15	4.6 (0)	3.0 (0)	1:1 (0)	45.11 ± 2.16	91.94 ± 0.28	0.495 ± 0.21	7.00 ± 0.35	89.65 ± 4.13	0.099 ± 0.02

2.2.3. Integrated Biodiesel Production and Purification in a Batch Reactor

The integrated biodiesel production and purification process was carried out in a laboratory double wall batch reactor (V = 10 mL, t = 2 h, 400 rpm, and T = 40 °C; Figure 1). Three different compositions of reaction media were used. In Experiment I, the synthesis of biodiesel was performed in an aqueous medium (0.01 mol L^{-1} phosphate buffer, pH 7.4, water content of 4.6% w/w) without DES addition. Experiment II was performed with DES addition at initial conditions used as the central point of the experimental plan (mass ratio of phases 1:1, mass fraction of water of 4.6%, and molar ratio of DES constituents of 1:3; Table 2), while Experiment III was performed at optimized conditions (mass ratio of phases ratio 1:1, mass fraction of water of 6.6%, and molar ratio of DES constituents of 1:3.5). All experiments were performed at the same molar ratio of oil and methanol (1:3.4) and at equal initial lipase concentrations ($\gamma_{E,0}$ = 0.1 mg mL^{-1}) as in the experiments performed for optimization purposes.

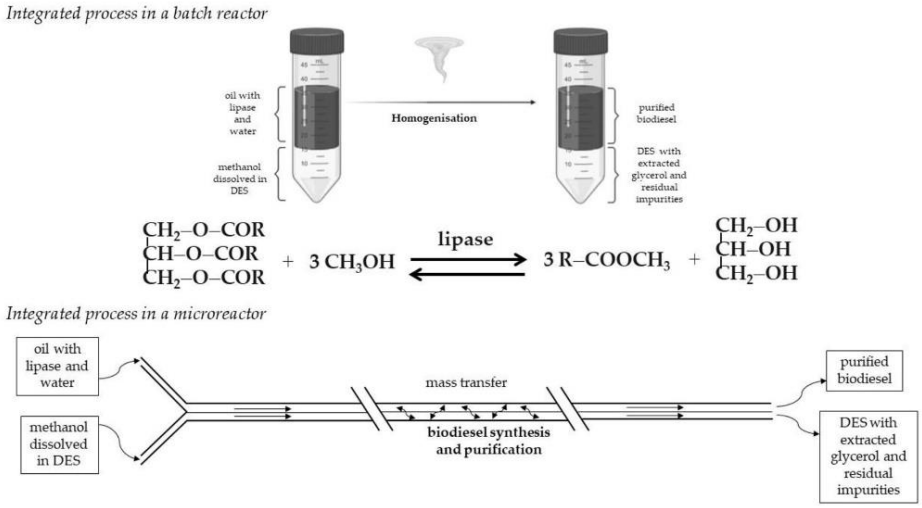


Figure 1. Schematic diagram of the integrated biodiesel production and purification in a batch reactor and in a microsystem.

2.2.4. Integrated Biodiesel Production and Purification Process in a Microsystem

The integrated biodiesel production and purification process was performed in a microsystem to investigate any potential enhancements of the process (Figure 1). The synthesis of biodiesel was carried out in a polytetrafluoroethylene (PTFE) tubular microreactor (length:diameter = 1.2 m:500 µm, with a total volume of 236 µL). The PTFE microreactor was equipped with a Y-shaped input. Two piston pumps (Harvard Apparatus, Holliston, MA, USA) were connected to the microreactor by PTFE tubes, with the first pump supplying oil mixed with lipase, while methanol mixed with a DES was fed with the second pump. The oil to methanol ratio (1:3.4) and enzyme concentration in the inlet stream ($\gamma_{E,0}$ = 0.1 mg mL^{-1}) were kept constant as in the batch processes. A microreactor was placed in a water bath heated to 40 °C to carry out the process at the optimum temperature for transesterification. The total flow was 1.96 µL min^{-1} (τ = 120 min).

2.2.5. Influence of Temperature on the Efficiency of Glycerol Extraction

In order to investigate the influence of temperature on the extraction efficiency, batch experiments were performed at 40 °C, 50 °C, and 60 °C. The integrated production and biodiesel purification process was carried out in a glass batch reactor with double walls (V = 10 mL). Appropriate weights of oil and methanol (molar ratio 1:3.4) were added in the eutectic solvent, while the reaction was started with the addition of an aqueous lipase suspension. The initial concentration of the enzyme in the reaction mixture was $\gamma_{E,0}$ = 0.1 mg mL^{-1}. The reactor was placed on a magnetic stirrer, and the reaction mixture was stirred at 400 rpm for 48 h. Afterwards, the reaction mixture was transferred to a separation funnel to separate the biodiesel phase from the deep eutectic solvent phase. The sample of the biodiesel phase was analyzed by gas chromatography.

2.2.6. Measurement of the Concentration of Fatty Acid Methyl Esters and Glycerol by Gas Chromatography

The concentration of fatty acid methyl esters (FAMEs) and glycerol was determined on a gas chromatograph equipped with a flame ionization detector (FID detector) and a Zebron ZB-Wax GC capillary column (length: 30 m; internal diameter: 0.53 mm; film thickness: 1.00 µm). Concentrations of FAME and glycerol were determined as described elsewhere [38]. For 1 min, the column was heated starting at a temperature of 180 °C, with heating to 230 °C at the rate of 5 °C min^{-1}, while the FID detector operated at 240 °C.

According to the method, the total duration of analysis for each sample was 15 min, and nitrogen with a flow rate of 1.97 mL min^{-1} was used as the carrier gas. A mixture of the FAME mix GLC-10 was used as a standard to identify the corresponding fatty acid esters. The sample for analysis was diluted 100-fold to obtain concentrations in the area covered by the calibration diagram. *N*-heptane was used as the solvent for sample preparation to determine the concentration of FAME, while ethanol was used as the solvent for sample preparation in the analysis of glycerol. After homogenization, the sample was filtered and analyzed. The retention times for the individual components were as follows: 7.74 min for palmitic, 10.59 min for stearin, 10.87 min for oleic, 11.58 min for linoleic, and 12.62 min for α-linolenic (linolenic) esters. The retention time for glycerol was 9.02 min, using the same method. To determine repeatability, all measurements were performed in triplicate.

2.2.7. Spectrophotometric Measurement of Glycerol Concentration

The concentration of glycerol in the sample was determined as described by Bondioli and Della Bella [39]. Briefly, 0.5 g of the biodiesel sample (±0.1 mg) was weighed into a 10-mL conical centrifuge tube. The sample was then dissolved in 2 mL hexane and 2 mL working solvent was added. The resulting mixture was then homogenized for 5 min and centrifuged for 15 min at 2000 rpm and 25 °C. After centrifugation, the upper layer (biodiesel) was removed and 0.5 mL of the lower layer was transferred to a 10-mL conical centrifuge tube. In this sample, 1.5 mL of working solvent (equal volumes of distilled water and 95% ethanol) and 1.2 mL sodium periodate solution were added. The resulting sample was homogenized for 30 s, followed by the addition of 1.2 mL of 0.2 mol L^{-1} acetylacetone solution. The conical centrifuge tubes were placed in a pre-thermostated water bath (Thermomix 1420, Braun, Melsungen, Germany) at 70 °C for 1 min, with manual stirring. After thermostating at 70 °C, the sample was transferred to a water bath at 20 °C for 2 min with manual stirring. The samples were then centrifuged at 2000 rpm and 25 °C for 1 min. Thus, 1 mL of the sample was obtained and transferred to plastic cuvettes and analyzed spectrophotometrically (UV-1601, Shimadzu, Kyoto, Japan) at a wavelength of λ = 410 nm. To determine repeatability, all measurements were performed in triplicate and the glycerol concentration was calculated based on the calibration curve.

2.2.8. Measurement of Lipase Activity

The measurement of lipase activity was based on the solvolysis of 0.0375 mol L^{-1} of 4-nitrophenyl acetate. Briefly, 100 µL of sample was added to 3900 µL of 0.05 mol L^{-1} Tris-HCl buffer, pH 8, and homogenized. After homogenization, 950 µL of the mixture was transferred to a UV cuvette previously thermostated at 40 °C in a water bath. The reaction was started by adding 50 µL of 0.0375 mol L^{-1} of 4-nitrophenyl acetate previously dissolved in acetonitrile. The enzyme activity was determined spectrophotometrically (UV-1800, Shimadzu, Kyoto, Japan) by measuring the change in absorbance at the wavelength of λ = 400 nm, with a total determination time of 20 s. To determine repeatability, all measurements were performed in triplicate.

2.2.9. Optimization of the Extraction Process by Response Surface Methodology (RSM)

Impacts of the three separate variables (water content (X_1), DES composition (X_2), and mass ratio of phases (X_3)) on the biodiesel yield (Y) were assessed by applying the Box–Behnken design implemented in Statistica version 10.0 (StatSoft Inc., Tulsa, OK, USA). The impact of independent variables was investigated at three levels (−1, 0, and 1) through 15 experiments according to the experimental design. The parameters of the second-order polynomial model were estimated to describe the experimental data (Equation (1), [40])

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i \cdot X_i + \sum_{i=1}^{3} \beta_{ii} \cdot X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} \cdot X_i \cdot X_j \quad (1)$$

where Y is the predicted response; β_0, β_i, β_{ii}, and β_{ij} are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, and X_i and X_j are the independent variables.

2.2.10. Artificial Neural Network (ANN) Modeling

Multi-layer perceptron (MLP) networks were developed in Statistica v.10.0 software (StatSoft Inc., Tulsa, OK, USA) for the prediction of biodiesel yield based on water content, DES composition, and mass ratio of phases. The artificial neural network (ANN) training was performed with separation of data into training, test, and validation sets in a 70:15:15 ratio. A back error propagation algorithm was applied for the model training to ensure the minimum value of the error function. The model performance was evaluated based on R^2 and root mean squared error (RMSE) values for training, testing, and validation.

3. Results

3.1. Optimization of Biodiesel Synthesis and the Glycerol Extraction Process

In order to optimize the integrated biodiesel synthesis and glycerol extraction process, the influence of three variables on biodiesel yield and extraction efficiency were analyzed at three levels.

The first chosen variable was the mass ratio of a reaction phase (oil, methanol, enzyme, and water) and a DES (ChCl:Gly or ChCl:EG) phase. According to Hayyan et al. [41], while purifying the produced biodiesel, biodiesel and the DES mass ratio have a significant impact on extraction efficiency, which is the highest for the 1:1 mass ratio. Therefore, a 1:1 mass ratio of a reaction phase (oil, methanol, enzyme, and water) and the DES phase was chosen as the initial value. To cover the entire range of phase ratios, the lower and higher levels were set to be 1:9 and 9:1, respectively.

The second analyzed variable was the mass fraction of water in the system. According to Merza et al. [42], water has a significant impact on biodiesel yield when using lipase as a catalyst, while water is necessary to keep the 3D structure of lipase. On the other hand, the water content should not be too high during the transesterification process because unwanted hydrolysis could occur [43]. Therefore, optimization of water content is necessary in order to obtain the highest possible yield.

Furthermore, based on previous research [34], the initial value was set at 4.6%, while the lower level was defined considering the water content in the original enzyme sample, which resulted in the minimal water content of 0.6% in the mixture. In order to keep the optimization step of 4%, the mass fraction of water was set to be 8.6% at the higher level.

The third chosen variable was the molar ratio of choline chloride and a hydrogen bond donor in a DES. Šalić et al. [33] highlighted that the extraction efficiency can be increased by changing the ratio of choline chloride and a hydrogen bond donor from 1:1 to 1:3. Therefore, the 1:3 molar ratio was chosen to be the initial value with step set to 1, and 1:2 and 1:4 as the lower and higher levels of the third variable, respectively.

The experimental plan obtained by the application of the Box–Behnken design implemented in the Statistica software package and the experimentally determined response values are shown in Table 2 for both investigated DESs.

It can be noticed that biodiesel yields are generally much higher in the ChCl:Gly system. According to Gorke et al. [44], who reported on higher lipase activity in ChCl:Gly compared to ChCl:EG, the reason could be the stronger hydrogen-bonding network in ChCl:Gly. Namely, ChCl:Gly has three hydroxyl groups (strong HBD sites), while ChCl:EG is composed of two hydroxyl groups [31]. Those HBD sites lower the chemical potential of DES components which makes DESs suitable as solvents. That way, the components, more specifically ethylene glycol or glycerol, no longer have a negative impact on the enzyme and that effect is, therefore, more pronounced with ChCl:Gly [43]. Furthermore, extraction efficiencies are also higher when using ChCl:Gly in most experiments. That agrees with research performed by Šalić et al. [33], where the same extraction efficiencies

were obtained for a significantly shorter residence time in continuous experiments, where ChCl:Gly was used as the reaction and extraction medium. As for the glycerol content, it can be observed that purity according to standards for the direct use of biodiesel, i.e., glycerol content lower than 0.02% [18], has been achieved in only one run (run 11—0.007%, Table 2). In other runs, glycerol content was significantly higher, which indicates the necessity of further optimization.

After the experiments were carried out, volume activity of lipase was measured in the phase containing DESs, because the enzyme, as well as glycerol and other possible by-products of transesterification, is being separated by them [31]. Based on the results shown in Figure 2, there is no noticeable trend that would indicate which DES is most suitable. However, the highest volume activity was measured in run 8 with the DES ChCl:Gly (9:1 mass ratio of phases, 8.6% of water, 1:3 DES molar ratio). That can be explained by the significant impact of a certain amount of water on the activity and stability of lipase [22]. Furthermore, activity was not measured in runs 5, 6, 9, and 10 due to gel formation caused by high DES excess (10:90 mass ratio of phases). Considering all the presented results, the ChCl:Gly DES can be considered as a more suitable reaction medium compared to the ChCl:EG DES. Additionally, change in enzyme volume activity was not noticed during single experiments (30 min).

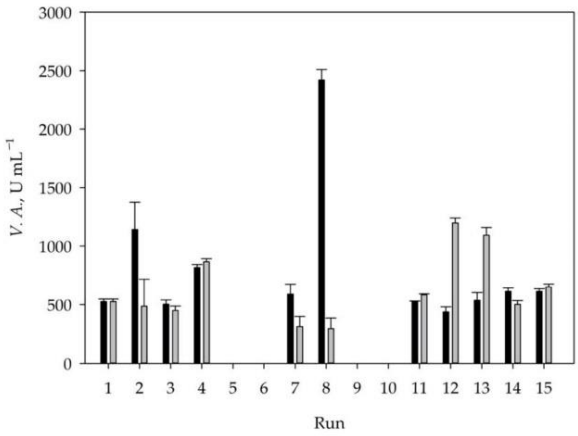


Figure 2. Volume activities of lipase for points of experimental plan according to the Box–Behnken experimental design in experiments with ChCl:Gly (•) and ChCl:EG (•).

In accordance with RSM, a second-order polynomial equation was used to describe the relationship between the response (biodiesel yield (Y)) and three variables (water content (X_1), DES composition (X_2), and the mass ratio of phases (X_3)). The estimated model parameters and ANOVA results are given in Table 3 and the RSM plot in Figure 3. According to Teng et al. [45], the variable has a significant impact on the response if $p < 0.05$. The results showed that water content in the linear ($p < 0.0001$) and quadratic term ($p < 0.0001$) has a significant positive effect on biodiesel yield in the ChCl:Gly system. Furthermore, it can be noticed that the phase mass ratio (X_3) has a significant negative effect in the linear term ($p = 0.0055$) and a significant positive effect in the quadratic term ($p < 0.0001$). The results also showed the significant positive effect of the $X_1 \cdot X_3$ interaction term ($p = 0.0009$). Similar results were obtained for the model describing biodiesel yield in the ChCl:EG system, where the water content in the linear term ($p < 0.0001$), the phase ratio in the linear term ($p < 0.0001$), and the $X_1 \cdot X_3$ interaction term ($p < 0.0001$) showed a significant positive effect on biodiesel yield.

Table 3. Analysis of variance of response surface methodology (RSM) models for biodiesel yield. (Sum of squares (SS), Degree of freedom (DF), Mean square (MS)).

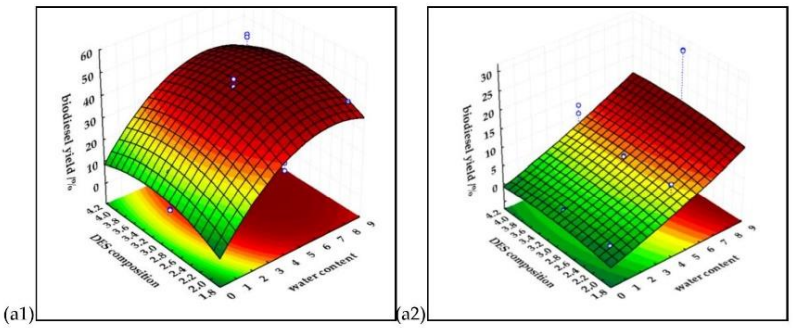
ChCl:Gly							
Source	**Coeff. ± st.er.**	**SS**	**DF**	**MS**	**F-Value**	***p*-Value**	R^2
Model		3347.14	9	371.90	31.43	<0.0001	0.9452
Intercept	17.98 ± 1.34						
X_1	49.80 ± 3.30	3133.08	1	3133.08	227.13	<0.0001	
X_2	2.67 ± 1.86	28.53	1	28.53	2.07	0.1658	
X_3	−5.78 ± 1.86	133.50	1	133.50	9.68	0.0055	
${X_1}^2$	24.07 ± 2.73	1069.34	1	1069.34	77.52	<0.0001	
${X_2}^2$	5.01 ± 1.37	185.62	1	185.62	13.45	0.0015	
${X_3}^2$	9.49 ± 1.37	665.74	1	665.74	48.26	<0.0001	
$X_1 \cdot X_2$	−1.81 ± 2.63	6.58	1	6.58	0.48	0.4974	
$X_1 \cdot X_3$	10.17 ± 2.63	206.73	1	206.73	14.98	0.0009	
$X_2 \cdot X_3$	3.82 ± 2.63	29.26	1	29.26	2.12	0.1608	
Residual		224.81	20	11.83			
Lack of fit		148.80	3	14.40	1.664	0.0667	
Pure error		76.02	17	4.47			
ChCl:EG							
Source	**Coeff. ± st.er.**	**SS**	**DF**	**MS**	**F-Value**	***p*-Value**	R^2
Model		1601.69	9	177.97	46.21	<0.0001	0.9541
Intercept	8.39 ± 0.71						
X_1	12.39 ± 1.75	193.22	1	193.22	50.17	<0.0001	
X_2	1.25 ± 0.98	6.24	1	6.24	1.62	0.2176	
X_3	11.14 ± 0.98	496.32	1	496.32	128.86	<0.0001	
${X_1}^2$	−1.39 ± 1.44	3.57	1	3.57	0.93	0.3473	
${X_2}^2$	0.75 ± 0.72	4.10	1	4.10	1.07	0.3143	
${X_3}^2$	−1.54 ± 0.72	17.43	1	17.43	4.52	0.0460	
$X_1 \cdot X_2$	0.43 ± 1.38	0.378	1	0.378	0.09	0.7574	
$X_1 \cdot X_3$	12.39 ± 1.38	307.42	1	307.42	79.82	<0.0001	
$X_2 \cdot X_3$	2.02 ± 1.38	8.16	1	8.16	2.12	0.1610	
Residual		177.03	20	3.85			
Lack of fit		155.47	3	8.49	1.458	0.0937	
Pure error		21.55	17	1.27			

To highlight the interactive effects of the independent variables on biodiesel yield, 3D response surfaces are presented in Figure 3. The plots were obtained by presenting the response variable versus two independent variables (the third was kept constant). For the ChCl:Gly system (Figure 3(a1–c1)), it can be noticed that biodiesel yield increases with the water content increase until the optimum water counter value is reached. Furthermore, biodiesel yield decreases with the increase in the mass ratio of phases. In case of the ChCl:EG system (Figure 3(a2–c2)), biodiesel yield increased with the water content increase, the DES composition increase, and the increase in the mass ratio of phases.

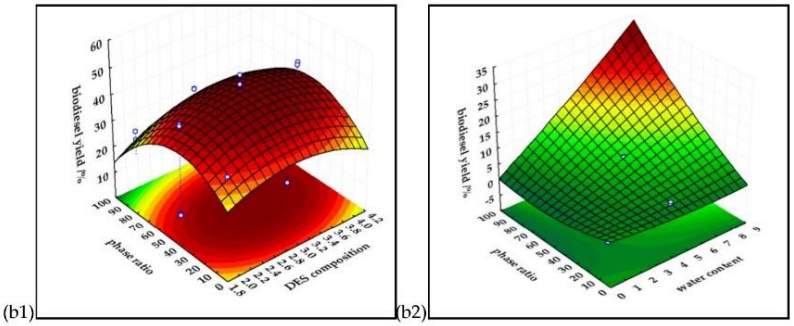
Based on the R^2 values of the developed RSM models (0.9452 for the ChCl:Gly system and 0.9541 for the ChCl:EG system), it can be concluded that the RSM models describe the experimental data with high precision. As previously presented by Le Man et al. [46], the model is considered adequate when $R^2 > 0.75$, which was achieved. Analysis of variance (ANOVA) showed that the proposed models were significant ($p < 0.05$) and that the F-values for the models were higher than the F-critical = 2.007 (F(ChCl:Gly) = 31.43, F(ChCl:EG) = 46.21).

Taking into account that the high R^2 value does not guarantee that the model will fit the data well, residual analysis was also performed. Results of the residual analysis are presented in Figure 4. Residuals were arranged nearly around the line (Figure 4(a1,a2)) and the histograms presenting the classification of residuals (Figure 4(c1,c2)) showed a specific bell shape, and therefore, the assumption on normality was convinced. Moreover,

by analyzing the plots showing residuals versus predicted values (Figure 4(b1,b2)), it can be noticed that residuals were randomly distributed, demonstrating good agreement between model and experimental data. Residual analysis also showed that the order of the experimental run cannot change the results because residuals alter close to zero (Figure 4(d1,d2)). The obtained results indicated the reliability of the developed response surface model for the analyzed range of input variables.



(**a**)



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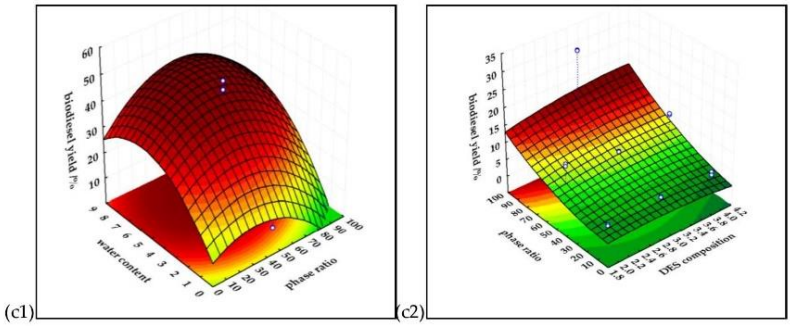

(**c**)

Figure 3. Response surface plot for biodiesel production and purification obtained by numerical optimization as a function of statistically significant interaction variables: (**a**) DES composition, (**b**) water content, and (**c**) ratio of phases.

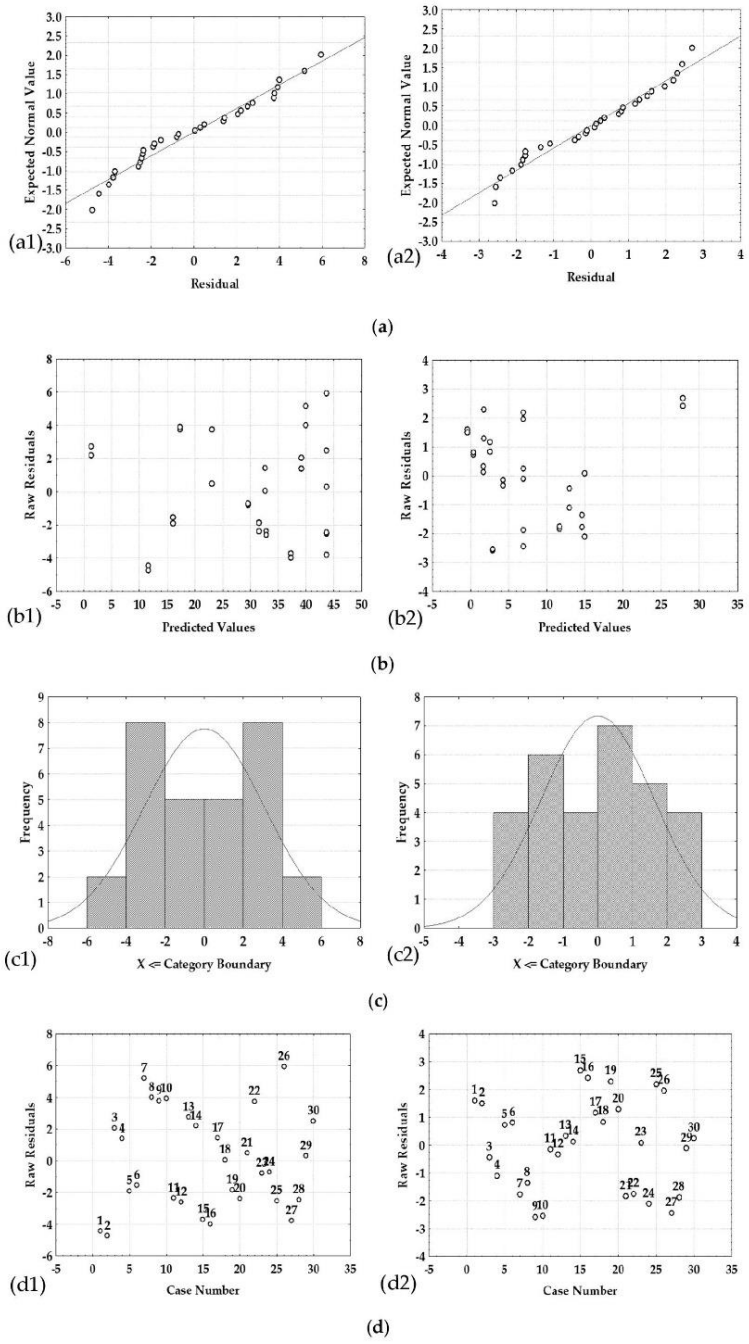

Figure 4. Residual analysis for the response surface regression of the biodiesel yield: (1) ChCl:-Gly, (2) ChCl:-EG. (**a**) Normal probability plot of the residuals; (**b**) residuals versus the predicted values; (**c**) histogram of the residuals; (**d**) residuals versus the order of the data.

Furthermore, optimization was performed based on the desirability profiles obtained from the RSM predicted values. The desirability scale in the range from 0 (undesirable) to 1 (very desirable) was used. Since the optimization of two processes was carried out, there were cases in which it was not possible to achieve the maximum response of both processes under the same conditions (process variables). In those cases, the optimal conditions were those that correspond to the maximum biodiesel yield since the values of glycerol extraction efficiency are generally high and their changes with the changes in process variables are not as pronounced as in the case of biodiesel yield. Therefore, the optimal conditions for integrated biodiesel synthesis and glycerol extraction regarding biodiesel yield were the following: water mass content 6.6%, molar ratio of DES constituents 1:3.5, and mass ratio of phases 1:1. The performance of the developed RSM model for the prediction of glycerol extraction regrading biodiesel yield was evaluated under selected optimal process conditions. As presented in Table 4 for experiment III, glycerol extraction regarding biodiesel yield of 43.54 ± 0.2% was obtained, which was not significantly different ($p > 0.05$) from the model-predicted value of 47.13%. It can be concluded that the RSM model can efficiently predict the glycerol extraction regarding biodiesel yield.

Table 4. Comparison of biodiesel yield, extraction efficiency, and glycerol content under different initial conditions of biodiesel synthesis and purification.

	Buffer		**ChCl:Gly**	
	Initial Conditions		**Optimal Conditions**	
	Experiment I	**Experiment II**	**Experiment III**	**Experiment IV**
		Batch reactor		Microsystem
Y, %	61.61 ± 1.99	43.01 ± 1.23	43.54 ± 0.2	45.33 ± 1.74
η, %	–	97.52 ± 0.9	99.54 ± 0.19	99.56 ± 0.13
w_G, %	7.84 ± 0.51	0.15 ± 0.11	0.027 ± 0.01	0.019 ± 0.003

3.2. Artificial Neural Networks (ANN) Modelling

In order to additionally enhance the description and prediction of biodiesel yield, MLP models were proposed. The networks selected based on their performance are given in Table 5. For the system with the ChCl:Gly, MLP 3-9-1 was selected as the optimum. The described ANN ensured good agreement between the experimental data and the model-predicted data for training, testing, and validation ($R^2 > 0.9900$). The selected MLP was characterized by three neurons in the input layer, nine neurons in the hidden layer, and one neuron in the output layer, and the hidden activation function was exponential, while the output activation function was the identity function. The ANN selected as the optimum for the prediction of biodiesel yield in the system with the ChCl:EG was MLP 3-8-1 and it showed very good agreement between the model and the experiment for training, testing, and validation ($R^2 > 0.9690$). In order to determine the influence of input variables on the output, a global sensitivity analysis was performed. A higher global sensitivity coefficient corresponds to a higher influence of the input. It can be noticed that water content has the most significant effect on the biodiesel yield for both systems (global sensitivity coefficient around 60%), followed by the phase ratio with sensitivity around 30% (Figure 5). As for the RSM model, the DES composition showed to be the least important variable.

Table 5. Architectures of artificial neural networks (ANNs) selected for prediction of biodiesel yield. Selected architectures are marked bold.

	Network Name	Training Perf./Training Error	Test Perf./Test Error	Validation Perf./Validation Error	Hidden Activation Function	Output Activation Function
ChCl:Gly	MLP 3-9-1	0.9977 0.0003	0.9968 0.0006	0.9906 0.0007	Exponential	Identity
	MLP 3-8-1	0.9977 0.0006	0.9855 0.0011	0.9339 0.0013	Exponential	Logistic
	MLP 3-5-1	0.9977 0.0004	0.9753 0.0016	0.9635 0.0019	Exponential	Logistic
	MLP 3-8-1	0.9977 0.0003	0.9844 0.0006	0.9812 0.0012	Exponential	Logistic
	MLP 3-9-1	0.9977 0.0004	0.9852 0.0016	0.9828 0.0019	Logistic	Logistic
ChCl:EG	MLP 3-8-1	0.9911 0.0007	0.9684 0.0008	0.9489 0.0017	Exponential	Exponential
	MLP 3-6-1	0.9913 0.0007	0.9741 0.0007	0.9689 0.0015	Exponential	Exponential
	MLP 3-7-1	0.9911 0.0007	0.9698 0.0008	0.9680 0.0017	Exponential	Exponential
	MLP 3-4-1	0.9852 0.0007	0.9688 0.0014	0.9664 0.0014	Exponential	Logistic
	MLP 3-8-1	0.9906 0.0006	0.9798 0.0007	0.9692 0.0008	Exponential	Exponential

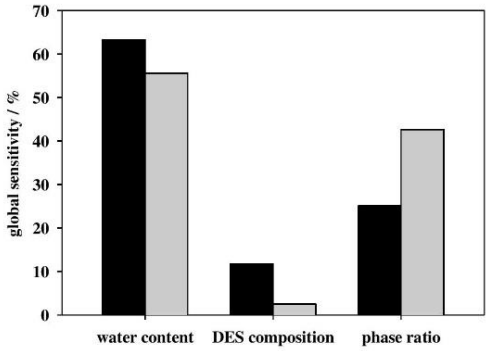

Figure 5. Global sensitivity analysis of optimal ANN architectures: (●) ChCl:Gly, (●) ChCl:EG.

3.3. Integrated Production and Purification of Biodiesel—Batch Reactor versus Microreactor

After the process optimization, three additional experiments on a laboratory shaker (t = 2 h, 400 rpm, and T = 40 °C) were performed. In Experiment I, the initial conditions were the same as in previous research [20,28]. After 2 h, the obtained yield was 61.61%, as presented in Table 4. In order to obtain biodiesel (Y > 96%), the process needs to be performed for 24 h [47]. As mentioned before, in order to enhance the process and to perform parallel biodiesel synthesis and purification, a DES was introduced in the system instead of a buffer. Two experiments were performed using a DES as the reaction and extraction medium. Experiment II was performed under initial conditions, while Experiments III and IV were performed under the optimal conditions.

As can be seen from results shown in Table 4, in both reaction systems, batch and microreactor, for experiments performed with a DES (Experiments II, III, and IV), a slightly lower yield in comparison to Experiment I was obtained. The reason for this is probably a decrease in enzyme activity for reactions performed with a DES. According to Xu et al. [48],

the constituting components of a DES can have an inhibitory effect on enzyme activity by changing the secondary structure of the enzyme.

On the other hand, although the biodiesel yield was lower, in all experiments performed with a DES, the mass fraction of glycerol in biodiesel was lower in comparison to Experiment I. Comparing the results of Experiments II and III, it is clear that the optimization goal was achieved, since both the yield and the extraction efficiency were higher in Experiment III conducted under optimal conditions. Furthermore, the mass fraction of glycerol achieved in Experiment III has a value close to that prescribed by the international standard EN 14214:2012+A2:2019 [6].

According to literature [29], extraction efficiency could be enhanced by using a higher temperature. Higher temperatures should reduce glycerol retention, as it was observed in the studies where the effect of temperature on glycerol removal was brought into relation with the concentration factor (CF). According to Saleh at al. [49], higher temperatures promote reaching the appropriate CF value sooner, so glycerol separates from the FAME-rich phase faster. Additionally, increasing the temperature can be another advantage for biodiesel purification, because higher temperatures facilitate the circulation of biodiesel by considerably decreasing its viscosity, reducing the cost of pumping in the meantime [50]. To examine the impact of temperature increase on extraction efficiency, the integrated process of biodiesel production and purification in a batch reactor was carried out at three different temperatures (40 °C, 50 °C, and 60 °C) under optimal conditions determined in experiments performed with ChCl:Gly. The highest yield of biodiesel was achieved at the temperature of 40 °C (temperature optimum for lipase activity). By increasing the temperature, the biodiesel yield decreases significantly. At a temperature of 50 °C, the yield was 1.42 times lower, while at 60 °C, it was 3.07 times lower in comparison to the value obtained at 40 °C. On the other hand, the extraction efficiency did not change significantly, indicating that enhancing the temperature does not have a positive effect on the integrated process.

As one of the solutions for the further intensification of the process, a change in the reactor type was proposed and the batch reactor was replaced by the tubular PTFE microreactor. It was expected that due to the specific design of the microreactor and the associated advantages in terms of the mass and energy transfer, an increased biodiesel yield and glycerol extraction efficiency could be expected. Therefore, integrated biodiesel production and purification was performed in a microsystem (Experiment IV). As it can be seen from results shown in Table 4, an increase in both yield and extraction efficiency was obtained in the microsystem. At the same time, free glycerol content was below the level prescribed by standards. The results indicated that the microsystem should be a focus for further process optimization.

4. Conclusions

An integrated biodiesel production process, composed of biodiesel synthesis from edible sunflower oil and methanol, catalyzed by the enzyme lipase from *Thermomyces lanuginosus*, with simultaneous extraction of glycerol using deep eutectic solvents as the reaction and extraction media was performed. The highest biodiesel yield and extraction efficiency were obtained in experiments performed with the ChCl:Gly DES, which was selected as a more suitable reaction and extraction medium for carrying out this integrated process in comparison to the ChCl:EG DES.

Optimal conditions for biodiesel synthesis and parallel glycerol extraction processes determined by numerical optimization and confirmed by an independent experiment were achieved at the mass ratio of phases of 1:1, the mass fraction of water of 6.6%, and a molar ratio of ChCl:Gly 1:3.5. The effect of temperature on the efficiency of glycerol extraction was found to be negligible. When the reaction was performed in a microsystem, additional process intensification was achieved, leading to the product that had free glycerol content below the international standards, which is a good base for further development of the single-stage biodiesel-integrated production/purification process on the microscale.

Additionally, the obtained results can be used for future research and sustainable development where waste oils, in combination with enzyme produced from waste feedstocks, could be applied for biodiesel production.

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List of Symbols and Abbreviations

t	Time (h)
T	Temperature (°C)
Y	Yield (%)
V	Reactor volume (mL)
X	Independent variables (-)
w	Mass fraction (%)
Greek letters	
β	Regression coefficients (-)
γ	Mass concentration (mg mL^{-1})
λ	Wavelength (nm)
η	Extraction efficiency (%)
τ	Residence time (s)
Abbreviations	
ANN	Artificial neural networks
ChCl:Gly	Choline chloride:glycerol
ChCl:EG	Choline chloride:ethylene glycol
DES	Deep eutectic solvent(s)
DF	Degree of freedom
E	Enzyme
FAME	Fatty acid methyl ester(s)
FID	Flame ionization detector
HBD	Hydrogen bond donor
HBA	Hydrogen bond acceptor
MLP	Multi-layer perceptron
MS	Mean square
PTFE	Polytetrafluoroethylene
RMSE	Root mean squared error
RSM	Response surface methodology
SS	Sum of squares

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Paper 5

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Kinetic Parameter Estimation and Mathematical Modelling of Lipase Catalysed Biodiesel Synthesis in a Microreactor

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Abstract: Development of green, clean, and sustainable processes presents new challenges in today's science. Production of fuel is no exception. Considering the utilisation of various renewable sources, the synthesis of biodiesel, characterised as more environmentally-friendly then fossil fuel, has drawn significant attention. Even though the process based on chemical transesterification in a batch reactor still presents the most used method for its production, enzyme catalysed synthesis of biodiesel in a microreactor could be a new approach for going green. In this research, edible sunflower oil and methanol were used as substrates and lipase from *Thermomyces lanuginosus* (Lipolase L100) was used as catalyst for biodiesel synthesis. Experiments were performed in a polytetrafluoroethylene (PTFE) microreactor with three inlets and in glass microreactors with two and three inlets. For a residence time of 32 min, the fatty acids methyl esters (FAME) yield was 30% higher than the yield obtained for the glass microreactor with three inlets. In comparison, when the reaction was performed in a batch reactor (V = 500 mL), the same FAME yield was achieved after 1.5 h. In order to enhance the productivity of the process, we used proposed reaction kinetics, estimated kinetic parameters, and a mathematical model we developed. After validation using independent experimental data, a proposed model was used for process optimization in order to obtain the highest FAME yield for the shortest residence time.

Keywords: lipase; biodiesel; microreactor; mathematical modelling

1. Introduction

Applications of some traditional methods like micro-emulsification, pyrolysis, blending, or mostly transesterification, and alkaline catalysis [1–5] for biodiesel production are well known and established. Although these processes are common and well investigated, there are still many disadvantages that could not be solved. Some of them are problems with catalyst recovery, low quality of glycerine created as a by-product of the reaction, consumption of high quantities of energy, generation of large amounts of wastewater, etc. [6,7]. In order to solve them, there is a continuous search for new production approaches and new technologies. Some of them are based on ultrasound technology [5], supercritical solvents [8,9], or simply on novel reactor design [10,11]. On the other hand, some studies suggest that the use of enzymes (particularly lipases) for the transesterification process could be a good alternative [12]. Using this approach, energy consumption would be reduced compared to alkaline catalysis, the purity of the produced biodiesel would be higher, no soap would be formed, mild reaction conditions would be ensured, and easy catalyst recovery, if an immobilized enzyme

is used, would be ensured, etc. [10]. This all makes the proposed process "greener", which is the necessary demand in order to reduce the ecological footprint of the industry [13]. As mentioned, aside from changing the production approach, another possibility is to change the technology. According to Budžaki et al. [14], the ideal process would be a continuous process that can eliminate and/or minimize the separation and purification steps. The mentioned authors claim that this overall technology is still waiting to be developed. Perhaps a step in that direction would be the application of continuous reactors that allow the purification and separation on a single unit. Microreactors are definitely such a new technology; reactors that enhance mass and heat transfer, increase reaction rates, reduce cost and energy consumption, generate lower waste streams, etc. [12,14]. Due to their design flexibly, different variations of microreactors have been used for biodiesel synthesis [15–18], but they were mostly focused on the use of a chemical catalyst [19–23]. Despite the fact that enzymatic catalysis offers many advantages, it is still not competitive with the chemical process. In order to become competitive, several challenges have to be resolved. The first one is the price of the enzyme. This could be resolved by the application of suspended or immobilized enzymes, for example the use of suspended/immobilized lipase from *Thermomyces lanuginosus* (TIL), which happens to be among the least expensive commercially available lipases [24]. The cost of the process could be even lower if raw, non-purified enzymes are used. The second challenge is time. Enzymatic reactions are usually slower than chemical reactions, so the reaction rate, along with the transfer of mass and heat, need to be enhanced. As mentioned, this problem could also be resolved with the application of microreactor technology [25,26]. The third challenge, as for all new processes, is scaling-up. The process itself requires intensive work and calculations for each step, going from the laboratory to the industry, usually with a continuous decrease of productivity by increasing volume. Also, complex scaling-up procedures can be avoided by using microreactors. In order to increase throughput when working with microreactors, there is no traditional dimension/size enhancement. By using external or internal numbering up (increasing the number of microreactors), capacities of microreactors can be enhanced significantly [27,28]. Another major advantage is related to the modelling of the system. Once the process is optimised on a single chip, all the process characteristics remain the same even after capacity enlargement/numbering-up.

In summary of everything mentioned above, it can be concluded that the application of microreactor technology in combination with the lipase from TIL could be a good alternative for biodiesel production catalysed with chemical catalysts on a macro scale.

That possibility was explored in this study. A polytetrafluoroethylene (PTFE) microreactor and two different glass microreactor configurations, with two different inlet strategies, were studied. The first glass microreactor was the one with two Y-shaped inlets that allowed the introduction of a stable emulsion of methanol and oil as one phase and the enzyme as the second phase. The second glass microreactor and a PTFE microreactor consisted of three Ψ-shaped inlets, where inlets were used to separately feed in three components, methanol, oil, and enzyme dissolved in buffer, respectively. Yields obtained in all reactor configurations were compared with results obtained in a batch reactor. In order to optimise the process, kinetic parameters were estimated and a 2D mathematical model composed of convection, diffusion, and kinetic terms was developed and used. The proposed model was also used to explore the influence of oil to methanol ratio, enzyme concentration, and numbering-up (the higher residence time) on overall process efficiency. Additionally, a 2D model for three-phase flow in a microchannel using the COMSOL Multiphysics computational fluid dynamics (CFD) package (4.3b, COMSOL, Inc., Burlington, MA, USA) was developed for biodiesel production. The objective was to investigate the reactive flow and to study the velocity properties of the three phases (oil, water, and methanol) that were introduced into the microchannel with the diameter of 1 mm.

2. Materials and Methods

2.1. Materials

Chemicals

Edible sunflower oil (Zvijezda, Croatia) was purchased from a nearby supermarket. The lipase from *Thermomyces lanuginosus* (Lipolase 100L), FAME. mix GLC-10, sodium dodecyl sulfate (SDS), isoamyl alcohol and iso-octane were purchased from Sigma-Aldrich Handels GmbH (Vienna, Austria). Tris(hydroxymethyl)aminomethane (TRIS), methanol, HCl, were purchased from BDH Prolabo (VWR, Lutterworth, UK). Chloroform and acetonitrile were purchased from Fisher Chemicals (Loughborough, UK). 4-nitrophenyil-acetate was purchased from Acros Organics (Fischer Scientific, Merelbeke, Belgium). Potassium dihydrogen phosphate (KH_2PO_4) and KOH were purchased from Lach:ner (Neratovice, Czech Republic). Dipotassium hydrogen phosphate (K_2HPO_4) was purchased from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Lipase Assay

Enzyme activity was determined by a test based on hydrolysis of 1.5 mol/L 4-nitrophenyl acetate. A total of 100 µL of the sample was added to 3900 µL of TRIS-HCl buffer and homogenized. 950 µL of this mixture was added to the UV cuvette. The test is started by adding 50 µL of 1.5 mol/L 4-nitrophenyl acetate (dissolved in acetonitrile). To determine the enzyme activity, spectrophotometer (Shimadzu UV–1601, Kyoto, Japan) was used. Determination time was 60 s, while the change of absorbance was measured at 400 nm. To confirm repeatability, all measurements were performed in triplicate. On 95% confidence interval, the results showed no significant difference.

2.2.2. Emulsion Preparation

The initial concentration of the SDS emulsifier was 0.1 g/L. The emulsion was prepared by mixing oil with enzyme dissolved in buffer in 8:1 ratio, and SDS was added as the selected emulsifier. The mixture was mixed on the laboratory shaker (Tehtnica, Vibromix 313EVT, Železnik Slovenia) for 15 min at 600 rpm.

2.2.3. Measurement of Fatty Acid Methyl Esters (FAME) and Glycerol Concentrations

In order to determine FAME concentration method described elsewhere was used [29]. The samples were prepared for analysis by gas chromatography (Shimadzu GC-2014, Tokyo, Japan) equipped with FID and Zebron ZB-wax GC capillary column (length 30 m, I.D. 0.53 mm and film thickness 1.00 µm, Phenomenex, Torrance, CA, USA). Carrier gas in this method was helium, at rate of 1.97 mL/min. In the method's total determination time of 15 min, measurement starts at the temperature of 180 °C for 1 min, after which at a rate of 5 °C/min, column is heating up to 230 °C. In order to identify peaks for corresponding esters of fatty acids, standard FAME mix GLC-10 was used. Retention times of fatty acids esters are as follows: 7.74 min for palmitic, 10.590 min for stearic, 10.867 min for oleic, 11.575 min for linoleic, and 12.615 min for linoleic. Glycerol determination was made with the same method and its retention time was 9.02 min. To confirm repeatability, all measurements were performed in triplicate. On 95% confidence interval, the results showed no significant difference.

2.2.4. Biodiesel Synthesis in a Batch Reactor

Biodiesel synthesis in a batch reactor was performed according to procedure described by Budžaki et al. [29]. Briefly, the reaction was performed in a batch reactor (V = 250 mL) by adding the enzyme (45 g of Lipolase 100 L stock solution diluted with 0.01 mol/L phosphate buffer at pH 7.4 in molar ratio 1:10) in to the reaction mixture comprised of 450 g of oil and 55.95 g of methanol to form 1:3.4

molar ratio. During the experiment optimal temperature of 40 °C was maintained by a water bath with a heat regulation system (Thermomix 1420, Braun, Germany). Samples of biodiesel were taken at different time intervals and the reaction was stopped by mixing the sample with an organic solvent (Marmur solution, chloroform:isoamyl alcohol = 24:1) cooled on ice. Collected samples were then filtered (Chromafil®AO-20/3; 0.2 µm, Macherey, Nagel GmbH, Düren, Germany) and analysed by gas chromatography.

2.2.5. Biodiesel Synthesis in Glass and PTFE Microreactors

In this work, three different reactors, a PTFE coil microreactor with three inlets (+-shape; length:width = 500 mm:1 mm with an internal volume of 392.5 µL) and two glass microreactors with different inlet configurations (Y-shape and Ψ-shape inlets, respectively) of microreactors (length: width:depth = 330 mm:250 µm:50 µm with an internal volume of 4.2 µL; Micronit Microfluidics B.V., Netherlands) were examined. In the first experimental set-up (Figure 1a), emulsion which was formed from oil and enzyme dissolved in buffer (in 8:1 ratio), with the addition of emulsifier, was placed into one syringe, while the second syringe was filled with methanol. In the second and third experimental set-up, for PTFE and glass microreactor with three inlets, all substrates were placed separately into stainless steel high-pressure syringes (8 mL, Harvard Apparatus, Holliston, MA, USA), first containing oil, second enzyme dissolved in buffer (0.01 mol/L potassium phosphate buffer pH 7.4) and a third one containing methanol (Figure 1b). Syringes were places on pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA). Syringes were connected with silica/PTFE tubes to both microreactors. In order to obtain optimal enzyme activity experiments were performed at 40 °C. This condition was secured by submerging the microreactor in a water bath with a heat regulation system (Thermomix 1420, Braun, Germany).

In all experiments, total flows were changed in an oil:methanol:enzyme ratio 10:1.24:1 so the influence on FAME formation could be monitored. Output streams from all microreactors, that contained remained substrates (oil, methanol, enzyme) and products (FAME, glycerol), were collected in vials. In order to stop the reaction via enzyme deactivation at the exit of the microreactor, outgoing silicate tubes were emerged in an organic solvent (Marmur solution, chloroform:isoamyl alcohol = 24:1) cooled on ice.

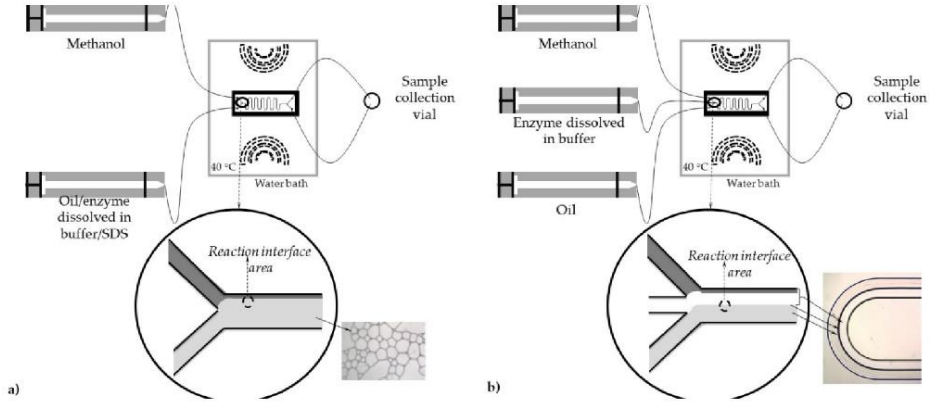

Figure 1. Schematic diagram of the microreactor system with (**a**) two inlets and (**b**) three inlets together with picture of formed flow pattern.

2.2.6. Kinetic Parameter Estimation

The kinetics of the reactions was investigated using the initial reaction rate method in a PTFE microreactor. The influence of each reaction compound on the initial reaction rate was monitored

by keeping the concentrations of other compounds constant. When the influence of fatty acids was monitored, concentration of methanol was set to be a constant ($\gamma_{i,\,methanol}$ = 40.36 mg/L) while the concentration of fatty acids was varied in the range 0–1488.53 mg/L. In the experiment where influence of methanol concertation on reaction rate was monitored, fatty acids concentration was set to be a constant ($\gamma_{i,\,fatty\ acids}$ = 1,488.15 mg/L) while methanol concentration was changed in the rage 0–40.36 mg/L.

2.2.7. Data Processing

The kinetic parameters were estimated by non-linear regression analysis from data collected from experiments in a microreactor. A software package SCIENTIST (MicroMath Scientist®, 3.0, MicroMath Scientific Software, Salt Lake City, UT, USA) with incorporated least square method was used to estimate the numerical values by fitting the kinetic model to the experimental data.

Mathematica 10 (Wolfram Research, Champaign, IL, USA) codes were developed and used for reactor model simulation and verification.

The CFD of the oil, methanol, and buffer phase was carried out using the finite element software COMSOL Multiphysics v. 4.3b.

2.3. Mathematical Modelling

2.3.1. Modelling of Biodiesel Transesterification

For the description and prediction of the biodiesel transesterification process in a microreactor 2D model was developed [30,31] considering convection in the flow (x) direction, diffusion in two directions (x and y) and kinetics of enzyme catalysed reaction (Michaelis–Menten kinetics).

Dimensionless partial differential equations for steady-state conditions in the single pass microreactor system with the associated boundary conditions are as follows (Equations (1)–(6)):

- Enzyme in aqueous phase (phase 1):

$$v_1 \cdot \frac{\partial \gamma_{E,1}}{\partial \xi} = \frac{D_{E/aq}}{W} \cdot \left(\frac{\partial^2 \gamma_{E,1}}{\partial \xi^2} + \frac{\partial^2 \gamma_{E,1}}{\partial \psi^2} \right) \quad (1)$$

$\gamma_{E,1}(0,\psi) = \gamma_{E,1,i}, \ -1 \le \psi \le 0$

$\frac{\partial \gamma_{E,1}\left(\frac{L}{W},\psi\right)}{\partial \xi} = 0, \ -1 \le \psi \le 0$

$\gamma_{E,1}(\xi,0) = K_{P,E} \cdot \gamma_{E,2}(\xi,0), \ 0 < \xi < \frac{L}{W}$

$\frac{\partial \gamma_{E,1}(\xi,1)}{\partial \psi} = 0, \ 0 < \xi < \frac{L}{W}$

- Enzyme in oil phase (phase 2):

$$v_2 \cdot \frac{\partial \gamma_{E,2}}{\partial \xi} = \frac{D_{E/oil}}{W} \cdot \left(\frac{\partial^2 \gamma_{E,2}}{\partial \xi^2} + \frac{\partial^2 \gamma_{E,2}}{\partial \psi^2} \right) \quad (2)$$

$\gamma_{E,2}(0,\psi) = 0, \ 0 \le \psi \le 1$

$\frac{\partial \gamma_{E,2}\left(\frac{L}{W},\psi\right)}{\partial \xi} = 0, \ 0 \le \psi \le 1$

$\frac{\gamma_{E,2}(\xi,0)}{\partial \psi} = \frac{D_{E/aq}}{D_{E/oil}} \cdot \frac{\gamma_{E,1}(\xi,0)}{\partial \psi}, \ 0 < \xi < \frac{L}{W}$

$\frac{\partial \gamma_{E,2}(\xi,1)}{\partial \psi} = 0, \ 0 < \xi < \frac{L}{W}$

- Methanol in aqueous phase (phase 1):

$$v_1 \cdot \frac{\partial \gamma_{\mathrm{M},1}}{\partial \xi} = \frac{D_{\mathrm{M/aq}}}{W} \cdot \left(\frac{\partial^2 \gamma_{\mathrm{M},1}}{\partial \xi^2} + \frac{\partial^2 \gamma_{\mathrm{M},1}}{\partial \psi^2} \right) - W \cdot V_{max} \cdot \frac{\gamma_{\mathrm{E},1} \cdot \gamma_{\mathrm{M},1} \cdot \gamma_{\mathrm{FA},1}}{\left(K_m^{\mathrm{M}} + \gamma_{\mathrm{M},1} \right) \cdot \left(K_m^{\mathrm{FA}} + \gamma_{\mathrm{FA},1} \right)} \tag{3}$$

$\gamma_{\mathrm{M},1}(0,\psi) = {}_{\mathrm{M},1,\mathrm{i}}, \ -1 \le \psi \le 0$

$\frac{\partial \gamma_{\mathrm{M},1}\left(\frac{L}{W},\psi\right)}{\partial \xi} = 0, \ -1 \le \psi \le 0$

$\gamma_{\mathrm{M},1}(\xi,0) = K_{P,\mathrm{M}} \cdot {}_{\mathrm{M},2}(\xi,0), \ 0 < \xi < \frac{L}{W}$

$\frac{\partial \gamma_{\mathrm{M},1}(\xi,1)}{\partial \psi} = 0, \ 0 < \xi < \frac{L}{W}$

- Methanol in oil phase (phase 2):

$$v_2 \cdot \frac{\partial \gamma_{\mathrm{M},2}}{\partial \xi} = \frac{D_{\mathrm{M/oil}}}{W} \cdot \left(\frac{\partial^2 \gamma_{\mathrm{M},2}}{\partial \xi^2} + \frac{\partial^2 \gamma_{\mathrm{M},2}}{\partial \psi^2} \right) - W \cdot V_{max} \cdot \frac{\gamma_{\mathrm{E},2} \cdot \gamma_{\mathrm{M},2} \cdot \gamma_{\mathrm{FA},2}}{\left(K_m^{\mathrm{M}} + \gamma_{\mathrm{M},2} \right) \cdot \left(K_m^{\mathrm{FA}} + \gamma_{\mathrm{FA},2} \right)} \tag{4}$$

$\gamma_{\mathrm{M},2}(0,\psi) = 0, \ 0 \le \psi \le 1$

$\frac{\partial \gamma_{\mathrm{M},2}\left(\frac{L}{W},\psi\right)}{\partial \xi} = 0, \ 0 \le \psi \le 1$

$\frac{\gamma_{\mathrm{M},2}(\xi,0)}{\partial \psi} = \frac{D_{\mathrm{M/aq}}}{D_{\mathrm{M/oil}}} \cdot \frac{\gamma_{\mathrm{M},1}(\xi,0)}{\partial \psi}, \ 0 < \xi < \frac{L}{W}$

$\frac{\partial \gamma_{\mathrm{M},2}(\xi,1)}{\partial \psi} = 0, \ 0 < \xi < \frac{L}{W}$

- Fatty acids in aqueous phase (phase 1):

$$v_1 \cdot \frac{\partial \gamma_{\mathrm{FA},1}}{\partial \xi} = \frac{D_{\mathrm{FA/aq}}}{W} \cdot \left(\frac{\partial^2 \gamma_{\mathrm{FA},1}}{\partial \xi^2} + \frac{\partial^2 \gamma_{\mathrm{FA},1}}{\partial \psi^2} \right) - W \cdot V_{max} \cdot \frac{\gamma_{\mathrm{E},1} \cdot \gamma_{\mathrm{M},1} \cdot \gamma_{\mathrm{FA},1}}{\left(K_m^{\mathrm{M}} + \gamma_{\mathrm{M},1} \right) \cdot \left(K_m^{\mathrm{FA}} + \gamma_{\mathrm{FA},1} \right)} \tag{5}$$

$\gamma_{\mathrm{FA},1}(0,\psi) = 0, \ -1 \le \psi \le 0$

$\frac{\partial \gamma_{\mathrm{FA},1}\left(\frac{L}{W},\psi\right)}{\partial \xi} = 0, \ -1 \le \psi \le 0$

$\frac{\gamma_{\mathrm{FA},1}(\xi,0)}{\partial \psi} = \frac{D_{\mathrm{FA/oil}}}{D_{\mathrm{FA/aq}}} \cdot \frac{\gamma_{\mathrm{FA},2}(\xi,0)}{\partial \psi}, \ 0 < \xi < \frac{L}{W}$

$\frac{\partial \gamma_{\mathrm{FA},1}(\xi,1)}{\partial \psi} = 0, \ 0 < \xi < \frac{L}{W}$

- Fatty acids in oil phase (phase 2):

$$v_2 \cdot \frac{\partial \gamma_{\mathrm{FA},2}}{\partial \xi} = \frac{D_{\mathrm{FA/oil}}}{W} \cdot \left(\frac{\partial^2 \gamma_{\mathrm{FA},2}}{\partial \xi^2} + \frac{\partial^2 \gamma_{\mathrm{FA},2}}{\partial \psi^2} \right) - W \cdot V_{max} \cdot \frac{\gamma_{\mathrm{E},2} \cdot \gamma_{\mathrm{M},2} \cdot \gamma_{\mathrm{FA},2}}{\left(K_m^{\mathrm{M}} + \gamma_{\mathrm{M},2} \right) \cdot \left(K_m^{\mathrm{FA}} + \gamma_{\mathrm{FA},2} \right)} \tag{6}$$

$\gamma_{\mathrm{FA},2}(0,\psi) = {}_{\mathrm{FA},2,\mathrm{i}}, \ 0 \le \psi \le 1$

$\frac{\partial \gamma_{\mathrm{FA},2}\left(\frac{L}{W},\psi\right)}{\partial \xi} = 0, \ 0 \le \psi \le 1$

$\frac{\gamma_{\mathrm{FA},2}(\xi,0)}{\partial \psi} = K_{P,\mathrm{FA}} \cdot {}_{\mathrm{FA},1}(\xi,0), \ 0 < \xi < \frac{L}{W}$

$\frac{\partial \gamma_{\mathrm{FA},2}(\xi,1)}{\partial \psi} = 0, \ 0 < \xi < \frac{L}{W}$

A systems of partial differential equations were solved by using the 2D finite differences method. Partial derivatives were discretised on the static equidistant grid.

The diffusion coefficient ($D_{S/B}$) of methanol at 40 °C, was estimated according to Scheibel empirical correlation (Equation (7)) proposed by Li and Carr [32]:

$$D_{S/B} = \frac{8.2 \cdot 10^{-8} \cdot T}{\eta_B \cdot V_S^{1/3}} \left[1 + \left(\frac{3 \cdot V_B}{V_S}\right)\right]^{2/3} \quad (7)$$

The diffusion coefficient ($D_{S/B}$) of enzyme at T = 40 °C, was estimated according to Young empirical correlation [33] (Equation (8)):

$$D_{S/B} = 8.34 \cdot 10^{-8} \left(\frac{T}{\eta_B \cdot M_S^{1/3}}\right) \quad (8)$$

Properties of the solutes and solvents that were used for calculations are presented in Table 1.

Table 1. Properties of methanol, water (buffer) and lipase.

Solute (S)	Solvent (B)	T (K)	V_S (mL/mol)	V_B (mL/mol)	M_S (g/mol)	η_B (mPa·s)
Lipase	Water (buffer)	313.15	-	18.069 [34]	33,400 [35]	0.654
Methanol	Water (buffer)	313.15	38.5 [36]	18.069 [34]	32.04	0.654
Lipase	Methanol	313.15	-	38.5 [36]	33,400 [36]	0.445
Methanol	Methanol	313.15	38.5 [36]	38.5 [36]	32.04	0.445

Additionally, the diffusion times of components diffusing from one phase to another were calculated according to Equation (9):

$$\tau_D = \frac{W^2}{D_{S/B}} \quad (9)$$

where W denotes width that components have to cross from one phase to another.

2.3.2. CFD Modelling

COMSOL Multiphysics 4.3b software was used for the solving of partial differential equations in order to obtain 2D velocity model of multiphase laminar flow in microchannel since COMSOL Multiphysics uses the finite element method together with adaptive meshing and error control using a variety of numerical solvers. The meshing is regarded to geometry division which is often triangular shaped (software default meshing) which can be modified to any shape or size by user. For this study the model was developed for room temperature conditions and the Navier–Stokes equations for incompressible fluids along with the continuity equation were used to model multiphase flow. The boundary conditions, that are commonly used, were also assumed for this model: no-slip at the walls, fully developed laminar flow, defined velocities for the inflow of each phase, and zero relative pressure for the outflow. The Navier–Stokes equations for the conservation of momentum used (Equation (10)):

$$\rho \frac{\partial u}{\partial t} + \rho u \cdot \nabla u = -\nabla p + \nabla \cdot (u(\nabla u + (\nabla u)^T) + F_v \quad (10)$$

and the continuity equation for conservation of mass (Equation (11)):

$$\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho u) = 0 \quad (11)$$

For the meshing one of the default options that the Comsol Multiphysics 4.3b provides was chosen in term of physics controlled finer triangular mesh which consisted of 48,138 domain elements and 4850 boundary elements since preliminary results with normal grid which consisted of 30804 domain elements and 3877 boundary elements gave less accurate results. Additionally, extra fine and extremely fine mesh were tested and the results showed no difference from the finer mesh which was selected.

3. Results and Discussion

Biodiesel was produced from edible sunflower oil, using the enzyme Lipolase 100L (*V.A.* > 100,000 U/mL), in a batch reactor (V = 250 mL), a PTFE (length:width = 500 mm: 1 mm with an internal volume of 392.5 µL), and a glass microreactor (length:width:depth = 330 mm:250 µm:50 µm with an internal volume of 4.2 µL) with two different microreactor inlet configurations. Based on previous experiments, every configuration has some pros and cons, so the first step was to examine which configuration yields better FAME and, consequently, which one is economically more viable (lower usage of substrates, shorter preparation of experimental set-up, and implementation).

3.1. Biodiesel Production

First, the biodiesel was synthetized in a batch reactor by repeating the experimental process described in the paper by Budžaki et al. [29]. As can be seen from Figure 2, the highest fatty acids methyl esters (FAME) yield of 97.70% ± 1.49 was achieved after 24 h in the one-step transesterification reaction of edible sunflower oil performed at 40 °C. At the end of the process, the results corresponded to those obtained by Budžaki et al. [29].

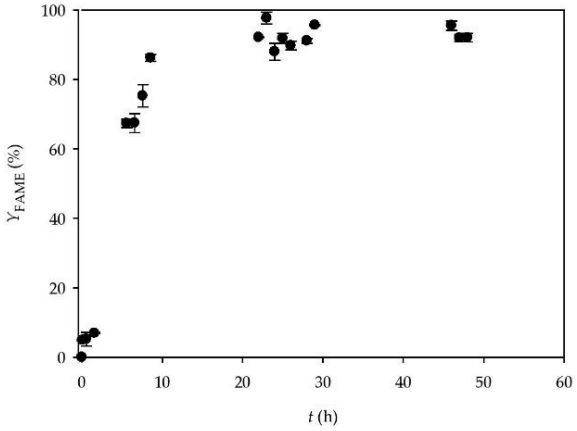

Figure 2. Lipase catalysed biodiesel synthesis in a batch reactor.

The next step was production on a micro scale. As mentioned in the materials and methods section, based on two microreactor inlet configurations, it was possible to introduce the reaction components in two different ways. The first possibility was to form a stable emulsion of two components (methanol and oil, buffer and oil, or methanol and buffer) and introduce them as one phase, while the third reaction component would be introduced as a separate phase. The second possibility would be to introduce each component separately.

3.1.1. Two Inlets Strategy

In one of our previous studies [18], different approaches to reactant supply were investigated when a microsystem (PTFE/Teflon tubular microreactor, length:width = 500 mm:1 mm, internal volume 392.5 µL) with two inlets was used for biodiesel production. In order to introduce three components into a microreactor with two inlets, two components had to be mixed together. Three different strategies of feeding components into the microchannel were investigated. The first one was based on an enzyme and methanol mixture which was introduced as one phase, and oil was the second phase. In the second strategy, oil and methanol were mixed together using an emulsifier (SDS) and introduced into a microchannel as one phase, and the enzyme dissolved in buffer was the second phase. In the third strategy, oil and the enzyme also formed a mixture using an emulsifier (Triton X-100, Sigma-Aldrich

Handels GmbH, Darmstadt, Germany) and the methanol was introduced separately. In the mentioned research, similar FAME yields were observed for all performed experiments.

Although the FAME yield was similar in all three experiments, two of the proposed systems had a disadvantage. When working with a system where methanol and the enzyme formed a mixture, it was noticed that after a longer period of activity, the methanol deactivates the enzyme (k_d = 0.0037 ± 0.00002 min^{-1} [18]). Although this was not an obstacle for the short laboratory experiments, in case of the long continuous processes, this would cause a significant problem. As mentioned in that research, to resolve this problem, it was necessary to separate the methanol and the enzyme from the inlet feeding container. Since the oil is immiscible with both methanol and the enzyme/buffer, it was necessary to introduce an additional component, the emulsifier. After the formation of stable emulsions, buffer/oil/SDS, and methanol/oil/Triton X-100 experiments were performed. A system composed of methanol/oil/Triton X-100 was rejected because the emulsion was not stable enough for a longer period of time, again leading to the same problem in the case of continuous production. In the end, the system where the enzyme/oil/SDS emulsion was fed into the reactor as one of the inlet streams and methanol as the second one, was proposed as the best one.

In the present study, the same strategy was applied (Figure 1a). Before the sample collection started, the flow profile was monitored under the microscope (Motic B1-220A, binocular, Weltzar, Germany). It was noticed that a mixture of oil and buffer (due to larger viscosity and density, as well as faster flow rate) occupies more than half of the microchannel volume. The flow was mostly stable and parallel during the measuring, but the frequency of the instabilities increased by increasing the residence time. Due to viscosity, the flow went from parallel to churn flow (Figure 1a). The results of the lipase catalysed biodiesel synthesis in a microreactor are presented in Figure 3. Up to 15% of the FAME yield was reached for the residence time of 4 min. The comparison with the results from the batch reactor confirmed that the reaction was 15 times faster in a microreactor, since the same FAME yield in a batch reactor was recorded after 1 h.

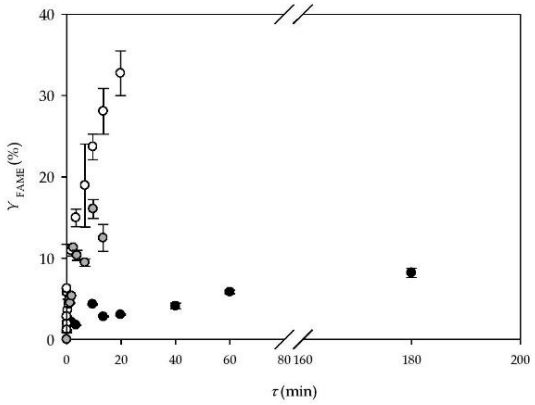

Figure 3. Lipase catalysed biodiesel synthesis in a glass microreactor with (**a**) two inlets (●), and (**b**) three inlets (○) and a polytetrafluoroethylene (PTFE) microreactor with tree inlets (●).

3.1.2. Three Inlets Strategy—Glass Microreactor

As can be noticed, an additional component (SDS) was introduced into the system in the previous experiment. Even though this component does not have a negative effect on the production process (no enzyme inhibition), it presents a problem for the product purification step, since it has to be removed from the final product. An additional problem was the enzyme that was a part of the oil phase, and at the end, a part of the biodiesel. In this way, it was not possible to reuse the enzyme (i.e., via recirculation) and it also had to be removed from the final product. In order to avoid these

problems, a microreactor with three inlets was proposed. All microreactor characteristics (volume, length, depth, width, and outlet) were the same as for the microreactor with two inlets, for the purpose of comparing the impact of the feeding strategy. Before the experiment was performed, it was again crucial to define the inlet strategy, which means defining the position of each component inlet.

When working with a microreactor with three inlets, there are three possibilities to introduce all three components of a reaction mixture: a) oil, b) methanol, and c) enzyme dissolved in buffer are fed in the middle channel and the other two components are fed from the edge inlets (Figure 4). In order to maximise the mass transfer, it was necessary to determine the right inlet position for all three components.

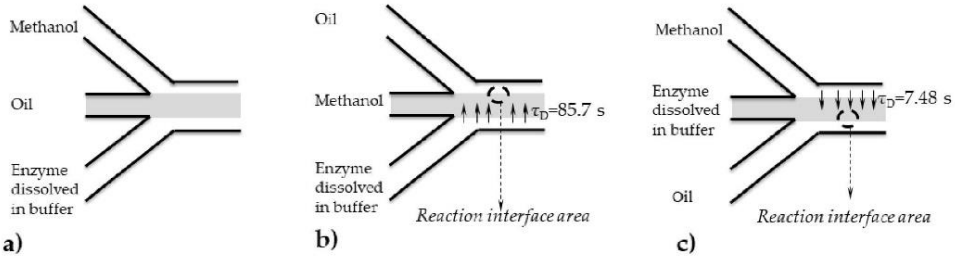

Figure 4. Inlet scheme with three different component inlet strategies, when (**a**) oil, (**b**) methanol, and (**c**) enzyme dissolved in buffer are fed in the middle channel.

The first option (Figure 4a), when oil is fed in the middle, was rejected, since oil is immiscible with both methanol and buffer. This would create an oil barrier that would not allow methanol to diffuse to the enzyme-active site on the opposite sight of the microchannel, so the reaction would be blocked. Based on this, it was assumed that a significant mass transfer will take place only through the interface area between the methanol and the buffer phase if they are placed next to each other. Once this mixture is formed, a reaction would take place on the second interface area, formed between the mixture and the oil. In other to determine which option is better, B or C (Figure 4), diffusion coefficients were calculated using the empirical correlation (Equations (7) and (8)) and data collected from literature.

As shown in Table 2, the diffusion coefficient for methanol was calculated to be higher than the one for the enzyme, meaning it will diffuse faster than the enzyme to another phase. Due to the size of both components, the obtained result was as expected. Additionally, combining the calculated values and partial differential equations (Equations (1)–(6)), without the kinetic part of the equation, a numerical simulation of the concentration profiles in a microreactor channel for methanol and lipase was obtained. Results confirming faster methanol diffusion are presented in Figure 5. As can be seen, the methanol diffuses almost completely to aqueous phase, even at the very beginning of the microchannel, which is important, since all the components have to be available at the interface area between the oil and the aqueous phase for the start of the reaction.

Table 2. Estimated diffusivities and average diffusion time for methanol and lipase at 40 °C.

Solute (S)	Solvent (B)	$D_{S/B} \cdot 10^{-9}$ (m^2/s)	τ_D (s)
Lipase	Water (buffer)	12.40	-
Methanol	Water (buffer)	2.09	7.48
Lipase	Methanol	18.22	85.7
Methanol	Methanol	1.71	-

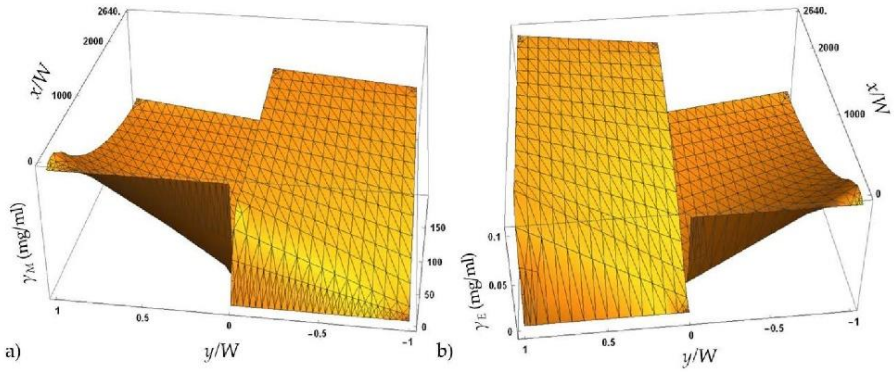

Figure 5. Diffusivity profile for (**a**) methanol and (**b**) enzyme in a microreactor channel for overall flow rate of Φ = 0.03817 µL/min.

Based on the obtained results, it was determined that the proposed option c), where the enzyme dissolved in buffer was placed in the middle, and oil and methanol on edge, was the favourable option.

Before biodiesel synthesis was performed in a microreactor, and in order to calculate diffusion time, the flow profile and flow stability along the length of the microchannel were investigated. The flow profile was monitored for different flow velocities using a microscope (Motic B1-220A, binocular, Weltzar, Germany). By feeding the components in the oil:methanol:enzyme ratio of 10:1.24:1 (based on successfully performed experiments in a macroreactor published elsewhere [29]), it was noticed that the oil phase (due to different physical properties) occupies half of the microchannel volume (Figure 1b).

In order to determine the space of the microchannel occupied by each phase for different flow rates exactly, a series of pictures were taken and analysed for the position at the beginning of the microchannel. As mentioned, it was noticed that the oil phase occupies half of the microchannel width (125 µm), while the mixture of methanol and buffer occupies the other half of the microchannel (125 µm). Due to fast methanol diffusion (calculated by using Equation (9) to be 7.5 s (Table 2)), according to calculated diffusion times, for all the residence times higher than 7.5 s, a two-phase system becomes a monophase system, with a width of 125 µm. As for the flow profile, a parallel, mostly stable fluid flow was developed from the entrance to the exit of the microchannel. Due to high oil viscosity, slight instabilities were noticed in different periods, but the fluid flow quickly become stable again.

Based on all results, it can be concluded that a favourable residence time for the reaction is over 10 s. In that time, both substrates are positioned closely (on the interface area, A = 1.66 nm^2, in the middle of the channel) and are available to the enzyme-active site so the reaction can be performed.

The results of the lipase catalysed biodiesel synthesis in a microreactor with three inlets are presented in Figure 3. A FAME yield of up to 32% was reached for the residence time of 20 min. In comparison, when the reaction was performed in a batch reactor (V = 250 mL), the same FAME yield was achieved after 1.5 h. Comparing the obtained results with the results from when a two-inlet microreactor was used, the same trend was noticed for all residence times, indicating that there is no difference in microreactor performance despite the inlet feeding strategies. Considering all the advantages of separate feeding for each component: no addition of new components, stable flow from the entrance to the exit of the microchannel, which makes the separation of biodiesel from the other component and consequently easier purification and potential enzyme recirculation possible, a microreactor with three inlets is the better choice.

3.1.3. Three Inlets Strategy—PTFE Microreactor

Even though both reactions performed in glass microreactors showed promising results in comparison to the batch reactor, one major problem was noticed during both experiments. Due to the small dimensions of the microchannels, clogging occurred, making the further work impossible. According to Poe et al. [37], when working with small diameter solids (for example enzyme dispersion) in a microreactor, or with highly viscous solvents (like oil), clogging can occur. In order to resolve this problem, a PTFE tubular microreactor with a larger diameter (1 mm) was tested for biodiesel production. The same microreactor type was successfully used in one of previous experiments [18] and no clogging formation was noticed.

Based on the results obtained for the glass microreactor in this research, the three inlets strategy, where the enzyme dissolved in buffer was placed as the middle inlet stream, was implemented for the PTFE microreactor. The obtained results are presented in Figure 3. The FAME yield of 8.9% was noticed for the residence time of 3 h, which was significantly lower in comparison to both glass microreactors. The reason for this is the different hydrodynamic and mass transfer rate that increases with the reduction of the channel size. On the other hand, if the reaction is performed in a PTFE tubular microreactor, but with a high excess of methanol, a FAME yield of 98% can be obtained in 2 h [18]. This indicated that the proposed process needs additional optimization.

In order to do so, a 2D mathematical model was developed considering the convection in the flow (x) direction, the diffusion in two directions (x and y), and the kinetics of the enzyme catalysed reaction (Michaelis–Menten kinetics) (Equations (1)–(6)).

In order to simplify the mathematical model, some assumptions were made:

1. The reaction occurs in the interphase area between phases.
2. Even though triacylglycerols, diacylglycerols, monoacylglycerols, and free fatty acids are present in the mixture, they could be treated as a single constituent [38].
3. Methanol is the main inhibitor of the enzyme, but due to the fact that the reaction is performed in a continuously operated tubular microreactor where methanol is constantly removed from the mixture, the inhibition effect was neglected [39].
4. The limiting step of the reaction was considered to be hydrolysis, but according to literature, if the percentage of water in the process is between 2–20%, the reaction is shifted towards transesterification. In the present research, the water content was calculated to be below 8% (w/w), so the hydrolysis reaction was also neglected.
5. It was assumed that the flow is laminar and parallel from the beginning of the microchannel.

The kinetic parameters were estimated from independent experiments performed in a microreactor, the mathematical model was validated and used to estimate the best process conditions for the experiment performed in a PTFE tubular microreactor.

3.2. Kinetic Parameter Estimation

The influence of fatty acids and the methanol concentration on the initial reaction rate was investigated to estimate the kinetic parameters of the Michaelis–Menten kinetic model. The experiments were performed for the residence time of 0.6 min in a continuously operated microreactor. The results are presented in Figure 6.

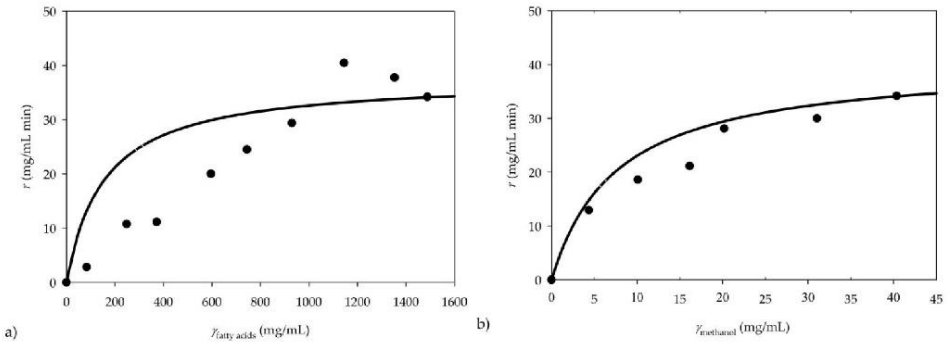

Figure 6. Kinetics of transesterification: dependence of the initial reaction rate on concentration of (**a**) fatty acids and (**b**) methanol.

The kinetic parameters were estimated by non-linear regression, using the experimental results and double substrate Michaelis–Menten kinetics, and determined to be V_{max} = 44.71 ± 1.89 mg/mL min, $K_{m\ fatty\ acids}$ = 155.02 ± 121.86 mg/mL and $K_{m\ methanol}$ = 7.56 ± 2.77 mg/mL. In order to analyse the importance of individual kinetic parameters included in the model, the Fourier amplitude sensitivity test (FAST) was applied. The effects of the kinetic parameters on the reaction rate, *r*, were analysed (data not shown). It was determined that V_{max} has the highest effect on the reaction rate in comparison to other parameters.

The obtained kinetic parameters were compared with data from the literature. Unfortunately, there is limited data on the Michaelis–Menten enzyme kinetics of the transesterification in a batch reactor using lipase from *Thermomyces lanuginosus* for biodiesel production. On the other hand, the kinetics were studied for many other lipases from different sources, but again with a different approach (mostly using Bi-Bi Ping-Pong kinetics) so a comparison was impossible. A detailed kinetic model of biodiesel production by free lipase *Callera Trans L* was described by Firdaus et al. [40], but with a different approach. On the other hand, Cheirsilp et al. [41] investigated the kinetics of transesterification of palm oil and ethanol for fatty acid ethyl ester production using Lipase PS (*Pseudomonas* sp.), Sun et al., [42] studied the kinetics of the transesterification of palm oil and dimethyl carbonate for biodiesel production, and Vey et al. [43] analysed the kinetics of LipozymeR for the transesterification of Jatropha curcas oil.

Due to the absence of literature data, it was assumed that due to the large surface to volume ratios, interfacial interactions play a significant role in the microreactor and define the predominantly laminar flow and stronger diffusion control compared to large scale reactors. As a consequence, the kinetic constants obtained in microreactor experiments could be different from those estimated using data collected from microreactor experiments [44].

3.3. Velocity Model of Multiphase Laminar Flow in a Microreactor

Since it was not possible to observe the flow profile under the microscope for the PTFE tube (not transparent), like it was done for the glass microreactor (Figure 1b prior to model validation, it was necessary to prove the assumption mentioned in Section 3.1.3., meaning that the flow is parallel and stable from the beginning of the microchannel. As mentioned before, for the meshing, one of the default options provided by the Comsol Multiphysics was chosen (Figure 7a), in terms of physics, a controlled finer triangular mesh, which consisted of 48,138 domain elements and 4850 boundary elements after preliminary results with normal a grid, which consisted of 30,804 domain elements and 3877 boundary elements, provided less accurate results. Additionally, an extra fine and an extremely fine mesh were tested, and the results showed no difference from the finer mesh which was selected. The results, in term of the velocity profile in the microchannel, are presented in Figure 7b.

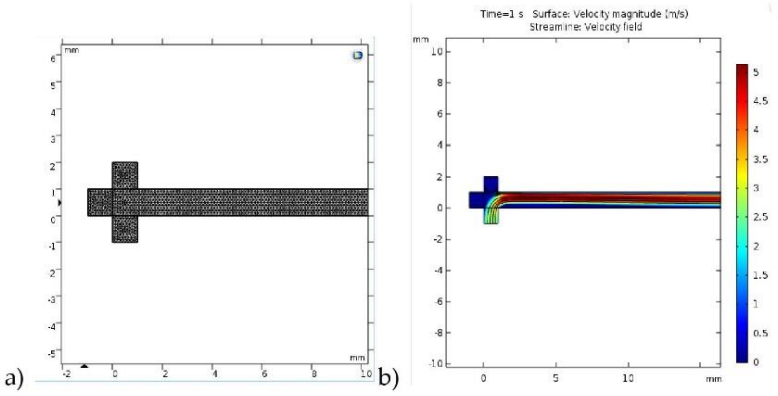

Figure 7. Computational fluid dynamics (CFD) simulation of velocity profile in microsystem (**a**) grid used for the simulation, and (**b**) velocity profile.

Since the initial flow rates of the three phases that were introduced into the microchannel differ, the velocity profiles provided details on how they interconnect. The lower (bottom) inflow represents the oil phase with the initial flow rate of 2.6 m/s, while the left inflow is the water phase with the initial flow rate of 0.26 m/s, and the upper (top) inflow is the methanol phase with the initial flow rate of 0.33 m/s. Even though the velocity streamlines are directed to the top of the microchannel at the start of the microchannel due to the fact that the oil phase had almost 10-fold higher initial value, after a certain length of microchannel, an almost normal flow throughout the microchannel is observed in terms of the even distribution of velocities, slightly leaning towards the top of the microchannel, which is to be expected. This sort of a simulation can sometimes be of great importance, not only to observe the velocities, but also to see whether there are dead spaces in the microchannel, like in this case with the small portion of the lower microchannel after the oil inflow.

3.4. Model Validation

In order to validate the proposed mathematical model (Equations (1)–(6)), two independent experiments preformed in a microreactor were used. Two different inlet concentrations of methanol were used, where a concentration of 40.36 mg/L was introduced to the microreactor in experiment 1, and the inlet methanol concentration was 10-fold higher in experiment 2 [18].

The experimentally obtained data was compared with the model simulation results and it can be noticed that the model describes the trend of the experimental data very well (Figure 8).

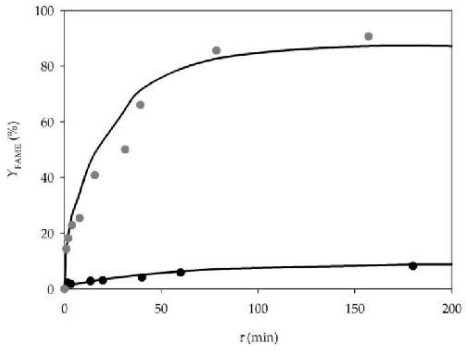

Figure 8. Validation of proposed mathematical model for lipase catalysed transesterification in a PTFE microreactor for different inlet methanol concentrations; 403.6 mg/L (●), 40.36 mg/L (●), model (—).

Based on the experimental results and the model predictions, the obtained model could be used for further process optimisation.

3.5. Process Optimization

The proposed mathematical model was used for the optimisation of the transesterification process catalysed by lipase, in order to collect data for further research. Model-based process optimisation is a fast and effective tool for the prediction of influence of different process parameters on process efficiency. The same approach was previously described by Santana et al. [19], where a mathematical model was used for the simulation of sunflower esterification with ethanol in the presence of sodium hydroxide.

In theory, there are several process parameters that could be altered in order to enhance the process productivity. The first one is certainly the effect of residence time. Therefore, the effect of residence time on process efficiency was analysed first. As shown in Table 3, no significant change in transesterification efficiency was noticed by prolonging residence time.

Table 3. Optimisation of the process by model simulation (values in brackets are results obtained from laboratory experiments).

Effect of Residence Time		Effect of Enzyme Concentration		Effect of Oil to Methanol Ratio	
τ (min)	Y_{FAME} (%)	γ_E (mg/mL)	Y_{FAME} (%) (τ = 180 min)	Oil:Methanol Molar Ratio	Y_{FAME} (%) (τ = 180 min)
180	8.849 (8.18)	0.1	8.849 (8.18)	1:3.4	8.849 (8.18)
270	9.729	0.2	9.505	1:17	40.938
360	10.245	0.3	10.282	1:34	87.179 (89.56)
450	10.583		-		

Another parameter that has an effect on process productivity is enzyme concentration. Model simulation results showed the positive influence of the enzyme concentration increase on the FAME content. An increase from 8.849% to 10.282% was achieved when the enzyme concentration in the inlet stream was increased from 0.1 mg/mL to 0.3 mg/mL. On the other hand, considering the price of the enzyme, this increase in yield is not sufficient to justify the application of larger amounts of the enzyme.

As presented in Figure 8, by comparing the yields obtained for the molar ratio of 1:34 [18] and for the ratio of 1:3.4, it is obvious that by enhancing the oil to methanol ratio, a higher FAME yield in shorter residence time can be achieved. As can be seen from the data shown in Table 3, the increase of the molar ratio of oil to methanol has the biggest impact on the FAME yield, in comparison to other process parameters. Thus, as a reference for future work, additional research on the effect of the oil to methanol ratio is recommended.

4. Conclusions

In this research, edible sunflower oil and methanol were used as substrates and lipase from *Thermomyces lanuginosus* (Lipolase L100) was used as a catalyst for biodiesel synthesis. Experiments were performed in microreactors equipped with two or three inlets, and correspondingly, two different feeding strategies were used. For the residence time of 32 min, fatty acids methyl esters (FAME) yield was higher than 30% for the experiment performed in a microreactor equipped with three inlets. For comparison, when the reaction was performed in a batch reactor (V = 250 mL), the same FAME yield was achieved after 1.5 h. On the other hand, this microreactor type was discarded due to clogging and a PTFE tubular microreactor was proposed as a better solution for biodiesel production. The proposed mathematical model predicted the trend of enzyme catalysed transesterification in a microreactor very well and it was used for further process optimisation. The mathematical model simulation results indicate that the residence time is the most significant process parameter.

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Nomenclature

A	Interface area (mm^2)
c	Concentration (mmol/L)
$D_{S/B}$	Diffusion coefficient (m^2/s)
F_v	Volume force vector (N/m^3)
K_m	Michaelis–Menten constant (mmol/L)
L	Microchannel length (m)
M_B	Molecular weight of solvent (g/mol)
p	Pressure (Pa)
T	Temperature (°C, K)
v	Linear velocity (m/s)
u	Velocity vector (m/s)
V	Reactor volume (µL)
V.A.	Volumetric activity (U/mL)
V_{max}	Maximal reaction rate (U/mL)
V_B	Molar volume of the solute (mL/mol)
V_S	Molar volume of the solvent (mL/mol)
x	Microchannel length (mm)
y	Microchannel width (mm)
Y	FAME yield (%)
W	Half-width of microchannel (mm)
Greek letters	
ρ	Solution density (kg/m^3)
η_B	Dynamic viscosity (kg/(m·s))
ξ	Dimensionless independent variables, x/W
τ	Residence time (s)
τ_D	Diffusion time (s)
υ_ξ	X-directional velocity (m/s)
Φ	Flow rate (µL/min)
ψ	Dimensionless independent variables, y/W
Abbreviations	
aq	Aqueous phase
B	Buffer
E	Enzyme
FA	Fatty acids
i	Inlet
M	Methanol
MetOH	Methanol phase
S	Solute

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Paper 6

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Model-to-model: Comparison of mathematical process models of lipase catalysed biodiesel production in a microreactor

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ABSTRACT

Based on the experimental results and model simulation it was shown that Bi-Bi Ping Pong mathematical process model is not suitable for the description of the lipase catalysed biodiesel production in a microreactor. Therefore, three additional mathematical process models were proposed. Prior to transport model development, the reaction rates were described with double substrate Michaelis-Menten kinetics, Bi-Bi Ping-Pong kinetics, and Hill kinetics. The Hill kinetic model was proposed as the best kinetic model based on the model selection criterion. In order to validate the proposed mathematical process models, biodiesel synthesis in a microreactor was performed at four different initial process conditions. In all validation experiments, free fatty acid concentration and enzyme concentration were kept constant and the oil to methanol ratio in the inlet streams was altered. An increase of biodiesel yield was observed for the higher methanol concentration in the system. If a large excess of methanol was used (oil to methanol ratio 1:90) the yield was higher than 90% for the residence time of only 40 min. In comparison to the batch process, where the yield of 96% was achieved for 48 h, this was a significant improvement. Two out of three proposed mathematical process models described experimental data very well for all analysed residence times. Considering the level of complexity and accuracy, a mathematical process model of steady-state two parallel plug flow reactors was proposed as the optimum solution for the mathematical description of enzymatic biodiesel synthesis performed in a microreactor.

1. Introduction

The development of sustainable processes/experiments is time, manpower, and money consuming. In order to justify sustainability even at the laboratory scale, mathematical modelling is being implemented in the overall experiment development/design even at the very beginning. The main task of modelling is to design experiments in order to replace hours spent in the laboratory with rapid computer simulations to get the right predictions of process behaviour. Nowadays, the major points in development are sustainability and the use of environmentally friendly technologies and compounds. A widely used approach in this direction is replacing chemical processes with bioprocesses wherever that possibility exists. Although they are more challenging to develop and design, the enzymatic reaction path offers some advantages compared to chemical reactions. The most important ones are milder reaction conditions, less energy consumption, possible reduced operational cost, while separation and purification of the product (and by-products) is somewhat easier (Guldhe et al., 2015).

Transesterification is the most prominent technology for biodiesel production aside from micro-emulsification, pyrolysis, and direct blending; mostly due to a better quality of produced fuel (Gebremariam and Marchetti, 2017). Regardless of the different industrial biodiesel production processes available, there is still a shortage of biodiesel on the market. The enzymatic transesterification processes from various oil and fat feedstock types are nowadays more and more used in biodiesel production. Amongst all available commercial enzymes, lipases are proposed by researchers as an alternative to conventional chemical catalysts in biodiesel production, thus forming new green chemistry procedures. They are one of the most used commercial biocatalysts, present in cosmetics, food processing, chemical synthesis, and pharmaceutical and detergent industries. The reason behind this broad versatility is a dual hydrolytic and synthetic activity, which is the foundation for carrying out the reactions of esterification (of free fatty acids – FFA) and transesterification (of oils-triglycerides – TG) (Shah et al., 2003).

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List of symbols and abbreviations

Symbols

$D_{S/B}$	diffusion coefficient, m^2/s
K	equilibrium constant, -
K_D	rate constant for the hydrolysis of diacylglycerols, g/mmol h
K_{Es}	rate constant for the hydrolysis of fatty acid methyl esters, g/mmol h
K_M	rate constant for the hydrolysis of monoacyglycerols, g/mmol h
K_m	saturation constant, mg/mL
K_P	distribution coeffcient, -
K_T	rate constant for the hydrolysis of triacylglycerols, g/mmol h
L	microchannel lenght, m
M_B	molecular weight of solvent, g/mol
n	exponent, -
r	reaction rate, mg/mL min
T	temperature, °C, K
V	volume, mL
$V.A.$	volumetric activity, U/mL
V_B	molar volume of the solute, mL/mol
V_B	molar volume of the solute, mL/mol
V_D	rate constant for the esterification s of diacylglycerols, g/mmol h
V_{Es}	rate constant for the esterification of fatty acid methyl esters g/mmol h
V_M	rate constant for the esterification of monoacyglycerols, g/mmol h
V_{max}	maximal reaction rate, mg/mL min
V_S	molar volume of the solvent, mL/mol
V_S	molar volume of the solvent, mL/mol
V_T	rate constant for the esterification of triacylglycerols, g/mmol h
W	half-width of microchannel, mm
x	microchannel length, mm
y	microchannel width, mm

Greek letters

ρ	density, g/mL
η	kinematic viscosity, Pa s
γ	mass concentration, mg/mL
ξ	dimensionless independent variables, x/W, -
ψ	dimensionless independent variables, y/W, -
τ	residence time, s
υ	x-directional velocity, m/s
Φ	total flow rate, µL/min

Abbreviations

aq	aqueous phase
B	solvent
E	enzyme
FA	fatty acid
FAME	fatty acid methyl ester
FFA	free fatty acid
G	glycerol
i	inlet
M	methanol
S	solute
TG	oil-triglyceride

Aside from changing the catalyst in biodiesel synthesis, there is another new emerging trend where conventional processes, mostly batch processes, are being replaced by continuous processes. The big advantage of shifting to flow synthesis is better process control, cost and energy reduction, etc. To emphasize the benefits of those systems even more, microreactor technology has been recognised as a potential solution for the intensification of biodiesel production (Xie et al., 2012). Microreactor technology has numerous advantages over conventional continuous processes. Some "external" characteristics are better process control due to their size, portability, and easier manageability, reduced footprint of the reactor, flexibility in capacity and design, which is a special subject of interest, where forming new production concepts through numbering-up instead of scaling-up occurs. Regarding "internal" characteristics, large surface to volume ratios are very important, since they provide very effective heat and mass transfer (Žnidaršič-Plazl and Plazl, 2009). An additional advantage of microreactors is the small dimension of channels, most commonly in the range of 10–100 μm. In that micro-region, diffusion limitations of enzymes and substrates could be reduced (Tušek et al., 2012a).

When talking about the design of enzyme catalysed biodiesel production, there are a lot of initial conditions that are known to be important, such as reactor type, reactor dimensions, initial substrate concentrations, flow rates, etc. Furthermore, a very important part in the development of enzyme catalysed biodiesel production is the knowledge about enzyme kinetics. Enzyme kinetic models, with the addition of mass balances, represent a complete picture of the enzyme reaction path and the base of enzymatic bioprocess design (Vasić-Rački et al., 2003). If the mathematical process models are set in a way in which they can describe the experimental data in a satisfactory way, they can provide guidelines for future experimental work through simulated reaction progress for different conditions (Firdaus et al., 2016).

Even though the synthesis of biodiesel catalysed by lipase is of great industrial interest and has been present in the literature (Soumanou and Bornscheuer, 2003; Iso et al., 2001; Chen et al., 2009; J. Sun et al., 2013), most of the kinetic studies cover only the process of esterification of FFA (Janssen et al., 1999; Marty et al., 1992; J.B.A. van Tol et al., 1995; J.B.A. van Tol et al., 1995; Gumel and Annuar, 2016). Some of the breakthroughs in the area of lipase catalysed transesterification are studies by Liu et al. (Liu et al., 2014), where the kinetic model of biodiesel production from waste cooking oil was established. Firdaus et al. (Firdaus et al., 2016) also developed the transport model for evaluating different reaction scenarios for biodiesel production. However, these two models were set-up based on batch experiments. To develop a mathematical process model in a microreactor, it is necessary to determine kinetic parameter values from microreactor experiments. Although the kinetics should be the same in the same processes, no matter the reactor type, if the collected data reflects the true kinetics of a process, this is usually not the case. The reason is that micro-scale differs from conventional macro-scale, in terms of different surface to volume ratios, shorter residence time, and higher mass transfer rate (Jensen, 2001), overall, different transport phenomena are presented in those types of reactors. In other words, if the kinetics are different across reactor scales, that indicates that measured kinetics are apparent, a combination of true kinetics lumped with one or several transport phenomena, respectively. Even if it seems that developing a process model for microreactor systems would be a challenge, previous successful work done by Tišma et al. (Tišma et al., 2009), where the two-dimensional mathematical process model was developed, gives valuable insight on how to address this challenge.

In one of our previous studies (Gojun et al., 2019), successful biodiesel production was performed in a microreactor. In order to optimise the process , kinetic parameters were estimated based on the assumption that the enzyme follows Michaelis-Menten kinetics and a 2D mathematical transport model was developed. In order to simplify the process model, several assumptions were made

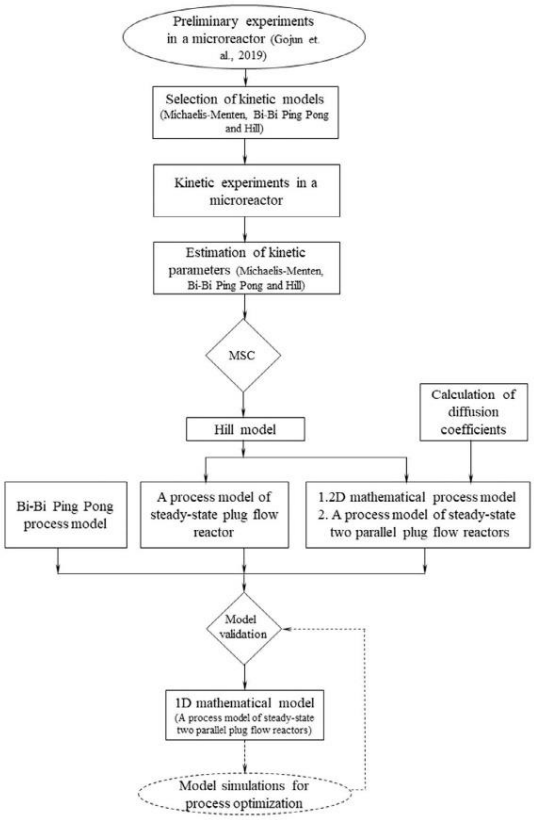


Fig. 1. The methodology flow chart.

together with the one that there is no reverse reaction (hydrolysis). In the present study, a microchannel width was downsized to 500 μm in order to enhance mass transfers and reduce diffusion time. As said previously, conditions change by changing the scale. Because of that, it was necessary to estimate the kinetic parameters again. To describe the process as realistically as possible, kinetic parameters of the reverse reaction - biodiesel hydrolysis, were also estimated in this study. The reaction rates were modelled with a double substrate Michaelis-Menten kinetics, Bi-Bi Ping-Pong kinetics, and Hill kinetics, and the results were compared. Finally, three different mathematical process models, one 2D and two 1D were developed, validated, and the best model for describing biodiesel transesterification in a microreactor was proposed. Model validation was performed using data from independent experiments, in which oil to methanol ratio was altered. Additionally, the obtained results were compared with the Bi-Bi Ping-Pong mathematical process model commonly used for mathematical modelling of biodiesel synthesis (Janssen et al., 1999; Liu et al., 2014; Al-Zuhair et al., 2007; Haigha et al., 2014; Gunawan et al., 2017; Azócar et al., 2014; Price et al., 2014; S. Sun et al., 2013) (Fig. 1).

2. Materials and methods

2.1. Materials

Edible sunflower oil (Zvijezda, Croatia) was purchased in a local store. Lipolase 100 L from *Thermomyces lanuginosus*, F.A.M.E. mix GLC-10, *iso*-amyl alcohol, and *iso*-octane were purchased from Sigma-Aldrich (Austria). Hydrochloric acid, methanol, and tris(hydroxymethyl)aminomethane (TRIS) were purchased from BDH Prolabo (United Kingdom). *p*-nitrophenyl acetate was purchased from Acros Organics (Fischer-Scientific, Belgium). Potassium dihydrogen phosphate (KH_2PO_4) was purchased from Lach:ner (Czech Republic), and dipotassium hydrogen phosphate (K_2HPO_4) was purchased from Merck (Germany). *Iso*-propanol, choline chloride, acetone, *n*-hexane, and acetonitrile were purchased from VWR Chemicals (BDH Prolabo, France). Glycerol was purchased from Kemika (Croatia) and ethanol from Gram mol d.o.o. (Croatia). All chemicals used were of analytical grade with the exception of edible sunflower oil.

2.2. Methods

2.2.1. Lipase assay

The enzyme activity test was based on the solvolysis of 0.0375 mol/L of *p*-nitrophenyl acetate (*p*NPA) (Gojun et al., 2019). Firstly, the sample (100 μL) was added to the 0.05 mol/L of TRIS-HCl buffer pH 8 (3900 μL) and the mixture was homogenized. Subsequently, 950 μL of the mixture was pipetted into the UV-cuvette which was previously thermostated to 40 °C in a water bath (Thermomix 1420, Braun, Germany). The reaction was started by adding 50 μL of 0.0375 mol/L *p*NPA which was dissolved in acetonitrile. Enzyme activity was determined spectrophotometrically (UV-1601, Shimadzu, Japan) by measuring the changes of absorbence at the wavelength of 400 nm, with the total determination time of 20 s. To ascertain repeatability, all measurements were made in triplicate.

2.2.2. Measurement of fatty acid methyl esters and glycerol concentrations

Fatty acid methyl esters (FAME) and glycerol concentrations were measured using a gas chromatograph (Shimadzu GC-2014, Japan) equipped with FID and the Zebron ZB-wax GC capillary column (length 30 m, I.D. 0.53 mm and film thickness 1.00 μm, Phenomenex, USA) using a method described elsewhere (Budžaki et al., 2015). Briefly, measurements start at the temperature of 180 °C for 1 min, with the column heating up to 230 °C, at the rate of 5 °C/min, while the FID detector works at 240 °C. The total duration of analysis for each sample was 15 min, and nitrogen was used as the carrier gas at the rate of 1.97 mL/min. The F.A.M.E. mix GLC-10 was used as a standard. *n*-heptane was used as the solvent for the preparation of the samples, if the FAME concentration was analysed while ethanol was used as a solvent for preparation of samples, if glycerol was analysed. The sample was homogenized, filtered (non-sterile Hydrophobic PTFE Syringe filters, pore: 0.45 μm, diameter: 25 mm, Macherey-Nagel GmbH and Co., Germany), and analysed on the GC. To confirm repeatability, all measurements were performed in triplicate.

2.2.3. Influence of organic solvents on enzyme activity

Organic (O) solvents (*n*-hexane, *n*-heptane, and *iso*-octane) were mixed separately with a suspension of lipase enzymes (γ_E = 0.1 mg/mL (enzyme concentration was determined by the Bradford method), V.A. = 89,000 U/mL), suspended in a 0.1 mol/L potassium-potassium phosphate buffer pH 7.4 (P) in the volume ratio of P:O = 1: 0.1, 1: 0.3, 1: 1, 1: 2.5, and 1: 9. Following incubation at 40 °C for 10 min, the enzyme activity was measured following the method described in Section 2.2.1.

2.2.4. Biodiesel synthesis in a batch reactor

The batch biodiesel production process was performed in a double-walled glass reactor (V = 250 mL). According to the method described by Budžaki et al. (2015), the synthesis was set-up with

edible sunflower oil and methanol, using Lipolase 100 L as a catalyst ($V_{reaction\ mixture}$ = 200 mL). Commercial enzyme Lipolase 100 L was suspended in a 0.1 mol/L potassium-potassium phosphate buffer pH 7.4, in a ratio of 1:10, so the initial lipase concentration was 0.1 mg/mL. The mass ratio of oil: methanol: lipase was 1: 0.174: 0.1. Under those conditions, the molar ratio of oil to methanol was 1:3.4, in order to secure a slight surplus of methanol in the reaction. The experiment was conducted for two days (48 h) with constant stirring (600 rpm) and temperature (40 °C). After production, biodiesel was purified in two process steps.

The first step in biodiesel purification was the transfer of the mixture obtained in the batch synthesis to a separation funnel. After 24 h, two layers were formed. The upper layer was mostly a mixture of FAME - biodiesel, while the lower layer contained glycerol, enzymes, water, and residual methanol. In the second step, biodiesel was purified using a deep eutectic solvent (DES). Biodiesel (the upper layer) was transferred from the separation funnel into a double-walled tank (V = 250 mL), where it was mixed with an equal mass of DES.

The DES was prepared by mixing accurately weighed masses of choline chloride and glycerol in a molar ratio of 1: 3.0. A beaker, which contained the required masses, was placed on a magnetic stirrer and thermostated at 50 °C. The stirring (200 rpm) was carried out for about 60 min, as long as a transparent and homogenous liquid was formed (ρ (T = 25 °C) = 1.225 g/mL, ρ (T = 40 °C) = 1.215 g/mL, η (T = 25 °C) = 0.283 Pa s, η (T = 40 °C) = 0.098 Pa s). The DES was then left to cool to room temperature and used for biodiesel purification.

Biodiesel purification with DES was carried out on a magnetic stirrer at 200 rpm and 50 °C for approximately 3 h (Petračić et al., 2017). The mixture was then again placed in a separation funnel for 24 h and the upper layer containing pure biodiesel was separated. Purified biodiesel was used in experiments performed in a microreactor, where the influence of initial biodiesel concentration on the reaction rate of hydrolysis was analysed.

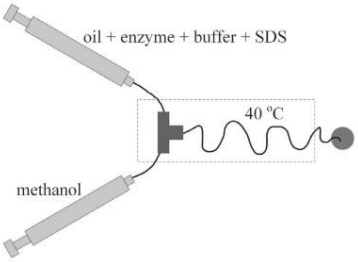
Water and enzymes were removed by filtering purified biodiesel through hydrophobic filters (non-sterile Hydrophobic PTFE Syringe filters, pore: 0.25 μm, diameter: 25 mm, Macherey-Nagel GmbH and Co., Germany).

2.2.5. Biodiesel synthesis in a microreactor

Biodiesel synthesis was carried out in a microreactor with two inlets (T-shape) and an internal volume of 235.62 μL (length: width: depth = 120 cm: 500 μm: 500 µm). Four experiments were performed with the different initial methanol concentration in the inlet stream (Table 1). In all experiments, an emulsion of oil ($\gamma_{fatty\ acids}$ = 946.52 mg/mL) and enzyme (γ_E = 0.1 mg/mL, V.A. = 89,000 U/mL) dissolved in a 0.1 mol/L potassium-potassium phosphate buffer with pH 7.4 was placed in one syringe and methanol was placed in the second syringe (Šalić et al., 2018). The emulsion was prepared by mixing oil and enzyme dissolved in a buffer, at the volume ratio 10:1. The emulsifier SDS was added to the 0.1 g/L concentration and the mixture was mixed in a laboratory shaker (Tehtnica, Vibromix 313EVT, Železnik, Slovenia) for 25 min at 600 rpm. The prepared emulsion was stable for more than 7 days (Šalić et al., 2018). The filled syringes were placed on pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA) and connected with PTFE tubes to the microreactor. All experiments were performed at 40 °C by submerging the microreactor in a water bath with a heat regulation system (Thermomix 1420, Braun, Germany) to achieve optimal enzyme activity.

In each experiment, inlet stream flow ratios were set based on required oil: methanol molar ratio and kept constant throughout the experiment, while total flow rates changed (Φ = 1.3–702 µL/min). That way, the influence of methanol on FAME formation at different total flow rates (retention times) could be examined.

Table 1
Experimental set-up for biodiesel synthesis in a microreactor, concentrations of methanol and oil: methanol molar ratio.



	methanol concentration, mg/mL	oil: methanol molar ratio
Experiment 1	30.60	1: 3.4
Experiment 2	90.10	1: 10
Experiment 3	246.17	1: 30
Experiment 4	836.97	1: 90

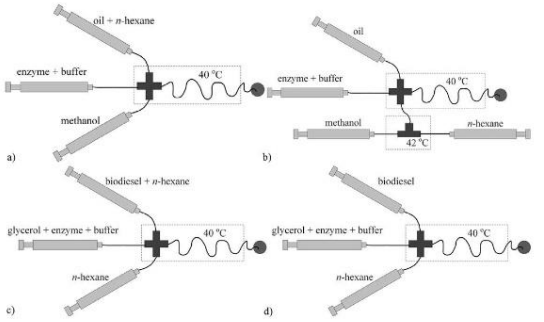


Fig. 2. Experimental set-ups used for kinetic measurements in a microreactor. Determination of the effect of a) fatty acids (oil), b) methanol, c) FAME (biodiesel) and d) glycerol concentration on reaction rate.

Samples were collected in vials with the Marmur solution (chloroform: *iso*-amyl alcohol = 24: 1) and cooled on ice to deactivate the enzyme and stop the reaction at the exit of the microreactor.

2.2.6. Kinetic parameter estimation

In order to estimate the kinetic parameters, the initial reaction rate method was adopted for experiments performed in a microreactor. Due to the fact that lipase has the ability to simultaneously catalyse hydrolysis, esterification, and transesterification (Budžaki et al., 2015), it was necessary to estimate the kinetic parameters of both reactions (transesterification and hydrolysis). When examining the effect of fatty acids and methanol concentration on the rate of the transesterification reaction, two experiments were carried out. Firstly, concentrations of fatty acids were changed in the range of 0 – 946.52 mg/mL while keeping the methanol concentration constant ($\gamma_{methanol}$ = 30.60 mg/mL). Different concentrations of fatty acids were obtained by dilution with *n*-hexane (Fig. 2a). In the second experiment, the concentration of fatty acids was kept constant ($\gamma_{fatty\ acids}$ = 946.52 mg/mL) and methanol concentrations were varied in the range of 0 – 30.60 mg/mL. Different concentrations of methanol were also obtained by dilution with *n*-hexane. Since methanol and *n*-hexane are not miscible at room temperature, it was necessary to preheat them at 42 °C to obtain a stable, homogenous mixture (Albaiti et al., 2016). For that purpose, an additional microreactor

(length: width: depth = 20 cm: 500 μm: 500 μm) was integrated into the system (Fig. 2b).

Also, a reverse reaction, hydrolysis, was investigated in two additional experiments by monitoring the influence of biodiesel and glycerol concentration on the rate of the hydrolysis reaction. In both experiments, *n*-hexane was introduced as a third phase to maintain the hydrodynamics of a system similar to that in the transesterification reaction. Biodiesel concentrations were changed in the range of 0 – 909.81 mg/mL, with the constant glycerol concentration of γ_{glycerol} = 90.00 mg/mL (Fig. 2c), while in the second case, glycerol concentrations were varied in the range of 0 – 90.00 mg/mL with the constant biodiesel concentration of $\gamma_{\text{biodiesel}}$ = 909.81 mg/mL (Fig. 2d). Different concentrations of biodiesel were obtained by dilution with *n*-hexane, while different glycerol concentrations were obtained by dilution with the reaction buffer. The retention time of 40.41 s was selected in all measurements in order to keep the conversion lower than 10%, which is one of the requirements of the initial velocity method.

2.2.7. Data processing

Non-linear regression was used for kinetic parameter estimation. Analysis was based on data collected from independent experiments in a microreactor. Numerical values were estimated by fitting the kinetic model to the experimental data by applying the least square method (incorporated in the software package SCIENTIST (MicroMath Scientist®, 3.0, MicroMath Scientific Software, Salt Lake City, UT, USA)). The reactor model simulation and validation were performed in Mathematica 10 (Wolfram Research, Champaign, IL, USA).

2.3. Mathematical modelling

2.3.1. Kinetic models for the reaction rates

The reaction rate of biodiesel transesterification was modelled with a double substrate Michaelis-Menten kinetics (Eq. (1)), Bi-Bi Ping Pong kinetics (Eq. (2)) and Hill kinetics (Eq. (3)) (Bisswanger, 2002).

$$r = V_{max} \cdot \frac{\gamma_A \cdot \gamma_B}{(K_{m,A} + \gamma_A) \cdot (K_{m,B} + \gamma_b)} \tag{1}$$

$$r = V_{max} \cdot \frac{\gamma_A \cdot \gamma_B}{K_{m,A} \cdot \gamma_A + K_{m,B} \cdot \gamma_B + \gamma_A \cdot \gamma_B} \tag{2}$$

$$r = V_{\max} \cdot \frac{\gamma_A^{n_1} \cdot \gamma_B^{n_2}}{\left(K_{m,A}^{n_1} + \gamma_A^{n_1}\right) \cdot \left(K_{m,B}^{n_2} + \gamma_B^{n_2}\right)} \tag{3}$$

2.3.2. 2D mathematical process model

The process of enzymatic biodiesel transesterification was described by a 2D mathematical process model including convection in the flow (x) direction, diffusion in two directions (x and y), and kinetics of the reaction (Gojun et al., 2019; Šalić et al., 2013; Žnidaršič-Plazl and Plazl, 2007):

Dimensionless partial differential equations for steady-state conditions with the associated boundary conditions describing the biodiesel production are as follows Eq. (4)-((13)):

- methanol in the aqueous phase (phase1):

$$\begin{aligned} \nu_1 \frac{\partial \gamma_{M,1}}{\partial \xi} = \frac{D_{M/aq}}{W} \cdot \left(\frac{\partial^2 \gamma_{M,1}}{\partial \xi^2} + \frac{\partial^2 \gamma_{M,1}}{\partial \psi^2} \right) \\ -W \cdot V_{\max 1} \cdot \frac{\gamma_{E,1} \cdot \gamma_{M,1}^{n1,1} \cdot \gamma_{FA,1}^{n2,1}}{\left(K_{m,M}^{n1,1} + \gamma_{M,1}^{n1,1}\right) \cdot \left(K_{m,FA}^{n2,1} + \gamma_{FA,1}^{n2,1}\right)} \\ +W \cdot V_{\max 2} \cdot \frac{\gamma_{E,1} \cdot \gamma_{FAME,1}^{n1,2} \cdot \gamma_{G,1}^{n2,2}}{\left(K_{m,FAME}^{n1,2} + \gamma_{FAME,1}^{n1,2}\right) \cdot \left(K_{m,G}^{n2,2} + \gamma_{G,1}^{n2,2}\right)} \end{aligned} \tag{4}$$

$$\gamma_{M,1}(0, \psi) = \gamma_{M,1,i}, \quad -1 \le \psi \le 0$$

$$\frac{\partial \gamma_{M,1}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad -1 \le \psi \le 0$$

$$\gamma_{M,1}(\xi, 0) = K_{P,M} \cdot \gamma_{M,2}(\xi, 0), \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{M,1}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

- methanol in the oil phase (phase 2):

$$\begin{aligned} \nu_2 \frac{\partial \gamma_{M,2}}{\partial \xi} = \frac{D_{M/oil}}{W} \cdot \left(\frac{\partial^2 \gamma_{M,2}}{\partial \xi^2} + \frac{\partial^2 \gamma_{M,2}}{\partial \psi^2} \right) \\ -W \cdot V_{\max 1} \cdot \frac{\gamma_{E,2} \cdot \gamma_{M,2}^{n1,1} \cdot \gamma_{FA,2}^{n2,1}}{\left(K_{m,M}^{n1,1} + \gamma_{M,2}^{n1,1}\right) \cdot \left(K_{m,FA}^{n2,1} + \gamma_{FA,2}^{n2,1}\right)} \\ +W \cdot V_{\max 2} \cdot \frac{\gamma_{E,2} \cdot \gamma_{FAME,2}^{n1,2} \cdot \gamma_{G,2}^{n2,2}}{\left(K_{m,FAME}^{n1,2} + \gamma_{FAME,2}^{n1,2}\right) \cdot \left(K_{m,G}^{n2,2} + \gamma_{G,2}^{n2,2}\right)} \end{aligned} \tag{5}$$

$$\gamma_{M,2}(0, \psi) = 0, \quad 0 \le \psi \le 1$$

$$\frac{\partial \gamma_{M,2}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad 0 \le \psi \le 1$$

$$\frac{\gamma_{M,2}(\xi, 0)}{\partial \psi} = \frac{D_{M/aq}}{D_{M/oil}} \cdot \frac{\gamma_{M,1}(\xi, 0)}{\partial \psi}, \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{M,2}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

- glycerol in the aqueous phase (phase1):

$$\begin{aligned} \nu_1 \frac{\partial \gamma_{G,1}}{\partial \xi} = \frac{D_{G/aq}}{W} \cdot \left(\frac{\partial^2 \gamma_{G,1}}{\partial \xi^2} + \frac{\partial^2 \gamma_{G,1}}{\partial \psi^2} \right) \\ -W \cdot V_{\max 1} \cdot \frac{\gamma_{E,1} \cdot \gamma_{M,1}^{n1,1} \cdot \gamma_{FA,1}^{n2,1}}{\left(K_{m,M}^{n1,1} + \gamma_{M,1}^{n1,1}\right) \cdot \left(K_{m,FA}^{n2,1} + \gamma_{FA,1}^{n2,1}\right)} \\ +W \cdot V_{\max 2} \cdot \frac{\gamma_{E,1} \cdot \gamma_{FAME,1}^{n1,2} \cdot \gamma_{G,1}^{n2,2}}{\left(K_{m,FAME}^{n1,2} + \gamma_{FAME,1}^{n1,2}\right) \cdot \left(K_{m,G}^{n2,2} + \gamma_{G,1}^{n2,2}\right)} \end{aligned} \tag{6}$$

$$\gamma_{G,1}(0, \psi) = \gamma_{G,1,i} = 0, \quad -1 \le \psi \le 0$$

$$\frac{\partial \gamma_{G,1}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad -1 \le \psi \le 0$$

$$\gamma_{G,1}(\xi, 0) = K_{P,G} \cdot \gamma_{G,2}(\xi, 0), \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{G,1}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

- glycerol in the oil phase (phase 2):

$$\begin{aligned} \nu_2 \frac{\partial \gamma_{G,2}}{\partial \xi} = \frac{D_{G/oil}}{W} \cdot \left(\frac{\partial^2 \gamma_{G,2}}{\partial \xi^2} + \frac{\partial^2 \gamma_{G,2}}{\partial \psi^2} \right) \\ +W \cdot V_{\max 1} \cdot \frac{\gamma_{E,2} \cdot \gamma_{M,2}^{n1,1} \cdot \gamma_{FA,2}^{n2,1}}{\left(K_{m,M}^{n1,1} + \gamma_{M,2}^{n1,1}\right) \cdot \left(K_{m,FA}^{n2,1} + \gamma_{FA,2}^{n2,1}\right)} \\ -W \cdot V_{\max 2} \cdot \frac{\gamma_{E,2} \cdot \gamma_{FAME,2}^{n1,2} \cdot \gamma_{G,2}^{n2,2}}{\left(K_{m,FAME}^{n1,2} + \gamma_{FAME,2}^{n1,2}\right) \cdot \left(K_{m,G}^{n2,2} + \gamma_{G,2}^{n2,2}\right)} \end{aligned} \tag{7}$$

$$\gamma_{G,2}(0, \psi) = 0, \quad 0 \le \psi \le 1$$

$$\frac{\partial \gamma_{G,2}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad 0 \le \psi \le 1$$

$$\frac{\gamma_{G,2}(\xi, 0)}{\partial \psi} = \frac{D_{M/aq}}{D_{M/oil}} \cdot \frac{\gamma_{G,1}(\xi, 0)}{\partial \psi}, \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{G,2}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

- enzyme in the aqueous phase (phase 1):

$$v_1 \cdot \frac{\partial \gamma_{E,1}}{\partial \xi} = \frac{D_{E/aq}}{W} \cdot \left(\frac{\partial^2 \gamma_{E,1}}{\partial \xi^2} + \frac{\partial^2 \gamma_{E,1}}{\partial \psi^2} \right) \quad (8)$$

$$\gamma_{E,1}(0, \psi) = 0, \quad -1 \le \psi \le 0$$

$$\frac{\partial \gamma_{E,1}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad -1 \le \psi \le 0$$

$$\gamma_{E,1}(\xi, 0) = K_{P,E} \cdot \gamma_{E,2}(\xi, 0), \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{E,1}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

- enzyme in the oil phase (phase 2):

$$v_2 \cdot \frac{\partial \gamma_{E,2}}{\partial \xi} = \frac{D_{E/oil}}{W} \cdot \left(\frac{\partial^2 \gamma_{E,2}}{\partial \xi^2} + \frac{\partial^2 \gamma_{E,2}}{\partial \psi^2} \right) \quad (9)$$

$$\gamma_{E,2}(0, \psi) = \gamma_{E,2,i}, \quad 0 \le \psi \le 1$$

$$\frac{\partial \gamma_{E,2}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad 0 \le \psi \le 1$$

$$\frac{\gamma_{E,2}(\xi, 0)}{\partial \psi} = \frac{D_{E/aq}}{D_{E/oil}} \cdot \frac{\gamma_{E,1}(\xi, 0)}{\partial \psi}, \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{E,2}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

- fatty acids in the aqueous phase (phase1):

$$v_1 \frac{\partial \gamma_{FA,1}}{\partial \xi} = \frac{D_{FA/aq}}{W} \cdot \left(\frac{\partial^2 \gamma_{FA,1}}{\partial \xi^2} + \frac{\partial^2 \gamma_{FA,1}}{\partial \psi^2} \right) - W \cdot V_{max1} \cdot \frac{\gamma_{E,1} \cdot \gamma_{M,1}^{n1,1} \cdot \gamma_{FA,1}^{n2,1}}{\left(K_{m,M}^{n1,1} + \gamma_{M,1}^{n1,1}\right) \cdot \left(K_{m,FA}^{n2,1} + \gamma_{FA,1}^{n2,1}\right)} + W \cdot V_{max2} \cdot \frac{\gamma_{E,1} \cdot \gamma_{FAME,1}^{n1,2} \cdot \gamma_{G,1}^{n2,2}}{\left(K_{m,FAME}^{n1,2} + \gamma_{FAME,1}^{n1,2}\right) \cdot \left(K_{m,G}^{n2,2} + \gamma_{G,1}^{n2,2}\right)} \quad (10)$$

$$\gamma_{FA,1}(0, \psi) = 0, \quad -1 \le \psi \le 0$$

$$\frac{\partial \gamma_{FA,1}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad -1 \le \psi \le 0$$

$$\frac{\gamma_{FA,1}(\xi, 0)}{\partial \psi} = \frac{D_{FA/oil}}{D_{FA/aq}} \cdot \frac{\gamma_{FA,2}(\xi, 0)}{\partial \psi}, \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{FA,1}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

- fatty acids in the oil phase (phase 2):

$$v_2 \frac{\partial \gamma_{FA,2}}{\partial \xi} = \frac{D_{FA/oil}}{W} \cdot \left(\frac{\partial^2 \gamma_{FA,2}}{\partial \xi^2} + \frac{\partial^2 \gamma_{FA,2}}{\partial \psi^2} \right) - W \cdot V_{max1} \cdot \frac{\gamma_{E,2} \cdot \gamma_{M,2}^{n1,1} \cdot \gamma_{FA,2}^{n2,1}}{\left(K_{m,M}^{n1,1} + \gamma_{M,2}^{n1,1}\right) \cdot \left(K_{m,FA}^{n2,1} + \gamma_{FA,2}^{n2,1}\right)} + W \cdot V_{max2} \cdot \frac{\gamma_{E,2} \cdot \gamma_{FAME,2}^{n1,2} \cdot \gamma_{G,2}^{n2,2}}{\left(K_{m,FAME}^{n1,2} + \gamma_{FAME,2}^{n1,2}\right) \cdot \left(K_{m,G}^{n2,2} + \gamma_{G,2}^{n2,2}\right)} \quad (11)$$

$$\gamma_{FA,2}(0, \psi) = \gamma_{FA,2,i}, \quad 0 \le \psi \le 1$$

$$\frac{\partial \gamma_{FA,2}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad 0 \le \psi \le 1$$

$$\frac{\gamma_{FA,2}(\xi, 0)}{\partial \psi} = K_{P,FA} \cdot \gamma_{FA,1}(\xi, 0), \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{FA,2}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

- FAME in the aqueous phase (phase1):

$$v_1 \frac{\partial \gamma_{FAME,1}}{\partial \xi} = \frac{D_{FAME/aq}}{W} \cdot \left(\frac{\partial^2 \gamma_{FAME,1}}{\partial \xi^2} + \frac{\partial^2 \gamma_{FAME,1}}{\partial \psi^2} \right) + W \cdot V_{max1} \cdot \frac{\gamma_{E,1} \cdot \gamma_{M,1}^{n1,1} \cdot \gamma_{FA,1}^{n2,1}}{\left(K_{m,M}^{n1,1} + \gamma_{M,1}^{n1,1}\right) \cdot \left(K_{m,FA}^{n2,1} + \gamma_{FA,1}^{n2,1}\right)} - W \cdot V_{max2} \cdot \frac{\gamma_{E,1} \cdot \gamma_{FAME,1}^{n1,2} \cdot \gamma_{G,1}^{n2,2}}{\left(K_{m,FAME}^{n1,2} + \gamma_{FAME,1}^{n1,2}\right) \cdot \left(K_{m,G}^{n2,2} + \gamma_{G,1}^{n2,2}\right)} \quad (12)$$

$$\gamma_{FAME,1}(0, \psi) = 0, \quad -1 \le \psi \le 0$$

$$\frac{\partial \gamma_{FAME,1}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad -1 \le \psi \le 0$$

$$\frac{\gamma_{FAME,1}(\xi, 0)}{\partial \psi} = \frac{D_{FAME/oil}}{D_{FAME/aq}} \cdot \frac{\gamma_{FAME,2}(\xi, 0)}{\partial \psi}, \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{FAME,1}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

- FAME in the oil phase (phase2):

$$v_2 \frac{\partial \gamma_{FAME,2}}{\partial \xi} = \frac{D_{FAME/oil}}{W} \cdot \left(\frac{\partial^2 \gamma_{FAME,2}}{\partial \xi^2} + \frac{\partial^2 \gamma_{FAME,2}}{\partial \psi^2} \right) - W \cdot V_{max1} \cdot \frac{\gamma_{E,2} \cdot \gamma_{M,2}^{n1,1} \cdot \gamma_{FA,2}^{n2,1}}{\left(K_{m,M}^{n1,1} + \gamma_{M,2}^{n1,1}\right) \cdot \left(K_{m,FA}^{n2,1} + \gamma_{FA,2}^{n2,1}\right)} + W \cdot V_{max2} \cdot \frac{\gamma_{E,2} \cdot \gamma_{FAME,2}^{n1,2} \cdot \gamma_{G,2}^{n2,2}}{\left(K_{m,FAME}^{n1,2} + \gamma_{FAME,2}^{n1,2}\right) \cdot \left(K_{m,G}^{n2,2} + \gamma_{G,2}^{n2,2}\right)} \quad (13)$$

$$\gamma_{FAME,2}(0, \psi) = \gamma_{FAME,2,i} = 0, \quad 0 \le \psi \le 1$$

$$\frac{\partial \gamma_{FAME,2}\left(\frac{L}{W}, \psi\right)}{\partial \xi} = 0, \quad 0 \le \psi \le 1$$

$$\frac{\gamma_{FAME,2}(\xi, 0)}{\partial \psi} = K_{P,FAME} \cdot \gamma_{FAME,1}(\xi, 0), \quad 0 < \xi < \frac{L}{W}$$

$$\frac{\partial \gamma_{FAME,2}(\xi, 1)}{\partial \psi} = 0, \quad 0 < \xi < \frac{L}{W}$$

2D finite differences method with discretisation on the static equidistant grid was used to solve the proposed system of partial differential equations.

Scheibel empirical correlation (Eq. (14)) (Li and Carr, 1997) was used for the estimation of the methanol and glycerol diffusion coefficients ($D_{S/B}$) at 40 °C

$$D_{S/B} = \frac{8.2 \cdot 10^{-8} \cdot T}{\eta_B \cdot V_S^{1/3}} \left[1 + \left(\frac{3 \cdot V_B}{V_S} \right) \right]^{2/3} \quad (14)$$

The Young empirical correlation (Young et al., 1980) (Eq. (15)) was used for the estimation of the enzyme, fatty acids, and the FAME diffusion coefficient ($D_{S/B}$) at $T = 40$ °C:

$$D_{S/B} = 8.34 \cdot 10^{-8} \left(\frac{T}{\eta_B \cdot M_S^{1/3}} \right) \quad (15)$$

2.3.3. 1D mathematical process model

2.3.3.1. A process model of steady-state two parallel plug flow reactors. A 1D mathematical process model based on an approximation of the microreactor with two parallel plug flow reactors, including the kinetics of the reaction as previously described by Jurinjak Tušek et al. Jurinjak Tušek et al., 2013), was used for the description and prediction of the biodiesel transesterification process in a microreactor. Equations for steady-state conditions are as follows (Eq. (16)-((20)):

- Mass balances for methanol concentration:

$$\begin{aligned}
v_1\frac{\partial \gamma_{M,1}}{\partial x} &= -\frac{D_{M/aq}}{W^2}\cdot(\gamma_{M,1}-\gamma_{M,2})\\
&\quad -W\cdot V_{max1}\cdot\frac{\gamma_{E,1}\cdot\gamma_{M,1}^{n1,1}\cdot\gamma_{FA,1}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_{M,1}^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA,1}^{n2,1}\right)}\\
&\quad +W\cdot V_{max2}\cdot\frac{\gamma_{E,1}\cdot\gamma_{FAME,1}^{n1,2}\cdot\gamma_{G,1}^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME,1}^{n1,2}\right)\cdot\left(K_{m,G}^{n2,2}+\gamma_{G,1}^{n2,2}\right)}\\
v_2\frac{\partial \gamma_{M,2}}{\partial x} &= \frac{D_{M/oil}}{W^2}\cdot(\gamma_{M,1}-\gamma_{M,2})\\
&\quad -W\cdot V_{max1}\cdot\frac{\gamma_{E,2}\cdot\gamma_{M,2}^{n1,1}\cdot\gamma_{FA,2}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_{M,2}^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA,2}^{n2,1}\right)}\\
&\quad +W\cdot V_{max2}\cdot\frac{\gamma_{E,2}\cdot\gamma_{FAME,2}^{n1,2}\cdot\gamma_{G,2}^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME,2}^{n1,2}\right)\cdot\left(K_{m,G}^{n2,2}+\gamma_{G,2}^{n2,2}\right)}
\end{aligned}\tag{16}$$

- Mass balances for glycerol concentration:

$$\begin{aligned}
v_1\frac{\partial \gamma_{G,1}}{\partial x} &= -\frac{D_{G/aq}}{W^2}\cdot(\gamma_{G,1}-\gamma_{G,2})\\
&\quad +W\cdot V_{max1}\cdot\frac{\gamma_{E,1}\cdot\gamma_{M,1}^{n1,1}\cdot\gamma_{FA,1}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_{M,1}^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA,1}^{n2,1}\right)}\\
&\quad -W\cdot V_{max2}\cdot\frac{\gamma_{E,1}\cdot\gamma_{FAME,1}^{n1,2}\cdot\gamma_{G,1}^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME,1}^{n1,2}\right)\cdot\left(K_{m,G}^{n2,2}+\gamma_{G,1}^{n2,2}\right)}\\
v_2\frac{\partial \gamma_{G,2}}{\partial x} &= \frac{D_{G/oil}}{W^2}\cdot(\gamma_{G,1}-\gamma_{G,2})\\
&\quad +W\cdot V_{max1}\cdot\frac{\gamma_{E,2}\cdot\gamma_{M,2}^{n1,1}\cdot\gamma_{FA,2}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_{M,2}^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA,2}^{n2,1}\right)}\\
&\quad -W\cdot V_{max2}\cdot\frac{\gamma_{E,2}\cdot\gamma_{FAME,2}^{n1,2}\cdot\gamma_{G,2}^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME,2}^{n1,2}\right)\cdot\left(K_{m,G}^{n2,2}+\gamma_{G,2}^{n2,2}\right)}
\end{aligned}\tag{17}$$

- Mass balances for enzyme concentration:

$$\begin{aligned}
v_1\frac{\partial \gamma_{E,1}}{\partial x} &= \frac{D_{E/aq}}{W^2}\cdot(\gamma_{E,2}-\gamma_{E,1})\\
v_2\frac{\partial \gamma_{E,2}}{\partial x} &= -\frac{D_{E/oil}}{W^2}\cdot(\gamma_{E,2}-\gamma_{E,1})
\end{aligned}\tag{18}$$

- Mass balances for fatty acids concentration:

$$\begin{aligned}
v_1\frac{\partial \gamma_{FA,1}}{\partial x} &= \frac{D_{FA/aq}}{W^2}\cdot(\gamma_{FA,2}-\gamma_{FA,1})\\
&\quad -W\cdot V_{max1}\cdot\frac{\gamma_{E,1}\cdot\gamma_{M,1}^{n1,1}\cdot\gamma_{FA,1}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_{M,1}^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA,1}^{n2,1}\right)}\\
&\quad +W\cdot V_{max2}\cdot\frac{\gamma_{E,1}\cdot\gamma_{FAME,1}^{n1,2}\cdot\gamma_{G,1}^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME,1}^{n1,2}\right)\cdot\left(K_{m,G}^{n2,2}+\gamma_{G,1}^{n2,2}\right)}\\
v_2\frac{\partial \gamma_{FA,2}}{\partial x} &= -\frac{D_{FA/oil}}{W^2}\cdot(\gamma_{FA,2}-\gamma_{FA,1})\\
&\quad -W\cdot V_{max1}\cdot\frac{\gamma_{E,2}\cdot\gamma_{M,2}^{n1,1}\cdot\gamma_{FA,2}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_{M,2}^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA,2}^{n2,1}\right)}\\
&\quad +W\cdot V_{max2}\cdot\frac{\gamma_{E,2}\cdot\gamma_{FAME,2}^{n1,2}\cdot\gamma_{G,2}^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME,2}^{n1,2}\right)\cdot\left(K_{m,G}^{n2,2}+\gamma_{G,2}^{n2,2}\right)}
\end{aligned}\tag{19}$$

- Mass balances for FAME concentration:

$$\begin{aligned}
v_1\frac{\partial \gamma_{FAME,1}}{\partial x} &= \frac{D_{FAME/aq}}{W^2}\cdot(\gamma_{FAME,2}-\gamma_{FAME,1})\\
&\quad -W\cdot V_{max1}\cdot\frac{\gamma_{E,1}\cdot\gamma_{M,1}^{n1,1}\cdot\gamma_{FA,1}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_{M,1}^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA,1}^{n2,1}\right)}\\
&\quad +W\cdot V_{max2}\cdot\frac{\gamma_{E,1}\cdot\gamma_{FAME,1}^{n1,2}\cdot\gamma_{G,1}^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME,1}^{n1,2}\right)\cdot\left(K_{m,G}^{n2,2}+\gamma_{G,1}^{n2,2}\right)}\\
v_2\frac{\partial \gamma_{FAME,2}}{\partial x} &= -\frac{D_{FAME/oil}}{W^2}\cdot(\gamma_{FAME,2}-\gamma_{FAME,1})\\
&\quad -W\cdot V_{max1}\cdot\frac{\gamma_{E,2}\cdot\gamma_{M,2}^{n1,1}\cdot\gamma_{FA,2}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_{M,2}^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA,2}^{n2,1}\right)}\\
&\quad +W\cdot V_{max2}\cdot\frac{\gamma_{E,2}\cdot\gamma_{FAME,2}^{n1,2}\cdot\gamma_{G,2}^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME,2}^{n1,2}\right)\cdot\left(K_{m,G}^{n2,2}+\gamma_{G,2}^{n2,2}\right)}
\end{aligned}\tag{20}$$

2.3.3.2. A process model of steady-state plug flow reactor. The second 1D mathematical process model was based on the assumption that there is no axial dispersion and no radial variations in velocity, concentration, and reaction rate Tušek et al., 2012a). Also, the mass transfer coefficient was assumed to be negligible. The proposed mass balances are as follows (Eqs. (21)-((24)):

- Mass balance for methanol concentration:

$$\begin{aligned}
&-v\cdot\frac{\partial \gamma_M}{\partial x}-V_{max1}\cdot\frac{\gamma_E\cdot\gamma_M^{n1,1}\cdot\gamma_{FA}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_M^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA}^{n2,1}\right)}\\
&+V_{max2}\cdot\frac{\gamma_E\cdot\gamma_{FAME}^{n1,2}\cdot\gamma_G^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME}^{n1,2}\right)\cdot\left(K_m^{n2,2}+\gamma_G^{n2,2}\right)}=0
\end{aligned}$$

$$\gamma_M(x=0)=\gamma_{M,i}\tag{21}$$

- Mass balance for glycerol concentration:

$$\begin{aligned}
&-v\cdot\frac{\partial \gamma_G}{\partial x}+V_{max1}\cdot\frac{\gamma_E\cdot\gamma_M^{n1,1}\cdot\gamma_{FA}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_M^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA}^{n2,1}\right)}\\
&-V_{max2}\cdot\frac{\gamma_E\cdot\gamma_{FAME}^{n1,2}\cdot\gamma_G^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME}^{n1,2}\right)\cdot\left(K_m^{n2,2}+\gamma_G^{n2,2}\right)}=0
\end{aligned}$$

$$\gamma_G(x=0)=\gamma_{G,i}\tag{22}$$

- Mass balance for fatty acids concentration:

$$\begin{aligned}
&-v\cdot\frac{\partial \gamma_{FA}}{\partial x}-V_{max1}\cdot\frac{\gamma_E\cdot\gamma_M^{n1,1}\cdot\gamma_{FA}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_M^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA}^{n2,1}\right)}\\
&+V_{max2}\cdot\frac{\gamma_E\cdot\gamma_{FAME}^{n1,2}\cdot\gamma_G^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME}^{n1,2}\right)\cdot\left(K_m^{n2,2}+\gamma_G^{n2,2}\right)}=0
\end{aligned}$$

$$\gamma_{FA}(x=0)=\gamma_{FA,i}\tag{23}$$

- Mass balance for FAME concentration:

$$\begin{aligned}
&-v\cdot\frac{\partial \gamma_{FAME}}{\partial x}+V_{max1}\cdot\frac{\gamma_E\cdot\gamma_M^{n1,1}\cdot\gamma_{FA}^{n2,1}}{\left(K_{m,M}^{n1,1}+\gamma_M^{n1,1}\right)\cdot\left(K_{m,FA}^{n2,1}+\gamma_{FA}^{n2,1}\right)}\\
&-V_{max2}\cdot\frac{\gamma_E\cdot\gamma_{FAME}^{n1,2}\cdot\gamma_G^{n2,2}}{\left(K_{m,FAME}^{n1,2}+\gamma_{FAME}^{n1,2}\right)\cdot\left(K_m^{n2,2}+\gamma_G^{n2,2}\right)}=0
\end{aligned}$$

$$\gamma_{FAME}(x=0)=\gamma_{FAME,i}\tag{24}$$

3. Results and discussion

3.1. Biodiesel synthesis and purification

The synthesis of biodiesel in a batch reactor was carried out to produce the necessary raw material - biodiesel, which was later used as a substrate in experiments performed to evaluate the kinetic parameters of the hydrolysis reaction. A one-step transesterification of sunflower oil was performed, and the FAME yield after 24 h was more than 96.5%, which is the minimum amount of FAME in biodiesel prescribed according to the norm EN 14214:2012 + A2:2019 (Liquid petroleum products). After the synthesis of biodiesel in the batch reactor, the resulting mixture was purified of unwanted products. This primarily referred to the removal of the inorganic (aqueous) phase, which in addition to water also contained lipase, and furthermore, the removal of glycerol

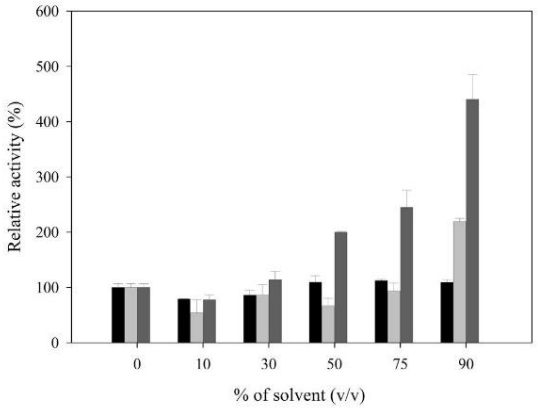


Fig. 3. Effects of organic solvent on lipase enzyme activity for different O: P ratios (■ *n*-hexane, ■ *iso*-octane, ■ *n*-heptane)).

from the organic phase. After synthesis, the reaction mixture contained around 8% (*w/w*) of water and 96.6 mg/mL of glycerol. The first purification step was purification in a separation funnel. After two stages of separation in a separation funnel, the concentration of glycerol in biodiesel decreased to 3.31 mg/mL. The crude biodiesel (upper layer in the separation funnel) was separated and further purified by DES (Petračić et al., 2017). After separation of DES from purified biodiesel in the separation funnel, the biodiesel sample was re-analysed by gas chromatography. The concentration of glycerol in this sample was below the detection limit of the method and can be considered negligible.

3.2. Influence of the organic solvent

Since oil and biodiesel are not miscible with water, it was necessary to find a suitable organic solvent in which oil and biodiesel could be dissolved to perform kinetic measurements. Apart from the solubility of the reactants in the solvent, another important criterion when selecting the appropriate solvent is the effect of solvent on the enzymatic activity. According to Wang et al. (Wang et al., 2009), the addition of organic solvents leads to a decrease of enzyme activity because solvents can reduce water activity around the enzyme. Based on previous measurements (data not shown) and literature data (Chaiyaso et al., 2012; Cervantes-Gonzales et al., 2014), three non-polar, hydrophobic organic solvents were selected based on their miscibility with methanol, oil, and biodiesel: *n*-hexane (log $P = 3.5$), *n*-heptane (log $P = 4.0$), and *iso*-octane (log $P = 4.7$) (Uttatree et al., 2010; Bharathiraja et al., 2019). According to Laane et al. (Laane et al., 1987), there is a connection between enzyme activity and polarity/hydrophobicity of organic solvents. They concluded that if the log P value is $<$ 2, low enzyme activity is to be expected. Moderate activity is expected in solvents having a log P between 2 and 4, while the highest activity is expected in non-polar solvents having log $P > 4$. The addition of solvents to biodiesel synthesis ensures better solubility of hydrophobic triglycerides and alcohol, significantly lowering the negative effect of short-chain alcohols on enzyme activity (Bharathiraja et al., 2019). Based on presented literature data, the expectation was that the activity would be intensified if *n*-heptane and isooctane are added into the mixture, while there would be a slight noticeable change in enzyme activity when *n*-hexane is used.

In order to test that assumption, organic solvents (O) were mixed with lipase (P). The influence of organic solvents on enzyme activity was measured. The results are presented in Fig. 3.

As can be seen, all three selected organic solvents have a positive effect on the activity of lipase. No reduction of enzyme activity was obtained even for the highest analysed O: P ratio 90% (mas.) of the organic solvent in the mixture. *Iso*-octane and *n*-heptane at the highest analysed O: P ratio noticeably increased the initial enzyme activity. Concerning those, *n*-hexane has a very low or negligible effect on the lipase activity, meaning that the activity of the enzyme was practically constant at the entire analysed O: P ratios. Based on the fact that *n*-hexane does not affect the activity of lipase for a wide range of O: P ratios and that all components included in biodiesel synthesis are mixable with it, *n*-hexane was selected as a solvent in kinetic measurements for the preparation of different initial concentrations of oil, biodiesel, and methanol.

3.3. Mathematical modelling

As mentioned in the introduction, in order to develop a mathematical process model of an enzymatic reaction, it is necessary to estimate the kinetic parameters of selected kinetic models. The reason behind this is that changing the scale of the reactor transport phenomena leads to a change in the measured kinetics, as it was demonstrated elsewhere (Šalić et al., 2013; Tušek et al., 2012b). Based on the literature, three kinetic models for the reaction rates, namely, Michaelis-Menten, Bi-Bi Ping-Pong, and Hill kinetic models, were selected. Michaelis-Menten and Bi-Bi Ping-Pong kinetic models were chosen because they are the mostly used models for describing the kinetics of multiple substrate enzymatic reactions. Michaelis-Menten kinetics assumes a substrate sequential displacement binding mechanism where all substrates have to bind to the enzyme before a product is formed. On the other hand, Bi-Bi Ping-Pong kinetics assumes a double displacement binding mechanism, meaning that one or more products are released before all substrates are bound to the enzyme. The Hill kinetic model was chosen based on the results of our previous research (Šibalić et al., 2020), where this model was superior in describing the biotransformation process of *p*-nitrophenyl palmitate hydrolysis catalysed bylipase. According to Marangoni (Marangoni, 1994), the lipase biotransformation mechanism is determined by three factors: enzyme conformational change, change on the substrate interface surface, and enzyme penetration into that surface interface. In order to describe all those changes, Hill introduced Hill constants in the equation. A Hill constant is often described as an index of the cooperativity of the process. Hill's constant n represents the number of substrate molecules associated with catalyst per catalytic cycle and it is an index of the cooperativity of the process. In the case where $n > 1$, positively cooperative binding occurs; for $n < 1$, negatively cooperative binding is observed, and if $n = 1$ binding is noncooperative – completely independent binding which is described by Michaelis–Menten and Bi-Bi Ping-Pong kinetic model. Obviously, estimated Hill's constant are the higher than 1 for both substrates in fatty acid methyl esters hydrolysis and for methanol in biodiesel synthesis indicating positively cooperative binding with enzyme. Contrary, estimated Hill's constant for fatty acids in biodiesel synthesis is lower than 1 indicating negatively cooperative binding with enzyme (Table 3). It is very important to define well the kinetic model of the process while on a microscale, due to the small size of microreactor channels, diffusion becomes negligible and kinetics of the reaction becomes the limiting factor of the process.

In addition to kinetic parameters, since the proposed 2D mathematical process model (Equations (4) - (13)) and a process model of steady-state two parallel plug flow reactors (Eqs. (16) - (20)) contain terms describing the diffusion of components in the axial direction, it was also necessary to determine the diffusion coefficients for the individual reactants and for the enzyme.

Table 2
Properties of lipase, glycerol, methanol, FAME and fatty acids together with estimated diffusivities at 40 °C.

Solute (S)	Solvent (B)	T (K)	η_B (mPa·s)	V_S (mL/mol)	V_B (mL/mol)	M_S (g/mol)	$D_{S/B}$ (m^2/s)
lipase	oil	313.15	34.52 (Correia et al., 2012)	–	–	33,400 (Gojun et al., 2019)	$2.35 \cdot 10^{-8}$
methanol	methanol	313.15	0.445 (Gojun et al., 2019)	38.5	38.5	–	$5.26 \cdot 10^{-5}$
lipase	biodiesel	313.15	14.69 (Hartman, 2015)	–	–	33,400 (Gojun et al., 2019)	$5.52 \cdot 10^{-8}$
glycerol	water (buffer)	313.15	0.654 (Gojun et al., 2019)	73.1	18.07	–	$1.70 \cdot 10^{-5}$
FAME	biodiesel	313.15	14.62 (Hartman, 2015)	–	–	279.71	$2.73 \cdot 10^{-7}$
fatty acids	oil	313.15	34.52 (Correia et al., 2012)	–	–	843.16	$8.01 \cdot 10^{-8}$

After calculating the diffusion coefficients and estimating the parameters of the kinetic models, the validation of the mathematical model of the process was performed using an independent set of experimental data obtained during the biodiesel biosynthesis in a microreactor for different retention times.

3.3.1. Calculation of diffusion coefficients

The rate of the enzymatic reaction is determined by the activity of the enzyme and the availability of the reactant to access the active sites of the enzyme Tišma et al., 2009). In systems where the reactant has to travel a great distance to the enzyme's active site, the diffusion effect on the overall reaction rate is significant. The use of microreactors in enzyme-catalysed reactions reduces the effect of diffusion on the overall reaction rate, and consequently, due to the small diffusion path, the effect of diffusion on the overall reaction rate is negligible. This effect is especially important in systems where the reaction mixture consists of multiple phases, such as in the case of the production of biodiesel catalysed by lipase or in the systems designed for kinetic parameter estimation. In the three-inlet configuration used for kinetic measurements (Fig. 2a and 2b), methanol diffuses into the buffer process stream containing the enzyme (and glycerol). In this way, all substrates are accessible to the active site of the enzyme and a biodiesel synthesis reaction occurs at the two-phase interface. Given the rapid diffusion of methanol, the system very quickly becomes a two-phase system from a three-phase system, and the reaction takes place along the entire length of the microchannel. Eqs. (14) and ((15) and the parameters listed in Table 2 were used to determine the diffusion coefficients of all the components that are involved in the transesterification process.

The calculated diffusion coefficients for methanol, fatty acids, glycerol, FAME and lipase at 40 °C are shown in Table 2. As can be seen, due to their size, methanol and glycerol will diffuse the fastest, followed by FAME in biodiesel, then the enzyme, and in the end, fatty acids in the oil.

3.3.2. Estimation of kinetic parameters – *biodiesel synthesis* vs *biodiesel hydrolysis*

As already mentioned, lipase has the ability to simultaneously catalyse hydrolysis, esterification and transesterification (Budžaki et al., 2015). Due to this property, it was necessary to estimate the kinetic parameters of both reactions (transesterification and hydrolysis) that occur in a biodiesel synthesis process. The influence of initial fatty acids, methanol, glycerol, and biodiesel concentrations on the reaction rate were measured to estimate the kinetic parameters of the proposed kinetic models. The kinetic parameters were estimated from independent data collected from the measurements in a microreactor. For the estimation of kinetic parameters, the concentration of one reactant was kept constant, while the concentration of the other was altered in the selected range. The dependence of the reaction rate on the initial concentration of fatty acids, methanol, FAME, and glycerol are shown in Fig. 4 and the obtained values of kinetic parameters in Table 3.

As can be seen, the dominant reaction is biodiesel synthesis-transesterification with an approximately 12-fold higher maximal reaction rate in comparison to biodiesel hydrolysis. The obtained results were expected, since according to the literature (Gojun et al., 2019), if the percentage of water in the process is between 2–20% (*w*/*w*), the dominant reaction is biodiesel synthesis (transesterification). The amount of water in all experiments performed in this research was 8% (*w*/*w*). Comparing the Michaelis-Menten kinetics parameters obtained in this research with the same parameters obtained in our previous research (V_{max} = 44.71 ± 1.89 mg/(mL min), $K_{m\ fatty\ acids}$ = 155.02 ± 121.86 mg/mL and $K_{m\ methanol}$ = 7.56 ± 2.77 mg/mL)) (Gojun et al., 2019), where two-fold larger diameter of the microreactor was used, a higher maximal reaction rate was obtained in this research. The reason behind this is the mass transfer rate increase connected with the reduction of the microchannel size, meaning that transport phenomena change. Consequently, components have to pass shorter lengths and diffuse faster, respectively, leading to higher reaction rates. Based on this, it could be concluded that additional downsizing would give different results in measured kinetics. On the other hand, it was noticed that further reduction of the diameter leads to significant channel blocking for biodiesel synthesis (Gojun et al., 2019).

As can be seen from Fig. 4, all kinetic models (Michaelis-Menten and Bi-Bi Ping-Pong kinetic models are overlapping) described the results of kinetic measurements well. To estimate which kinetic model described the obtained experimental data the best, the model selection criterion (MSC) was used as the trial function (built-in software package Scientist) (Zelić et al., 2004). The MSC was chosen because the proposed kinetic models have a different number of parameters that have to be estimated. The selected criterion attempts to represent the "information content" of a given set of parameter estimates by relating the coefficient of determination to the number of parameters (or equivalently, the number of degrees of freedom) that were required to obtain the fit. The most appropriate model will be the one with the largest MSC. Based on the obtained results, the Hill kinetic model describes the obtained data the best (Table 3).

3.3.3. Bi-Bi Ping-Pong mathematical process model

The Bi-Bi Ping-Pong mechanism is the most commonly used mechanism for the description of biodiesel synthesis (Janssen et al., 1999; Al-Zuhair et al., 2007; Haigha et al., 2014; Gunawan et al., 2017; Azócar et al., 2014; Price et al., 2014; Sun et al., 2013b; Budžaki et al., 2015). It includes substrate competition coupled with competitive inhibition by alcohol. Like the process models proposed in this research, they usually include reactions, biodiesel synthesis (transesterification), and hydrolysis. The main assumption of those process models is that the most important step is the esterification of fatty acids simultaneously followed by the fatty acid reaction with methanol. The mechanism used in this paper was the same one proposed by Liu et al. (2014). The mentioned authors proposed the Bi-Bi Ping-Pong mechanism based on the assumption that there are no mass transfer limitations, different substitutions of mono-, di, triglycerides and fatty acids are treated as a single constitute, methanol is considered to be the main inhibitor of the enzyme, and that the limiting step of the reaction are fatty acid esterification and glyceride hydrolysis. The Bi-Bi Ping-

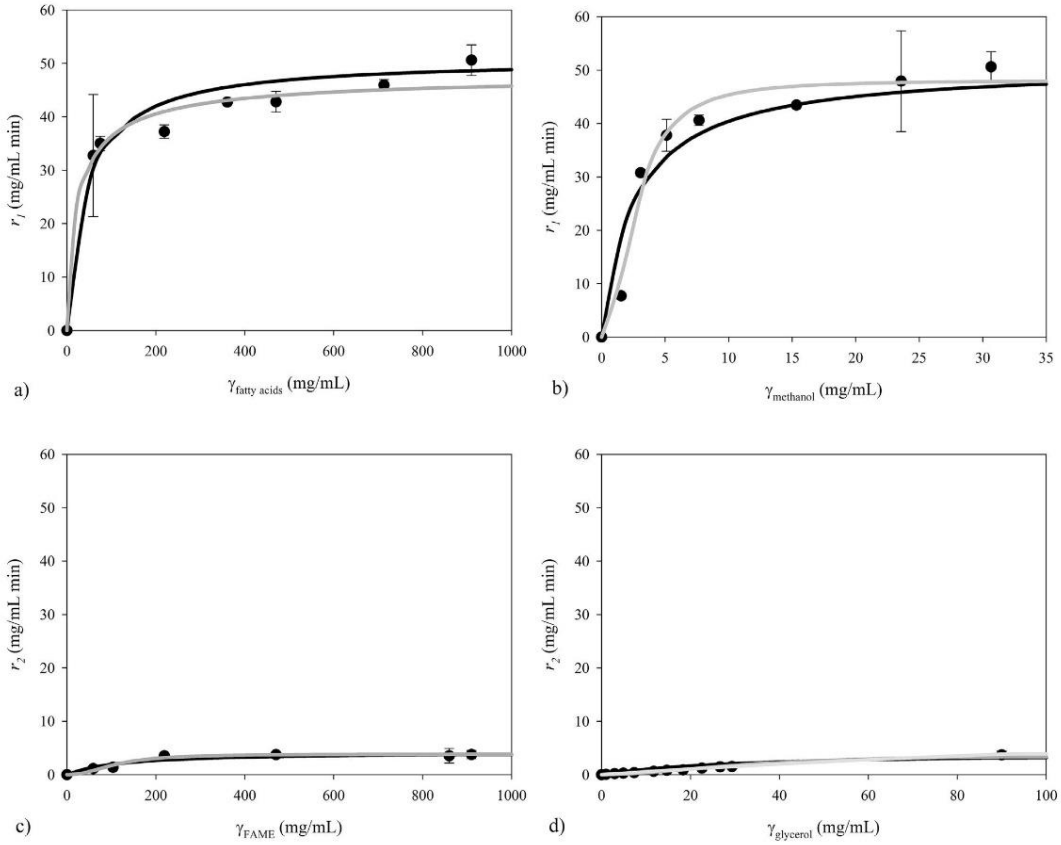


Fig. 4. Kinetics of biodiesel synthesis and FAME hydrolysis: dependence of the reaction rate on initial concentration of (a) fatty acids (b) methanol, (c) FAME, (d) glycerol (— Michaelis-Menten model, = = Bi-Bi Ping-Pong model, Hill model, • experimental data).

Table 3
Estimated kinetic parameters with standard deviations for biodiesel synthesis and biodiesel hydrolysis based on Michaelis-Menten, Bi-Bi Ping-Pong and Hill kinetics.

Biodiesel synthesis						
Model	V_{max} (mg/mL min)	$K_{m\ fatty\ acids}$ (mg/mL)	$K_{m\ methanol}$ (mg/mL)	n_1(-)	n_2(-)	MSC(-)
Michaelis-Menten	54.73±3.06	36.80±11.43	2.97±0.72	–	–	2.58
Bi-Bi Ping-Pong	54.94±3.15	40.52±12.88	3.10±0.77	–	–	2.58
Hill	48.13±1.89	24.28±11.00	2.73±0.26	0.80±0.27	2.16±0.42	3.18
Fatty acid methyl esters hydrolysis						
Model	V_{max} (mg/mL min)	$K_{m\ FAME}$ (mg/mL)	$K_{m\ glycerol}$ (mg/mL)	n_1(-)	n_2(-)	MSC(-)
Michaelis-Menten	4.21± 0.99	124.97± 12.36	32.45± 18.82	–	–	2.79
Bi-Bi Ping-Pong	4.31±0.49	121.11±17.52	39.62±15.37	–	–	2.79
Hill	3.88±0.17	110.64±16.36	33.01±3.38	2.22±0.49	1.91±0.32	3.27

Pong mathematical process model (Eqs. (24)-(29)) proposed by Liu et al. (2014) was used in this research to set-up the mathematical model, in order to describe biodiesel synthesis in a microreactor. Mass balances were transformed to mass balances that fit the plug flow reactor. As already mentioned, process conditions change when going from macro to micro and from a batch to a flow reactor. Based on that, kinetic parameters of the Bi-Bi Ping-Pong mathematical process model were estimated from experiments performed in a microreactor with 95% confidence (experiment with 1:3.4 oil to methanol molar ratio) and the results are present in Table 4.

Comparing the parameters estimated from the microreactor experiments and those obtained in a batch experiment (Liu et al., 2014), it can be observed that there is no significant difference between them. Both sets of parameters indicated that the hydrolysis of triglycerides (high K_T value) and the esterification step of fatty acids (high V_{ES} value) are the most important steps of the transesterification process.

3.3.4. Mathematical process model validation

With regard to validating the proposed mathematical process models and a comparison between them, four additional exper-

Table 4
Comparison of estimated and published (Liu et al., 2014) values for kinetic parameters of Bi-Bi Ping-Pong mathematical process model.

Parameters (g/mmol h)	Values	
	This research	(Liu et al., 2014)
K_T	5.975	8.746
V_T	0.076	0.154
K_D	1.279	1.027
V_D	0.064	0.122
K_M	0.361	0.397
V_M	0.144	0.910
K_{Es}	0.004	0
V_{Es}	6.709	7.827
Equilibrium constants		
K_1	2.267	1.688
K_2	0.001	0
K_3	2.270	2.640
K_4	0.001	0
K_5	2.228	2.369
K_6	0.001	0
K_7	5.126	8.246
K_8	5.184	9.576
K_9	1.163	0.783
K_{10}	16.661	16.343

iments of biodiesel synthesis in a microreactor were performed (Table 1). In each experiment, the oil to methanol ratio was increased starting from 1:3.4 up to 1:90. The first ratio (1:3.4) was chosen because the stoichiometric molar ratio in the biodiesel synthesis reaction is 1:3 and a slightly higher ratio value was set because the reaction is reversible. Further increase in the ratio was in favour of shifting the reaction in the synthesis direction, therefore a higher excess of alcohol is necessary. Additionally, higher amounts of alcohol enhance the contact between alcohol and triglycerides (Lee and Saka, 2010), thus breaking the glycerine-fatty acid linkages during the transesterification process (Miao and Wua, 2006). To summarize, higher methanol to oil ratios lead to a higher yield in shorter residence time and the formed product (biodiesel) is purer (Helwani et al., 2009). The optimum molar ratio differs based on the chosen catalyst (Musa, 2016). According to Barnwal and Sharma (Barnwal and Sharma, 2005), when transesterification is performed with an alkali-based catalyst, the optimum molar ratio of oil to methanol is 1:6. Under that condition, the yield of more than 98% is achieved. When working with acid-based transesterification, molar ratios can go really high. Banerjee and Chakraborty reported transesterification with oil to methanol molar ratio of 1:250 (Banerjee and Chakraborty, 2009). When enzymes are used as catalysts, molar ratios are usually in the lower range, usually up to 1:7.5 (Musa, 2016). The reason for this is the negative effect that short-chain alcohols have on enzyme activity. In general, regardless of the catalyst, according to Balat and Bala (Balat and Balat, 2008), the best molar ratio for biodiesel synthesis is in the range of 1:6–1:30. It should also be stressed that all mentioned molar ratios and general rules refer to transesterification in a batch reactor. Taking into consideration that mass transfer, kinetic and equilibrium control, reaction time, temperature, pressure, water content, etc. will play a major role in biodiesel synthesis (Behzadi and Farid, 2009), it could be expected that paradigms will change when transferring this reaction to flow reactors, especially microreactors.

Although lower ratios are more favourable for carrying out enzymatic biosynthesis, the chosen ratios in this research were 1:3.4, 1:10, 1:30, and 1:90, two of them being in the most favourable range and two extremes, one lower and one higher. The main assumptions for applying higher molar ratios is that due to the very short residence time, the enzyme and methanol will be in very short contact. Additionally, unlike in a batch reactor in which all components are in contact for a longer period of time, the great advantage of a microreactor is the continuous removal of all components. Also, by creating an emulsion between oil and enzymes, the enzyme is partially protected from the negative impact of methanol because the reaction occurs only on the interfacial area of the two phases.

Table 5
R^2 of analysed mathematical process models.

Mathematical model	Oil to methanol ratio			
	1:3.4	1:10	1:30	1:90
2D mathematical model	0.535	0.603	0.890	0.933
A model of steady-state two parallel plug flow reactors	0.330	0.653	0.891	0.942
A model of ideal plug flow reactor	0.108	0.454	0.508	0.910
Bi-Bi Ping-Pong model	0.148	0.253	0.216	0.264

As mentioned, based on all stated assumptions, four additional experiments were performed and the results are presented in Fig. 5. In all the experiments, the initial FFA concentration ($\gamma_{\text{fatty acids}} = 946.52$ mg/mL) and enzyme concentration ($\gamma_E = 0.1$ mg/mL, V.A. = 89,000 U/mL) were kept constant. Based on the oil to methanol ratio, the methanol concentration was increased as shown in Table 1.

As can be seen from the results (Fig. 5), by increasing the methanol concentration in the system, yield increased, and in the process where the large excess of methanol was used, the yield was over 90% for the residence time of only 40 min. In comparison, when the same reaction was performed in a batch reactor (Price et al., 2014; Gojun et al., 2020), 24–48 h was necessary to obtain the same yield. It is also important to point out that the reaction in the batch reactor was performed with 30-fold lower methanol concentration (molar ratio 1:3.4) to avoid methanol inhibition. Using the same enzyme, and replacing the methanol with ethanol, under 1:4 molar ratio and 45% (v/v) ethanol concentration, Rachmadona et al. (Rachmadona et al., 2020) achieved the yield of 97.43% at 40 °C and 24 h. On the other hand, when the obtained results are compared with the results obtained under the same initial conditions, except in a microreactor with the double diameter size (Price et al., 2014), the same yield was observed for 4-fold shorter residence time in the present research.

The comparison of model efficiency was evaluated based on R^2 as presented in Table 5. It can be noticed that, by increasing the oil to methanol ratio, the increase in R^2 was noticed for all four analysed models. The highest numerical value of R^2 for all four analysed oil to methanol ratios was obtained for the 2D process model followed by the process model of steady-state two parallel plug flow reactors and the process model of the ideal plug flow reactor. The lowest values of R^2 were obtained for the Bi-Bi Ping-Pong transport model. Taking into account the structure of the analysed process models and that a process model can be considered applicable if the determination coefficient describing the difference between the experimental values and the predicted model values exceeds 0.75 (Le Man et al., 2010), it can be concluded that the process model of steady-state two parallel plug flow reactors can reliably be used for the description of the biodiesel transesterification process at high oil to methanol ratios.

The Bi-Bi Ping-Pong mathematical process model describes the reaction performed in a microreactor for experiments performed at shorter residence times poorly (Fig. 5d). The similar was previously described by Jurinjak Tušek et al. (Jurinjak Tušek et al., 2013), where dispersion between experimental data and model predicted data at low residence times was explained by order of approximation. If a low order of approximation (1D model) is used for the description of the diffusion process in a microreactor it is not pos-

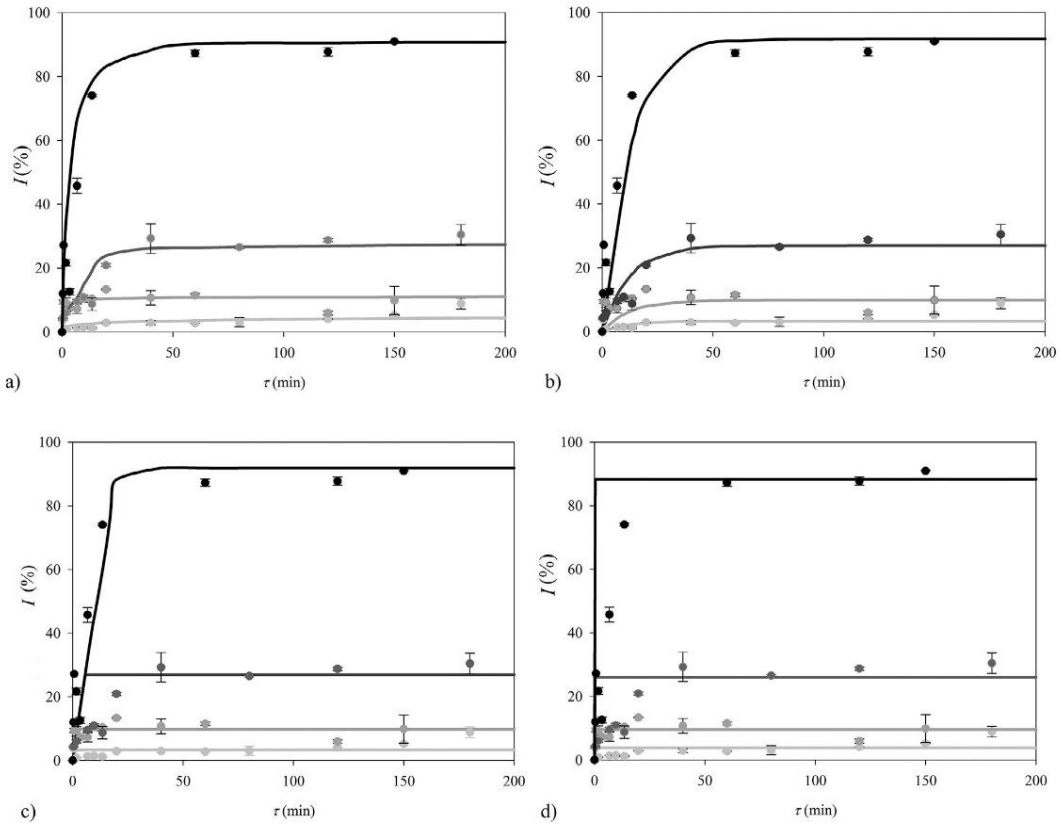


Fig. 5. Process model validation of biodiesel synthesis in a microreactor a) 2D mathematical process model, b) a process model of steady-state two parallel plug flow reactors, c) a process model of ideal plug flow reactor and d) Bi -Bi Ping-Pong mathematical process model (— mathematical model, ● 1:3.4 oil to methanol molar ratio, ● 1:10 oil to methanol molar ratio, ● 1:30 oil to methanol molar ratio, ● 1:90 oil to methanol molar ratio).

sible to precisely describe the transesterification process for short residence times. This all means that one of the beneficial aspects of running more complex and physically representative mathematical models is that transport effects can be separated from the reaction kinetics to get more intrinsic kinetics rates and parameters. This is exemplified by the shortcomings pointed out with using the Bi-Bi Ping-Pong mechanism to describe the entirety of the reactor process and moving from a conventional macroscale batch reactor to a microreactor with completely different transport limitations.

Based on the obtained results, it could be concluded that the Bi-Bi Ping-Pong mathematical process model is not accurate for the description of biodiesel synthesis in a microreactor, but could perhaps be used for rough predictions. The models proposed in this study are more suitable for more refined and precise predictions.

4. Conclusion

This paper presents the comparison of different mathematical process models used for the description of biodiesel synthesis in a microreactor catalysed by lipase. The Bi-Bi Ping-Pong mechanism is the most commonly used mechanism described in the literature for the description of biodiesel synthesis. On the other hand, as shown in this manuscript, it is not suitable for the description of the process on a micro level due to a low order of approximation. Amongst the three proposed mathematical process models in this research, titled the 2D mathematical process model, the process model of steady-state two parallel plug flow reactors, and the process model of steady-state plug flow reactor, the 2D mathematical process model and the process model of steady-state two parallel plug flow reactors showed good agreement between experimental results and model simulation. Additionally, the reaction rates for transesterification and hydrolysis were described with double substrate Michaelis-Menten kinetics, Bi-Bi Ping Pong kinetics, and Hill kinetics. Based on the model selection criterion, the Hill model was proposed as the best kinetic model for biodiesel production catalysed by lipase.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Martin Gojun: Investigation, Data curation, Writing - original draft. **Anabela Ljubić:** Investigation, Software, Data curation, Writing - original draft. **Matea Bačić:** Investigation, Software, Data curation, Writing - original draft. **Ana Jurinjak Tušek:** Software, Data curation, Writing - review & editing. **Anita Šalić:** Conceptualization, Methodology, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Bruno Zelić:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Paper 7

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Integrated microsystems for lipase-catalyzed biodiesel production and glycerol removal by extraction or ultrafiltration

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ABSTRACT

Competition among renewable energy sources in today's market is growing rapidly. Renewables have grown to the point where they are taking significant material market share from coal, gas, and uranium. Recently, biodiesel production has been intensified by lipase-catalyzed transesterification performed in microsystems. Another challenge in biodiesel production is the purification of the biodiesel. Common purification methods are usually more energy and time consuming than the biodiesel production itself.

In this work, an integrated biodiesel production process composed of lipase-catalyzed transesterification of sunflower oil and product purification was performed in microreactor and microseparator units connected in series. Glycerol, the by-product of the transesterification process, was removed from the reaction mixture by two different separation methods, deep eutectic solvent extraction and membrane separation. Different integrated setups were developed and evaluated in terms of FAME yield and purity. The most promising integrated process was found to be the one combining 2-inlets feeding strategy for biodiesel production in a microreactor with a microseparator connected in series, in which a choline chloride-glycerol deep eutectic solvent was used. In this integrated system, a FAME yield of 94% and a glycerol content below 0.02% (w/w) were achieved for the residence time of 20 min.

1. Introduction

The global energy market has been dynamic in recent decades, based on various trends that are emerging in terms of development of renewable technologies. Biodiesel, representative of renewable fuels and renewable alternative to fossil diesel, has found its place in the rapidly growing market [1,2]. Various industrial processes have been developed for biodiesel production (base blending, pyrolysis, micro-emulsification, and transesterification) [3], of which the transesterification process is the best known [4,5]. Although there is a wide variety of biodiesel production processes, the demand for biodiesel in the market continues to increase. Most of the solutions do not require the stacking of larger volumes of space of batch reactors, but a different approach, namely studying the possible intensification of the processes by combining miniaturization and flow chemistry [6]. Instead of thinking in large dimensions, intensification research went to the very small scale. Microreactors, microscale reactor systems fabricated using micro and precision engineering, offer some significant advantages over conventional meso and macroscale reactors, such as large surface to volume ratio and intensification of mass and energy transfer combined with short residence times. The combination of these advantages leads to higher conversions and productivities in microreactors in comparison with the same reactions which are carried out in macroreactors. Other advantages include significant reduction in implementation and operating costs, the volume of the reactor is also reduced along with the size of the plant, savings in capital and production costs can be achieved, less energy is consumed during the process compared to traditional reactor systems, etc. As with any other system, disadvantages must be considered as well. The most important disadvantages are clogging or fouling in the microchannels, leakage between the channels, and high manufacturing cost of the reactor. Another disadvantage for the industry is the increasing cost of analytical equipment required to ensure consistent product quality [7,8].

The intensification of biodiesel production through miniaturization is not sufficient to develop a sustainable and environmentally friendly process when the reaction requires aggressive chemicals used in chemical transesterification. Also, complex and

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long-term processes combined with the application of the large amount of fresh water are main shortcomings of biodiesel purification.

The emerging solution is to use biocatalysts for transesterification in combination with green solvents such as deep eutectic solvents (DES) for biodiesel purification. One of the most common enzymes in the industry is lipase (EC 13.1.1.3.). Lipase, a representative of the hydrolases, can simultaneously catalyse a number of reactions such as hydrolysis, transesterification, and esterification [9]. Lipase is mostly used in the transesterification process for several reasons: mild process conditions and possible use of waste oils as raw material, even without pretreatment. However, when working with enzymes, some disadvantages always must to be taken into account, such as the cost of the enzyme, the longer reaction time, its poor stability, and the deactivation of the enzyme by alcohols. Fortunately, lipases used for the transesterification process are among the least expensive commercially available enzymes. Reaction time is often limited by diffusion and this obstacle can be overcome in a microreactor due its aforementioned properties. Other disadvantages can be partly overcome by immobilization of the enzyme as it increases its stability and efficiency. Moreover, immobilization leads to easier separation of the catalyst, which can also reduce the costs of the process [7].

Another advantage of applying enzyme in the transesterification process is the very high purity and value of produced glycerol [10]. During biodiesel purification glycerol is the main challenge because glycerol in free form has a negative impact on the quality of biodiesel fuel. From an industrial point of view, the most common biodiesel purification method is the use of a decanter, followed by wet washing [11]. Although wet washing is a successful method, large amounts of wastewater are still produced after the purification process, as the method requires up to 10 L of water per 1 L of biodiesel produced [12]. Alternative methods had to be developed, and the use of DESs emerged as an example of green solvents. Due to their high biodegradability and low toxicity, DESs offer a solution to the economic and environmental obstacle for overall biodiesel production process [13]. To date, DESs have been successfully used for biodiesel purification (mainly for glycerol removal), but mainly in discontinuous separation systems [13,14].

In order to combine all stated advantages and to develop a sustainable enzymatically catalyzed biodiesel production and purification process on a microscale, the first step was to investigate the transesterification process. In our previous studies [4,15], different initial conditions and their influence on FAME yield were investigated. A lipase from *Thermomyces lanuginosus* (TIL) was used as a catalyst (both commercial and produced by solid-state fermentation) while edible or waste sunflower oil together with methanol were used as substrates. The highest yield obtained was 32% for residence time of 30 min in experiment where waste oil was used as a substrate and commercial enzyme as catalyst (for a molar ratio methanol:oil of 3.4:1). Although the reaction was faster in the microreactor than in a batch reactor [16,17]), the obtained yield was lower than the values specified by the EN 14214:2013 standard [5]. To shift the reaction towards biodiesel production and in the same time to keep short the residence time, increase of methanol:oil molar ratio can be considered as one of the options. The motivation for this approach has been found in acid-based transesterification where methanol:oil molar ratio was extremely high (up to 250:1 [18]), although in the enzyme based synthesis of biodiesel conducted so far this ratio has not been as high as 7.5:1 [19]. The reason for this is the negative effect of small alcohols like methanol on enzyme activity [20]. On the other hand, it should be taken into account that all the mentioned methanol:oil ratios were investigated and defined for batch reactors. It is to be expected that different phenomena, considering the activity of the enzyme lipase at high concentrations of ethanol, will occur when carrying out the reaction in flow systems, especially microflow reactors. Following that idea and based on our preliminary research [20], higher methanol:oil ratios were investigated in this research.

Several assumptions have been considered for the next phase of the research. The first assumption was based on the fact that all reaction components (methanol, oil and lipase) can be introduced separately into the microreactor. The second assumption was based on the short contact of methanol and enzyme which can be carried out using short retention times when conducting the experiment in a microreactor. Moreover, the reaction in the microreactor occurs at the interface area, so a large amount of the enzyme that did not diffuse towards that area will be protected from the methanol. The third assumption was consequence of the fact that in a microreactor experiments, all reaction mixture components are continuously removed from the reactor while fresh substrate and catalyst are feed in. Based on all those assumptions, the 4 molar ratios methanol:oil 3.4:1, 10:1, 30:1, and 90:1 were investigated [21]. By increasing the concentration of methanol in the system, the yield increased, and in the process where the largest excess of methanol was used, the FAME yield was over 90% for a residence time of only 40 min. Once a sufficient FAME yield was obtained the next step in the investigation was the biodiesel purification process. According to the literature [22], different approaches can be used for biodiesel purification, which are divided into wet and dry washing processes. In this stage of research focus was placed on glycerol removal. To investigate which approach is most suitable for glycerol removal on a microscale, three approaches were tested– washing with water [23], washing with DES [23,24] and membrane filtration [25]. Washing with water resulted in a separation efficiency of 75.19% for a residence time of only 8.3 s. Unfortunately, glycerol was not removed sufficiently, and based on the mathematical model, it was calculated that two cycles of purification are probably required to achieve standards. On the other hand, an efficiency of 98.35% for a residence time of only 13.61 s was obtained when DES was used in extraction, leading to the conclusion that DESs are a better solution than water for the biodiesel purification. Finally, four different membranes, polyethersulfone, polyacrylonitrile, polypropylene and regenerated cellulose were selected for biodiesel purification [25] and the polyacrylonitrile membrane showed the best performance with the possibility of reusing it in multiple cycles.

In this work, based on all that is mentioned above, the basic concept, development and realisation of an integrated microsystem for biodiesel production and purification is described. As described elsewhere [20], a continuous process in microsystems is easy to set up by simply connecting independent process stages in series. Fig. 1 shows the basic concept of the integrated microsystem, where the first micro unit serves as a microreactor, and the second micro unit as a microextractor for glycerol separation. In the further investigation, microextractor was replaced with a cross flow filtration unit where polyacrylonitrile membrane was used for glycerol removal in a fully integrated system.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Several listed chemicals were used to perform all experiments. Edible sunflower oil (Zvijezda, Zagreb, Croatia) was purchased from a local store. The waste cooking oil (WCO) used in this research was obtained by deep frying of potatoes. Enzyme lipase from *Thermomyces lanuginosus* (commercial name Lipolase 100 L), fatty acid methyl ester (FAME) mix GLC-10 and glutaraldehyde were purchased from Sigma-Aldrich Handels GmBH (Vienna, Austria).

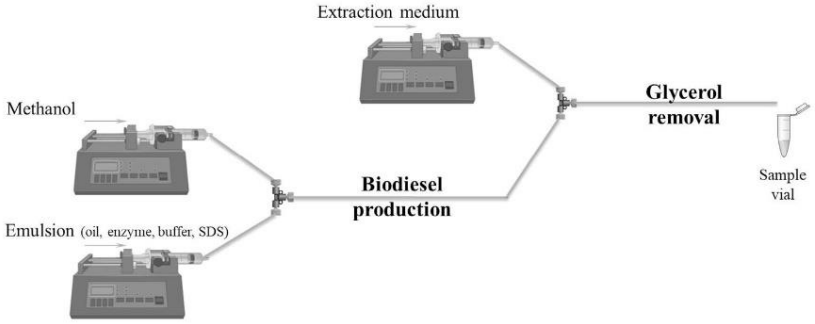

Fig. 1. Basic concept of an integrated microsystem for biodiesel production and glycerol removal.

Ethanol was purchased from Gram mol d. o.o. (Zagreb, Croatia) and glycerol was purchased from Kemika (Zagreb, Croatia). The 4-nitrophenyl acetate and choline chloride were purchased from Acros Organics (Geel, Belgium). Potassium dihydrogen phosphate (KH_2PO_4) and ethylene glycol were purchased from Lach-Ner (Prague, Czech Republic). The tris(hydroxymethyl)aminometal (TRIS), sodium dodecyl sulfate (SDS) and methanol were purchased from VWR Chemicals, BDH Prolabo (Lutterworth, United Kingdom). Dipotassium hydrogen phosphate (K_2HPO_4) was purchased from Merck (Darmstadt, Germany). All chemicals used were of analytical grade with the exception of edible sunflower oil, waste oil and enzyme lipase.

2.1.2. Microreactors and microseparators

The operating scale of all experiments performed in this work was the microscale; the diameter of the microsystems used was 500 or 1000 μm. Although microreactors can be made of glass, steel, and polymers [8], those used in this work were made of PTFE. The PTFE tubes had a constant internal volume of 235.62 μL, so the experiments can be compared with previous studies [21]. Based on different experimental setups, either 2-inlets strategy or 3-inlets strategy was used. Connections were realized as T-shaped or +-shaped, respectively. The syringes filled with substrates/enzyme were placed on piston pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA) and connected over T-shaped or +-shaped connections to the PTFE tube, that served as the microreactor (length: width: depth = 30 cm: 1000 μm: 1000 μm, internal volume 235.62 μL; or length: width: depth = 120 cm: 500 μm: 500 μm, internal volume 235.62 μL). Microseparators used in this work were the same PTFE tubes as microreactors (length: width: depth = 30 cm: 1000 μm: 1000 μm, internal volume 235.62 μL; or length: width: depth = 120 cm: 500 μm: 500 μm, internal volume 235.62 μL), and were also operated by piston pumps. Schemes for different experimental setups for biodiesel production are shown in Fig. 2.

2.1.3. Ultrafiltration unit

Purification of biodiesel by ultrafiltration was conducted in a membrane holder containing a PES membrane (13.4 cm^2, 10 kDa, Millipore, Germany), which served as separator of glycerol from biodiesel. The ultrafiltration unit was operated by piston pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA), which pumped two outlet streams, one containing purified biodiesel and a second containing mainly waste glycerol (Fig. 3b).

2.2. Methods

2.2.1. Emulsion preparation

The emulsion was prepared by mixing oil (edible sunflower oil or waste sunflower oil) and enzyme dissolved in a buffer in a volume ratio of 10:1. As described in detail in the work of Sharma et al. [26], the oil-in-water emulsion was formed using an ultrasonic homogenizer (SONOPULS mini20, Bandelin, Berlin, Germany) that sonicated the mixture for 15 min, at 50% amplitude and an operating frequency of 25 kHz. The emulsifier used was sodium dodecyl sulfate (SDS), at a concentration of 0.1 g/L. The homogeneous mixture with negligible creaming was recorded and used as substrate in all Experiments where a mixture of oil and enzyme was used as inlet stream (2-inlets strategy).

2.2.2. Preparation of magnetic nanoparticles and enzyme immobilization

Magnetic nanoparticles (γ-Fe_2O_3) were synthesized according to the procedure described elsewhere [27,28] and used as carriers for the immobilization of the enzyme. To initiate the surface activation, the magnetic nanoparticles were exposed to 2% aqueous glutaraldehyde solution for 2 h. After that, the particles were washed with deionized water and the enzyme (γ_{lipase} = 0.01 mg/mL) was added to attach over a 28 h period.

2.2.3. Preparation of DES

Choline chloride (ChCl) was dried in a vacuum concentrator (Savant SPD131DDA SpeedVac Concentrator, Thermo Scientific, USA) at 60 °C for 24 h before use. An anhydrous ChCl-Gly DES was prepared by mixing choline chloride (ChCl) and glycerol (Gly) in a molar ratio of 1:3.0 [29]. After determining the desired molar ratio and weighing each component, the components were mixed on a magnetic stirrer (MS-H-S, DLAB, Ontario, CA, USA) at 200 rpm and 50 °C. The process was carried out for at least 1 h until a homogeneous, transparent and colorless liquid was obtained.

2.2.4. Measurement of lipase activity

Lipase activity was determined spectrophotometrically (UV-1800, Shimadzu, Kyoto, Japan) by measuring the absorbance change at the wavelength of λ = 400 nm [30]. The total determination time was 20 s and the measurements were performed in triplicate. The sample was prepared by adding 100 μL of sample to 3900 μL of 0.05 mol/L Tris-HCl buffer. The measurement was started by adding 50 μL of 0.0375 mol/L 4-nitrophenyl acetate to the homogenized sample-buffer mixture. The amount of released 4-nitrophol was calculated using a molar extinction coefficient of 0.29866 L/(mmol·cm). One unit (U) was defined as the amount of

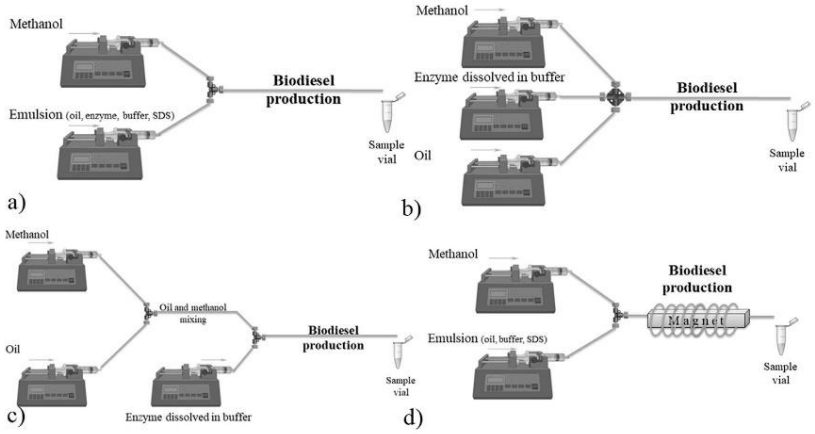

Fig. 2. Schemes of the used biodiesel production systems: a) 2-inlets strategy, b) 3-inlets strategy, c) 2 x 2-inlets strategy, d) 2-inlets strategy for biodiesel production catalyzed by enzyme immobilized on magnetic nanoparticles.

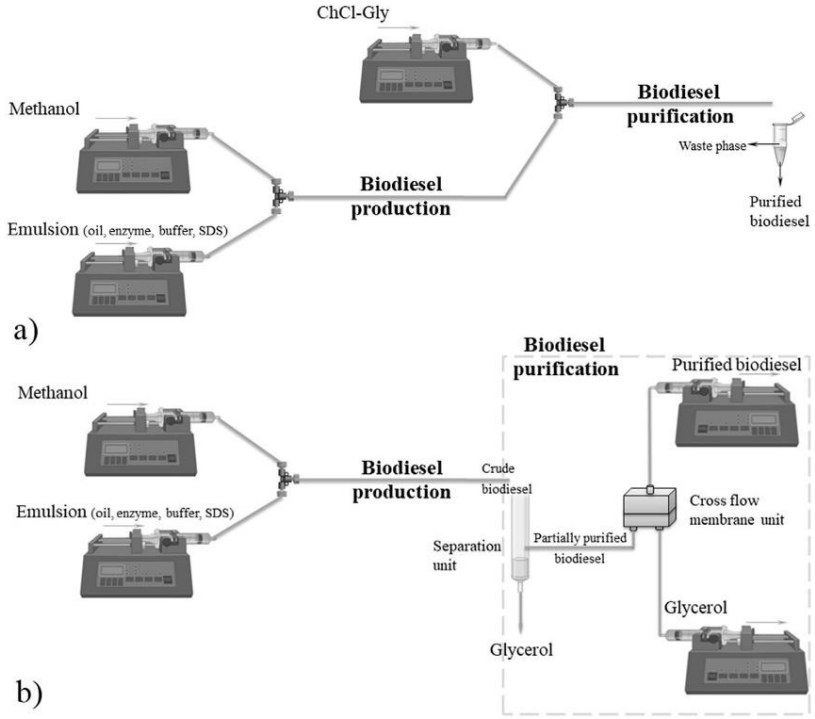

Fig. 3. Schemes of the used integrated systems: a) integrated microsystem composed of biodiesel production in a microreactor and microseparator for biodiesel purification by DES, b) integrated system composed of biodiesel production in a microreactor and membrane unit for biodiesel purification by ultrafiltration.

lipase that degrades 1 μmol of 4-nitrophenyl acetate in 1 min.

2.2.5. Determination of fatty acid methyl esters and glycerol concentrations

Fatty acid methyl esters (FAME) and glycerol concentrations were determined according to the method described by Budžaki et al. [16]. Briefly, the analysis of the samples was performed by gas chromatography (Shimadzu GC-2014, Tokyo, Japan) using heptadecanoic acid methyl esters as an internal standard. The GC was equipped with FID and Zebron ZB-wax GC capillary column (length

30 m, I.D. 0.53 mm and film thickness 1.00 μm, Phenomenex, Torrance, CA, USA). Nitrogen was used as a carrier gas in this method at rate of 1.97 mL/min. The measurement starts at a temperature of 180 °C for 1 min, heating the column to 230 °C at a rate of 5 °C/min; the total time of each analysis is 15 min. FAME yield was calculated as ratio of FAME formed per free fatty acids (FFA) in oil consumed by transesterification. FFA concertation in oil was determined according to the method described by Gojun et al. [4]. All samples were analyzed in triplicate. At a confidence interval of 95%, the results showed no significant difference.

2.2.6. Microreactor experiments for biodiesel production catalyzed by free lipase

Biodiesel synthesis was carried out in a PTFE microreactor (internal volume of 235.62 μL) with two inlets (T-shape) or three inlets (+-shape), depending on the feeding strategy. Proposed schemes for feeding strategies with 2-inlets and 3-inlets, are shown in Fig. 2a and b, respectively. A total of seven different experiments were performed and the corresponding experimental conditions are shown in Table 1.

In all experiments, methanol was fed separately to avoid inactivation of the enzyme. All performed experiments were first studied in a PTFE microreactor with diameter of 500 μm (Experiments 1–4). However, in the experiment where the emulsion was used as the inlet stream (Experiments 5 and 6), a 1000 μm diameter microreactor was used. Lipase activity was constant in all conducted experiments (S.A. = 723.34 ± 19.19 U/mg). All experiments were performed at 40 °C because lipase showed the highest activity at this temperature. Therefore, microreactors were immersed in a water bath with thermostat (Thermomix 1420, Braun, Germany) to achieve optimal enzyme activity.

Experiments 1–3 were based on 3-inlets strategy (Fig. 2b). Research started with Experiment 1, in which the stoichiometric ratio of the substrates (methanol:oil ratio of 3.4:1) was investigated and the experiment was performed for a residence time of 20 min. In the Experiment 2, the methanol:oil ratio was switched from stoichiometric to a ratio of 90:1. In the Experiment 3, the high methanol:oil ratio (90:1) was kept, but the order of inlets was switched in that way, that the inlet containing oil was placed in the middle between inlets for methanol and enzyme. In the Experiment 4, the high methanol:oil ratio was maintained and 2 x 2-inlets strategy was investigated (Fig. 2c). Briefly, the microreactor system in this experiment was composed of 2 T-shaped inlets connected in series. Methanol and oil streams were first mixed using T-shaped connection and the resulting stream (mixture of oil and methanol) was connected as the inlet of the second T-shaped connector where the lipase stream was the second inlet. Experiments 5 and 6 were based on 2-inlets strategy (Fig. 2a), where one inlet was an emulsion of oil and enzyme with added emulsifier, while the other inlet was methanol. Experiments 1–5 were performed with edible sunflower oil, while in Experiment 6 waste oil was used. Experiment 5 and 7 were performed under the same process conditions, the only difference was the biodiesel purification method used afterwords. The total flow in a microreactor experiments was altered at Φ = 3–700 μL/min.

Table 1
Experimental setups used for biodiesel synthesis.

Experiment	Strategy	PTFE tube diameter	Methanol:oil ratio	Oil source
1	3-inlets	500 μm	3.4:1	Edible oil
2	3-inlets	500 μm	90:1	Edible oil
3	3-inlets	500 μm	90:1	Edible oil
4	2 x 2-inlets	500 μm	90:1	Edible oil
5	2-inlets	1000 μm	90:1	Edible oil
6	2-inlets	1000 μm	90:1	Waste oil
7	2-inlets	1000 μm	90:1	Edible oil

2.2.7. Biodiesel production catalyzed by immobilized lipase

Biodiesel production catalyzed by immobilized lipase was performed using 2-inlets strategy. Methanol and oil were fed separately into a PTFE microreactor using piston pumps. The PTFE microreactor was wrapped around a permanent magnet and lipase immobilized on magnetic nanoparticles was loaded into system. A magnetic field was used to keep the lipase immobilized on magnetic nanoparticles in a PTFE microreactor. Methanol and oil were fed separately into a PTFE microreactor using piston pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA) (Fig. 2d). The initial specific activity of lipase was S.A. = 412.11 ± 6.31 U/mg. The whole system composed of a PTFE microreactor, a permanent magnet and pumps, was placed in the thermostat (MRC Orbital Shaker Incubator, Essex, UK) and the experiment was performed at a temperature of 40 °C. The Experiments were performed for different residence times ranging from 1 to 40 min.

2.2.8. Biodiesel purification/glycerol removal

Two main directions of biodiesel purification were studied: extraction of glycerol using DESs and removal of glycerol by ultrafiltration.

In the experiments where glycerol extraction was performed, ChCl-Gly based DES was used. A PTFE tube (length: width: depth = 30 cm: 1000 μm: 1000 μm, internal volume 235.62 μL) was used as a microseparator. Two inlet streams were connected to the microseparator by a T-shape connection. The first inlet stream was the biodiesel produced in the microreactor unit (Experiments 2, 5, 6 and experiment with immobilized lipase) and the second inlet was DES as the extraction medium. The streams were fed in the microseparator by piston pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA). Experiments were performed at different residence times and temperature of 25 °C.

The second purification method was based on the ultrafiltration. A homemade steal housing for ultrafiltration membranes was equipped with a PES membrane (13.4 cm^2, 10 kDa, Millipore, Germany). The produced biodiesel (Experiments 1, 3, 4, and 7) was fed into the membrane housing using a piston pump (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA). The upper outlet stream was purified biodiesel and the bottom outlet stream was a glycerol-rich waste stream. Both outlet streams were analyzed by GC.

2.2.9. Integrated systems

Two main types of integrated systems have been developed based on two purification methods. When the integrated process was composed of biotransformation and biodiesel purification by extraction, the experimental setup consisted of two PTFE tubes (Fig. 3a). Briefly, based on the feeding strategy used, syringes filled with substrates were placed on pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA) and connected to a tubular PTFE microreactor with either T-shaped or +-shaped connectors. The total flow in a microreactor was altered at Φ = 3–700 μL/min. The reaction mixture at the outlet of the microreactor was connected to a microseparator via T-shaped connector. DES was fed as a second inlet stream into a microseparator with a flow ratio DES:reaction mixture of 1:0.3. Samples were collected from the outlet stream of microseparator and analyzed by GC [15].

The integrated system which combines biotransformation and biodiesel purification based on ultrafiltration, had a slightly

different design. The biodiesel production part remained the same as in the previously described integrated system. The difference started with the outlet stream of the microreactor (reaction mixture) which was connected with a separation unit for the glycerol removal (Fig. 3b). The upper phase formed in the separation unit was transferred to the ultrafiltration module equipped with the PES membrane. One piston pump (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA) was used to pump out the purified biodiesel (upper stream) from the ultrafiltration module, while the second pump was used to pump out the glycerol-rich stream (bottom stream). To ensure that the biodiesel is successfully pumped out at the upper stream, the flow ratio upper:lower stream was kept at 4:1 (total flow $\Phi = 15$ μL/min). Samples from both streams were collected and analyzed by GC.

3. Results and discussion

3.1. Biodiesel production in a microreactor catalyzed by free lipase

The main drawback of lipase-catalyzed biodiesel production in batch systems is the inability to increase the methanol:oil ratio and consequently to shift the reaction equilibrium toward biodiesel formation. Namely, excess of methanol will lead to the fast enzyme deactivation [20]. An additional problem is the rate of the reaction, which is carried out in batch systems. Lipase-catalyzed biodiesel production in batch system has been described elsewhere. One of the examples is the research performed by Budžaki et al. [16], where a FAME yield of 96% was achieved after 48 h of lipase-catalyzed sunflower transesterification performed with stoichiometric amount of methanol. In the research perfomed by Price et al. [17], mathematical model was developed and used to predict biodiesel yield (90.8 ± 0.55%) when the enzymatic transesterification of rapeseed oil with methanol using CalleraTM Trans L (a liquid formulation of a modified *Thermomyces lanuginosus* lipase) was performed. The maximum biodiesel yield of 94.2 ± 1.3% under the optimal conditions (liquid lipase Eversa® transform 2.0 load 6%, water content 20%, 7:1 (mol/mol) ethanol to Semen Abutili (*Abutilon theophrasti* Medic.) seed oil, 11 h, 37 °C) was achieved in the research presented by Sun et al. [31].

There are several possible improvements for biodiesel production by lipase-catalyzed transesterification performed in a batch system, such as the use of a fed-batch reactor with methanol feed, or the use of reactor systems where the enzyme is in contact with methanol for a short time period, with microreactors appearing to be the most viable option.

Feasible biodiesel production from sunflower cooking oil or waste oil by enzymatic transesterification in microreactor systems has been shown elsewhere [4,21].

In our previous study [21], the methanol:oil ratio was switched from the stoichiometric ratio to a very high methanol excess. When the methanol:oil ratio was switched from 3.4:1 to a ratio of 90:1, the FAME yield increased from 5 to 90% for the same residence time ($\tau = 120$ min).

In this study, biodiesel was produced in different microreactors applying different feeding strategies. In Experiment 1, 3-inlets feeding strategy (Fig. 2b) was used to investigate the initial stoichiometric ratio (3.4:1). Under these conditions, a FAME yield of 35% was achieved. In order to improve process in term of FAME yield, Experiment 2 was performed with a high excess of methanol. Namely, the methanol:oil ratio was switched from 3.4:1 to a ratio of 90:1 and Experiment 2 was performed using the same 3-inlets strategy (Fig. 2b). Unfortunately, the obtained FAME yield was only 2% at a residence time of 20 min, probably due to the inactivation of lipase by the high excess of methanol. It is known, from the research performed in similar systems that lipase activity drops below 5% of its initial activity in about 30 min [20]. In the Experiment 3, the high methanol:oil ratio was kept, but the order of inlets was switched in that way, that the inlet containing oil was placed as the central inlet stream of the +-shape connector. The inlet with oil was placed in the central position to separate methanol and lipase streams and to prevent direct contact of methanol and lipase already at the entrance of the microreactor. Consequently, the deactivation of lipase by methanol was delayed and for aforementioned residence time ($\tau = 20$ min) a FAME yield of 46% was achieved.

This was the base for further improvement of the feeding strategy, which was realized in frame of the Experiment 4. Basically, the microreactor system in this experiment was combined of 2 T-shaped inlets connected in series. Methanol and oil streams were first mixed using T-shaped connection and the resulting stream (mixture of oil and methanol) was connected as the inlet of the second T-shaped connector where lipase stream was the second inlet. Using this configuration, the excess of methanol should not inactivate the lipase immediately. The FAME yield obtained using this feeding strategy was only 33% and was even lower than in the experiment performed using modified 3-inlets feeding strategy of Experiment 3.

Since all the previously mentioned experiments did not show satisfactory FAME yield, 3-inlets strategy was switched to 2-inlets strategy, using a mixture of oil and methanol (emulsion developed by adding SDS) as one inlet stream and methanol as the other inlet stream of a microreactor. Based on this result and our previous experiments [21], Experiment 5 was performed where 2-inlets feeding strategy was applied while maintaining the high methanol excess. It is important to clarify that this system was based on the formation of an emulsion of oil and lipase, which was used as one of inlet streams. Emulsion of oil and lipase probably preserved the lipase activity even though methanol was added in high excess. Experiment 6 was performed using the same feeding strategy as Experiment 5, but with one difference. Waste oil was used in emulsion formation and consequently for biodiesel production. The influence of residence time on FAME yield for 2-inlets feeding strategy applied in Experiments 5 and 6 are shown in Fig. 4.

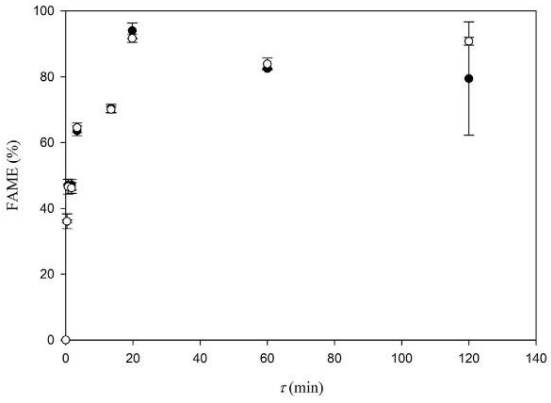

Fig. 4. Influence of residence time on FAME yield for biodiesel production catalyzed by lipase performed in a microreactor using 2-inlets feeding strategy (● - edible oil as an oil source in biodiesel production (Experiment 5), ○ – waste oil as an oil source in biodiesel production (Experiment 6)).

3.2. Biodiesel production in a microreactor catalyzed by lipase immobilized on magnetic particles

Lipase immobilized on magnetic particles in combination with the use of a microreactor placed in the magnetic field was an additional experimental setup used for biodiesel production. The idea behind this experiment is related to the prevention of lipase wastage during biodiesel production in a microreactor. In Experiments 1–7, lipase in free form was used and consequently, was washed-out from a microreactor during continuous biodiesel production. The use of lipase immobilized on magnetic nanoparticles in a microreactor placed in a magnetic field will maintain enzyme in the reactor while all other components of the reaction mixture should pass through microreactor. In this way, lipase can be reused and the continuous biodiesel production will be economically sustainable considering that the cost of the enzyme is the most important issue for lipase-catalyzed biodiesel production. In addition, the purification steps following the bioproduction should be easier and simpler to perform.

Experiments at different residence times with lipase immobilized on magnetic nanoparticles in a microreactor placed in a magnetic field were performed and the results are shown in Fig. 5. The main problem in this experiment was the activity of the immobilized lipase. After immobilization on magnetic nanoparticles, the lipase activity maintains only 57% of initial activity (S.A. = 412.11 ± 6.31 U/mg). The FAME yield was highest for relatively short residence time and at the residence time of 3.5 min it was 35% (Fig. 5). Unfortunately, the FAME yield decreased at longer residence times, most likely due to washing of lipase from the magnetic nanoparticles and the inactivation by methanol. Nevertheless, the initial results of biodiesel production with lipase immobilized on magnetic nanoparticles were promising and surely base for further process optimization.

3.3. Integrated systems

Clear improvement of continuous biodiesel production in a microreactor in comparison with batch reactor (96% FAME yield in 48 h [16,17]), is shown in this and in our previous research [4]. Briefly, by increasing the concentration of methanol in the system the FAME yield will increase. It was observed that in the process where a large excess of methanol was used, the FAME yield was over 90% for a residence time between 20 and 40 min [20,21]. This

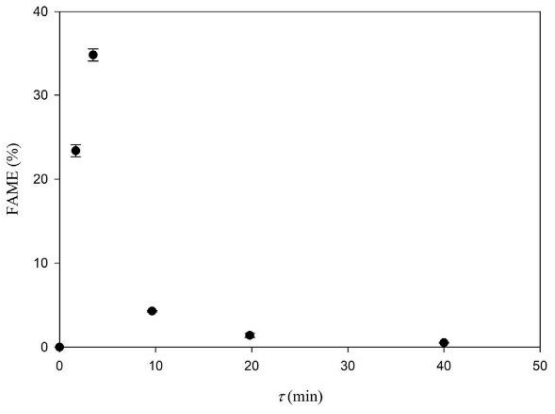

Fig. 5. Influence of residence time on FAME yield for biodiesel production catalyzed by immobilized lipase performed in a microreactor.

was only possible in a microreactor configuration where a mixture of oil and methanol (emulsion developed by adding SDS) was used as one inlet stream and methanol as the second inlet stream. Purification of produced biodiesel is mainly related to the removal of glycerol. That process has been recently extensively studied [32] in macroextractors [24] and microextractors [23] using water [23] or DESs as solvents. Glycerol-based and ethylene glycol-based DESs were mainly used in experiments where DES was used as solvent [23,33]. Other approach is based on use of solvents such as hexane, 2-methyltetrahydrofuran (2-MeTHF), and cyclopentyl methyl ether (CPME) in combination with column chromatography to extract glycerol [34]. In order to avoid addition of different solvents in the process line, dry methods can also be applied such as ultrafiltration [25,35] or membrane contactor for liquid–liquid extraction [36]. Based on results of our previous research [23,25], two main directions in biodiesel purification were further developed: extraction of glycerol using DES and glycerol removal by ultrafiltration.

As an additional improvement of the biodiesel production process, two experimental setups combining production and separation steps in series were developed. Basically, biodiesel production in a microreactor was directly connected with two types of biodiesel purification investigated in our previous research: extraction by DES [23,24] and purification by ultrafiltration [25]. In our previous research, biodiesel is firstly produced, partially purified for 48 h by sedimentation and used as initial material in two different purification methods. It is important to note, that in all previous purification studies [20,23–25], the glycerol content was reduced below the limit according to EN 14214:2013 [5] (free glycerol <0.02% (w/w)). In this research, the biodiesel produced in a microreactor was directly fed to the purification unit and in the overall, process was significantly shorter.

3.3.1. Integrated system with glycerol removal by DES based extraction

The extraction Experiments were mainly based on the assumption that DESs can be a very good alternative extraction medium in comparison with water (process of wet washing), which is still the most commonly used in industrial processes of biodiesel purification. In our previous studies [23], ChCl-Gly DES has been shown to be the best extraction medium for biodiesel purification, which mainly refers to the removal of glycerol.

The base for the development of integrated microsystems in this research was mainly found in the research of Šalić et al. [20]. In that research, a mixture of oil and lipase in the form of an emulsion was used as one of the two substrates, as this form was shown to protect the lipase activity from inactivation due to a high methanol excess. A very high FAME yield of 95% was achieved with a residence time of less than 1 h. The direct in-line unit for biodiesel production, the unit for biodiesel purification by the means of using ChCl-Gly DES reduced the glycerol content to 0.02% (w/w), the maximum limit according to the standards. The breakthrough of that research was definitely the first proposal for an integrated microsystem, consisting of two microchips connected in series, for biodiesel production and purification. Of course, that system had some drawbacks that this work tried to address. The most important disadvantage is the reusability of the enzyme. This problem can be solved either by enzyme recirculation or enzyme immobilization, which was addressed in this work. Other problems include the use of emulsifiers, elimination of phases and separation of pharmaceutical grade glycerol from the waste stream.

In the experimental setups using DESs, it was found that ChCl-Gly DES was much more effective compared to ChCl-EtGl DES [23]. The glycerol content was even lower with ChCl-Gly DES compared to ChCl-EtGl DES, with shorter residence time ($\tau = 20$ min).

3.3.2. Integrated systems with glycerol removal by ultrafiltration

While extraction as a purification method was successful, another approach for glycerol removal based on ultrafiltration was investigated. The integrated system was based on the studies of Sokač et al. [25], where the PES membrane was successfully applied for the glycerol removal resulting in glycerol concentrations in the purified biodiesel in accordance with EN 14214:2013 [5] standard (free glycerol <0.02% (w/w)).

As previously described, after biodiesel production in a microreactor, reaction mixture was fed to separation unit for glycerol removal (Fig. 3b). In order to ensure that the biodiesel is successfully pumped through the membrane as the upper stream, the upper stream/bottom stream flow-ratio was kept at 4:1 to achieve a uniform flow of purified biodiesel. The integrated experiment was performed for different residence times and the results are shown in Fig. 6. The highest FAME yield of 90.7% was obtained for a residence time of 20 min.

All results of the integrated processes are summarized in Figs. 7 and 8. The FAME yield obtained in different integrated systems is shown in Fig. 7. The highest FAME yield was achieved in experiments where 2-inlets feeding strategy was combined by DES based purification (Experiment 5 and 6). Taking into consideration confidence intervals, FAME yield achieved in those experiments was within range defined by biodiesel quality standards. Similar results were achieved in experiment where 2-inlets feeding strategy was integrated with ultrafiltration. Obviously, 3-inlets feeding strategy is not an appropriate method to achieve high FAME yields despite the method used for biodiesel purification (Experiments 1–3). The same is for 2 × 2 inlets feeding strategy, the low FAME yield obtained during production cannot be increased by the integrated purification. Although promising, the integrated biodiesel production based on lipase immobilized on magnetic nanoparticles needs further optimization (Experiment 8).

The glycerol content (w/w) in different integrated experiments is also analyzed (Fig. 8). Obviously, the highest biodiesel purities were obtained in the experiments with low FAME yields. On the other hand, both purification methods were quite successful, even in the integrated experiments where the FAME yield was high. Experiment 5 was identified as the best integrated experimental setup. Namely the highest FAME yield of 94 ± 3.1% and the glycerol content below 0.02% (w/w) (which is the maximum glycerol content according to EN 14214:2013 [5]) were obtained in the integrated system in which the 2-inlets feeding strategy was combined with the DES based extraction.

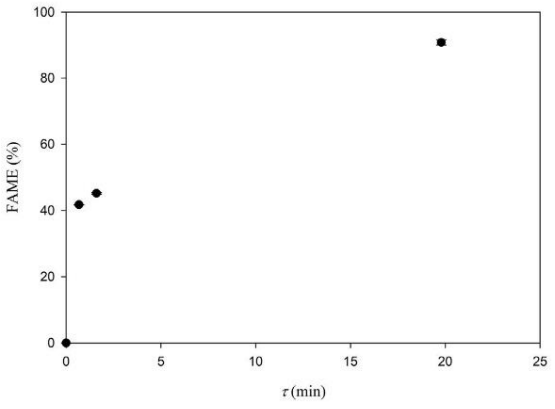

Fig. 6. Influence of residence time on FAME yield for integrated systems with biodiesel purification by ultrafiltration.

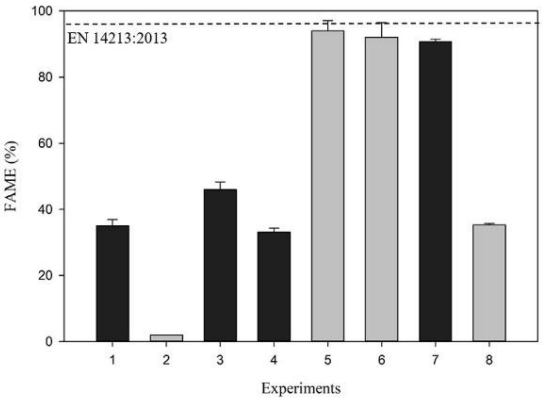

Fig. 7. FAME yield accomplished for different integrated systems (biodiesel purification by: ■ – ultrafiltration, ■ – DES based extraction).

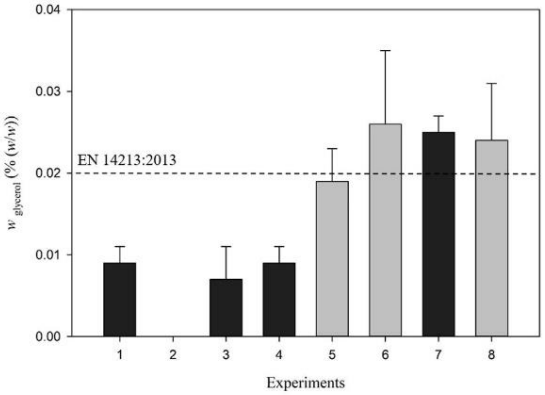

Fig. 8. Glycerol content in biodiesel for different integrated systems (■ – ultrafiltration unit, ■ – DES as extraction medium).

4. Conclusion

In this paper, several integrated microsystems composed of biodiesel production and purification have been developed. Although promising, biodiesel production using lipase immobilized on magnetic nanoparticles requires further optimization. The best integrated microsystem was the set-up where 2-inlet feeding strategy for biodiesel production was combined with DES based extraction. In this integrated system, a FAME yield of 94% and glycerol content in the purified biodiesel, lower than 0.02% (w/w), for the residence time of 20 min, were achieved. The reduction of reaction time was a significant improvement in comparison with the same process performed in a batch reactor (96% FAME yield for 48). This confirms the initial assumption that an integrated microsystem composed of production and purification steps connected in series could be the solution for intensification of biodiesel production process. On the other hand, additional step has to be included in the integrated process, namely, methanol recovery and recirculation. Without methanol reuse proposed integrated

production and purification process is not environmentally and economically feasible.

CRediT authorship contribution statement

Martin Gojun: Investigation, Methodology, Visualization, Data curation, Writing – original draft. **Anita Šalić:** Methodology, Visualization, Writing – review & editing. **Bruno Zelić:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CURRICULUM VITAE

Martin Gojun [redacted] Primary education he acquired while attending the elementary school "Cvjetno naselje" in Zagreb. In 2007, he enrolled in the 4th language grammar school in Zagreb, where in addition to languages he showed exceptional interest in natural sciences, especially chemistry. After graduating from high school in 2007, he enrolled in the desired study of Environmental engineering at the Faculty of Chemical Engineering and Technology, University of Zagreb (Faculty). He completed his undergraduate studies in Environmental engineering at the Faculty in 2015.

In addition to studying, he was also interested in raising the quality standard of student and during four years as a member of the Student Council, he performed several functions, such as student Ombudsman, Deputy President and President of the Student Council.

In 2016, the Faculty nominated him for a special Rector's Award for participation and winning the award at MSST X. By the decision of the Rector, he received a special Rector's recognition on the occasion of the University of Zagreb Day on November 3rd 2016. He graduated from Faculty in 2017.

In addition to his notable academic work, he also participated in numerous sports events as a table tennis player. In six years of study, he won 24 medals representing the Faculty, winning two Most Valuable Player (MVP) awards.

During 2018, he worked in CROTEH Ltd as an R&D engineer. In September on 2018, he returned to the Faculty, to enroll on project led by Prof Bruno Zelić, PhD, dealing with production of biodiesel in microsystems. During the spell of 4 years, he published as co-author 7 scientific papers and participated in 15 international and national scientific conferences with oral and poster presentations.

Scientific papers:

M. Gojun, M. Bačić, A. Ljubić, A. Šalić, B. Zelić, Transesterification in microreactors - overstepping obstacles and shifting towards biodiesel production on a microscale, Micromachines 11 (2020) e457.

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