BICATALYTIC CONVERSION OF GLYCEROL TO VALUE-ADDED PRODUCTS

by

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Dedicated to my parents, Elena and Vladimir Sotenko

Summary

The objective of this study was to develop a tandem bio- chemical transformation of a feedstock material to value-added products. The chosen example is microbial fermentation of glycerol with subsequent esterification of the intermediate 1,3-propanediol with fatty acids. The use of biphasic aqueous/organic medium for the bi-catalytic system is the key feature of this study.

In the first part of this work, batch and continuous fermentations of glycerol by *Clostridium Butyricum* bacteria were optimized to increase productivity of 1,3-propanediol. In the second part, several catalysts were screened for mono- and biphasic transformation of 1,3-propanediol. It was discovered that enzymes are the most suitable catalysts for the tandem reactions of glycerol to 1,3-propanediol derivatives compared to chemical catalysts. Biphasic enzymatic esterification of 1,3-propanediol was further optimized using *Rhizomucor miehei* lipase. Finally, a segmented flow tubular reactor and hollow fiber membrane contactors were designed and tested as a concept tandem reactor. The hollow fiber reactor with *Rhizomucor miehei* lipase immobilized onto polypropylene membrane was found to be the most effective in the biphasic linoleic acid/aqueous esterification of 1,3-propanediol.

In general, the demonstrated approach and the developed system can be easily utilized in the biorefinery processes to avoid extraction and purification of the intermediate products, thus reducing time, energy and emissions.

Acknowledgements

I would like to thank my supervisor Professor Alexei Lapkin for this great opportunity to continue my study as a PhD student first at the University of Bath and then at the University of Warwick. Professor A. Lapkin directed my work with valuable advice, fresh ideas, discussions of results and constant support in all my difficulties.

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Declaration

Signature of the author:

Maria V. Sotenko

Contents

List of abbreviations and symbols

A-15 – Amberlyst - 15

AA – acetic acid

An – anisole

$AmNTf_2$ – methyltrioctylammonium bis(trifluoromethylsulfonyl) imide

B – batch

BA – butyric acid

BES – bis-2-ethyl-hexyl-sebacate

$BmimNTf_2$ – 1-buthyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide

BPh – dibutyl-phthalate

$BuPyNTf_2$ – 1-buthylpyridinium bis(trifluoromethylsulfonyl) imide

Bz – benzene

CALB – *Candida Antarctica* lipase B

CLEA – cross-linked enzyme aggregates

DA – decanoic acid

dBE – dibutyl ether

dHE – dihexyl ether

dPhE – diphenyl ether

Euc – eucalyptol

FB – fed-batch

Gly – glycerol

HF – hollow fiber

IL – ionic liquid

KPi – potassium phosphate

LA – linoleic acid

LacA – lactic acid

N-435 – Novozyme 435

OS – di-octyl-sebacate

P – α-pinene

PD – 1,3-propanediol

PES – polyethersulfone

EB – ethylbenzene (phenetol)

PLLA – polylactic acid

ROP – ring-opening polymerization

PP – polypropylene

PTFE – polytetrafluoroethylene

T – α-terpinene

Tetr – tetradecane

THF – tetrahydrofuran

α – phase volume ratio

a_w – water activity

C – concentration, mol L^{-1}

D – dilution rate, h^{-1}

E_a – activation energy, kJ mol^{-1}

ξ – fractional conversion

F – flow rate, ml min^{-1}

K_m – Michaelis constant

K_i – inhibition constant

K – equilibrium constant

L – length, cm

N – number of membranes

n – number of moles, mol

μ_{max} – maximum growth rate

P – partition coefficient

Q – productivity, g L^{-1} h^{-1}

r – reaction rate

S – interfacial area, cm^2

V – volume, L

V_{max} – maximum reaction rate, mol h^{-1} g^{-1}_{cat}

x – conversion

Y – yield, mol mol^{-1}

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Introduction

Highly oxygenated and often low purity bio-feedstocks are difficult to convert into the desired products by conventional chemical catalysis. However they are suitable substrates for biotechnological processes with bacteria and yeasts as catalysts, which can selectively ferment them to a range of simpler molecules. These molecules can then be further used as building blocks for synthesis of useful higher-value products by traditional catalysts.

Transformation of feedstocks into industrially important chemicals can be accomplished within a biorefinery – sequential bio- and chemical conversion of feedstocks for food, fuels, chemicals, heat and power applications. However, the main disadvantages of sequential processing are time, energy and materials-consuming extraction and purification of the intermediate products of the individual steps, such as the fermentation processes. This also means an increase in waste, solvents and, therefore, a contribution to the detrimental impact of multi-stage industrial processes on the environment. To meet the demands of intensification and optimisation of bioprocesses a new methodology has emerged, that integrates bio- and chemo-catalysis within a single reaction system.

In general, the main idea of developing a combined bio-chemical system is in the combination of the principles of biology, chemistry and engineering. But the main scientific challenge is mutual incompatibility of bio- and chemical catalytic processes, caused by toxicity of chemicals (products, by-products, solvents or chemical catalysts) for bio-systems, low catalytic activity due to low temperature and pressure, low system stability and selectivity, *etc*. The problem of compatibility can be resolved by spatial and/or temporal separation of both

systems within a single reaction space. Supported catalysts, immobilized cells, biphasic media or selective membranes are considered as potential routes to achieve the desired result.

The main aim of this project is to develop a tandem bio-chemical transformation of glycerol to useful products. Thus, the vision of this project is to bring the theory to practice and to evaluate the key principles of tandem bio-chemical systems.

Glycerol, as a by-product of transesterification of plant oils for biodiesel production, has become a relatively cheap and widespread feedstock material, which is attractive for biotransformations. The most well studied conversion of glycerol to value-added products is its microbial fermentation to 1,3-propanediol which is used in the synthesis of polymers [1]. At the same time this process involves extraction of 1,3-propanediol from a diluted aqueous solution, which is a significant technological challenge [2].

We propose a sequential transformation of 1,3-propanediol formed *via* a biocatalytic fermentation by a catalyst integrated within the same reactor system. The problem of different operating conditions and mutual toxicity of bio- and chemical catalysts can be resolved by using a biphasic reaction medium. In order to achieve the main goal, several tasks need to be undertaken, such as:

1) optimization of the glycerol fermentation by *Clostridium butyricum*,
2) screening effective catalyst-solvent pairs for 1,3-propanediol transformation, and
3) design of a reactor system for the combined two-phase process.

1 Literature review

This literature review provides a short survey of biotechnology with a closer look at non-aqueous and biphasic biocatalysis, immobilized biocatalysts and membrane bioreactors, which are directly relevant to this study. Several approaches combining multi-step bio-chemical transformation within a single system are discussed, and some examples of such systems are presented. Within the main goal of the project, glycerol is considered as a feedstock for the synthesis of useful products. A detailed review of its microbial fermentation to 1,3-propanediol is followed by discussion of the problem of 1,3-propanediol extraction from the fermentation broth. Commercial processes of 1,3-propanediol synthesis are also mentioned. A separate chapter is devoted to different possible routes of 1,3-propanediol transformation to value-added products with a particular attention to catalysts for esterification of 1,3-propanediol with acids. Enzymatic polycondensation is considered in detail.

1.1 Biocatalysis as alternative to conventional catalysis

There is an increasing demand for environmentally friendly technologies and processes in the chemical industry due to the growing concern about the irrevocable harmful anthropologic impact on the environment. In some cases traditional organic/inorganic catalysts or stoichiometric reactions could be replaced by biocatalytic routes, which offer environmental benefits [3-6]. Taken from the environment, biocatalysts seem to satisfy the criteria of green chemistry [7] in terms of biodegradability, high selectivity, mild reaction conditions and low amounts of waste and by-products. Biocatalysis has a much longer history than chemical catalysis, dating back to ancient times, when bacteria were used to produce alcohol and milk-

based products, whereas the oil of vitriol as a catalyst of alcohol esterification was discovered by alchemists only in the 15th century [8]. Since then, many biocatalysts were discovered and many biocatalytic processes were established.

Firstly, biochemistry was widely adopted for the food, agriculture, paper, leather, textiles industries and waste treatment [5, 9-13]. Two of the largest processes estimated at tens of millions tones per year are the production of fructose syrup [9] and bioethanol [14]. These two processes have several common steps, when first alpha-amylase is used to liquefy starch and then glucoamilase further hydrolyzes starch to glucose. Glucose further undergoes isomerisation by glucose-isomerase, leading to fructose syrup, or is fermented to ethanol by yeasts. Fructose is sweeter in comparison to glucose. Hence, different mixtures of glucose and fructose are used as sugar substitutes in food and beverages, although there is still a great controversy about their safety to human health. Ethanol is a highly demanded product in the food industry, and also used as a pure or blended (20-25 %) fuel. In the last decade production of bioethanol from non-food lignocellulosic biomass attracted significant, but the key problem of cellulose degradation to glucose still remaines [15].

Secondly, many biocatalytic processes are developed for production of pharmaceuticals and fine chemicals, such as antibiotics, vitamins, pharmaproteins, steroids, amino and organic acids, enzymes and polysaccharides [16-19]. Thus, a significant cost-reduction was achieved by changing the chemical production of riboflavin to a biotechnological route due to substitution of the multi-step process with a one-step fermentation that has a lower environmental impact [3]. *Bacillus subtilis* harvested on glucose (DSM, Germany) yields 85 %

of riboflavin, which crystallises in the fermentation medium, and can be easily separated. In another example the biosynthesis of acrylamide (**Figure 1.1**) replaced the acid-catalysed process. The synthesis, established by Mitsubishi Rayon, Japan, is carried out in a semi-batch reactor with *Rhodococcus* bacteria immobilized onto a poly(acrylamide) gel [16]. The substrate concentration is maintained below 3 % to avoid its inhibitory effect on the bacteria.

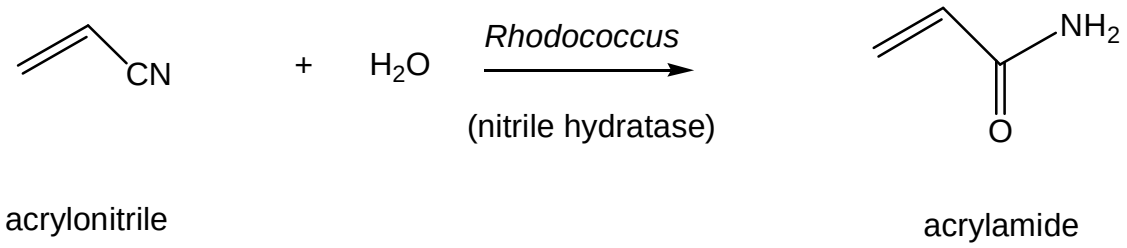


Figure 1.1. A scheme of acrylamide biosynthesis.

The chemical synthesis of vanillin starts with toxic phenol and involves carcinogenic dimethyl sulfate. A potential biosynthetic route to vanillin (**Figure 1.2)** involves first recombinant *E. coli*, which consumes glucose and produces vanillic acid, which is then converted to vanillin by aryl aldehyde dehydrogenase isolated from *Neurospora crassa* [16].

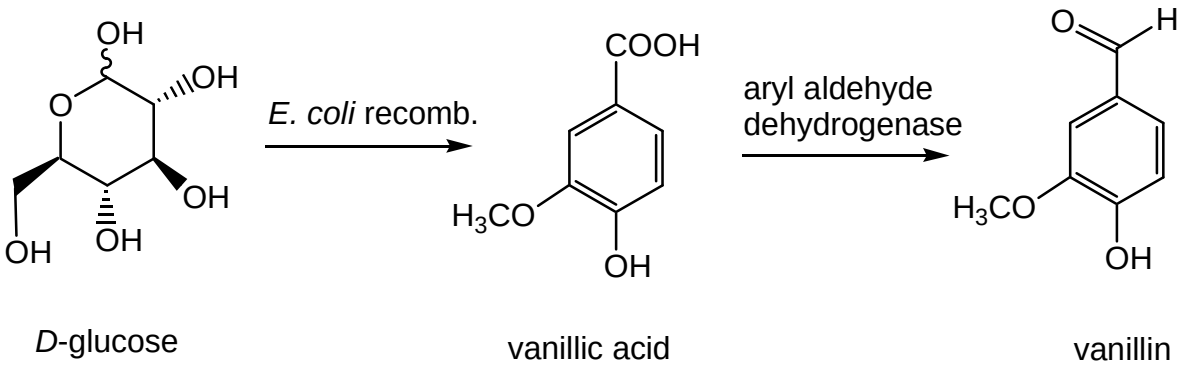


Figure 1.2. A scheme of biosynthetic route to vanillin.

Due to concerns about dwindling fossil fuels and increasing CO_2 emissions, biocatalysis began to play a significant role in chemistry and production of renewable and clean energy sources, such as biodiesel, bioethanol and biohydrogen [4, 6, 20]. In many cases biocatalysis has advantages when compared to traditional chemical routes. For example, industrial production of biodiesel by alkali catalysts (KOH, NaOH) is currently under intense development. The process is characterized by low cost of the catalysts and high reaction rates. At the same time the catalysts are sensitive to water and to the presence of free fatty acids, and need to be neutralized and washed away after completion of a reaction, leaving product glycerol of low quality. The enzymatic transesterification of oils by *Mucor miehei*, *Candida antarctica*, *Rhizopus oryzae*, *Pseudomonas cepacia* lipases offers such advantages as catalyst reusability, higher stability towards water and free fatty acids, low reaction temperatures, less waste and a lower environmental impact [6]. Enzymatic transesterification of oils was intensively investigated and several approaches were developed to overcome the problem of the inhibitory effect of methanol on lipases. These are: immobilization of the enzymes to increase their stability, the use of other acyl-acceptors (methyl acetate, ethyl acetate) or alcohols (ethanol, 2-propanol), dosing of methanol to maintain its low concentration, and development of methanol-tolerant lipases [21]. Enzymatic synthesis of biodiesel is particularly attractive for conversion of waste cooking oils with high concentration of water and free fatty acids (2.5-5.5 wt %). Enzymes are tolerant to the residues, whereas traditional inorganic catalysts undergo saponification.

Biotechnological processes with bacteria, yeasts and fungi are still dominant over enzymatic biotransformations (**Figure 1.3**), but mainly due to the food and beverage sectors of industry [18, 22]. The applications of enzymes vary from food, detergents to pharmaceutical and

chemical industries. The process set up for the whole-cell biocatalysis has lower costs of catalyst production compared to that of enzymatic processes due to no need for enzyme and cofactors isolation [23]. Also, whole cells are characterized by the ability to regenerate cofactors *in situ* and by multiple enzymes chains. However, bacteria or yeasts generally will not tolerate high concentrations of a substrate or a product, have limited compatibility with organic solvents and can generate unwanted side products [24].

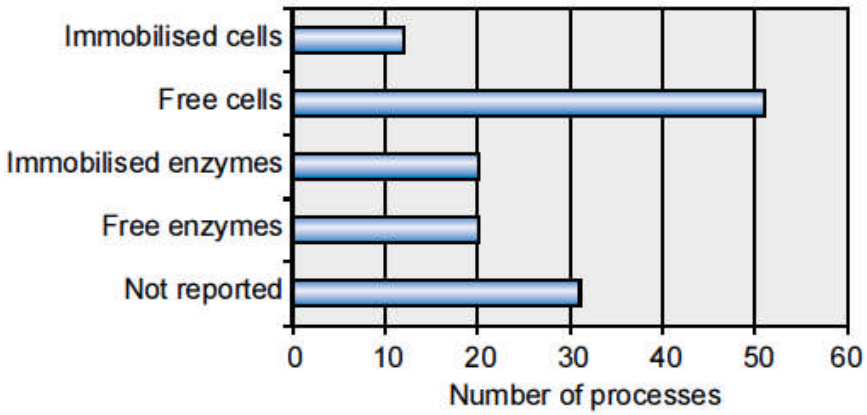


Figure 1.3. Distribution of biotechnological processes between bacterial and enzymatic [18].

Most bioprocesses are based on batch and fed-batch reactors, and fewer on continuous plug-flow reactors [18]. There are a number of problems with large-scale biocatalytic processes, such as low product concentrations, difficulties of heterogeneity and maintenance of fermentation parameters (concentration of nutrients, temperature, pH, pO_2).

Based on the two types of reactors - batch and flow - their several modifications are used in biocatalysis: stirred tank, fed-batch reactor, bubble column, airlift reactor, packed and

fluidized reactors (**Figures 1.4** and **1.5**). Membranes can be used in all types of the reactors as an additional filtration unit or a functional part of the complete system.

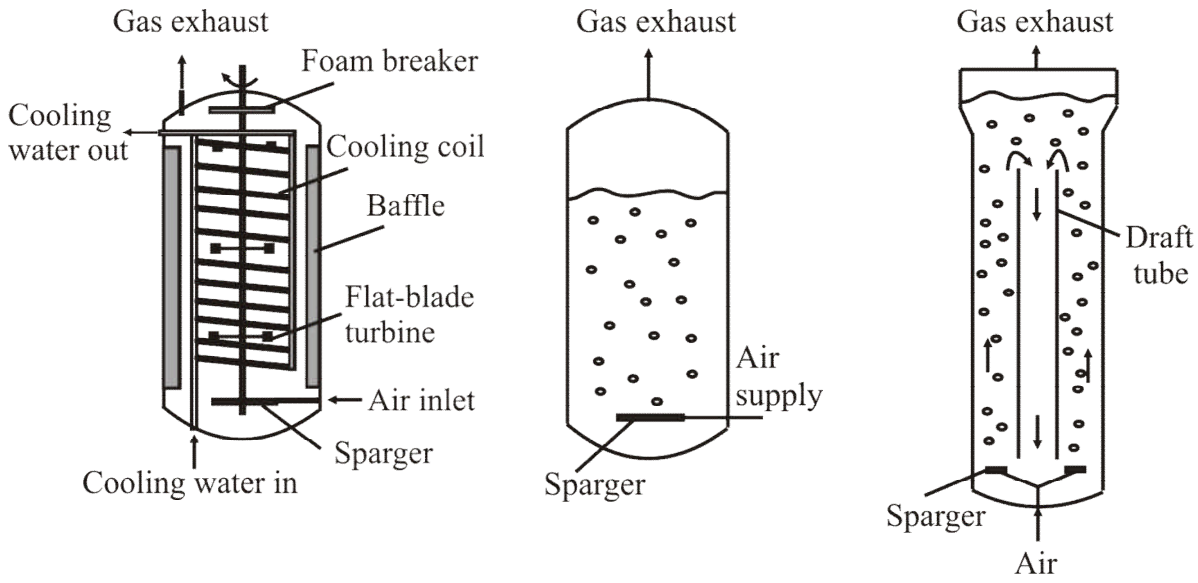


Figure 1.4. Schematic diagrams of the different types of bio-reactors: stirred tank, bubble column and air-lift reactors (adapted from [25]).

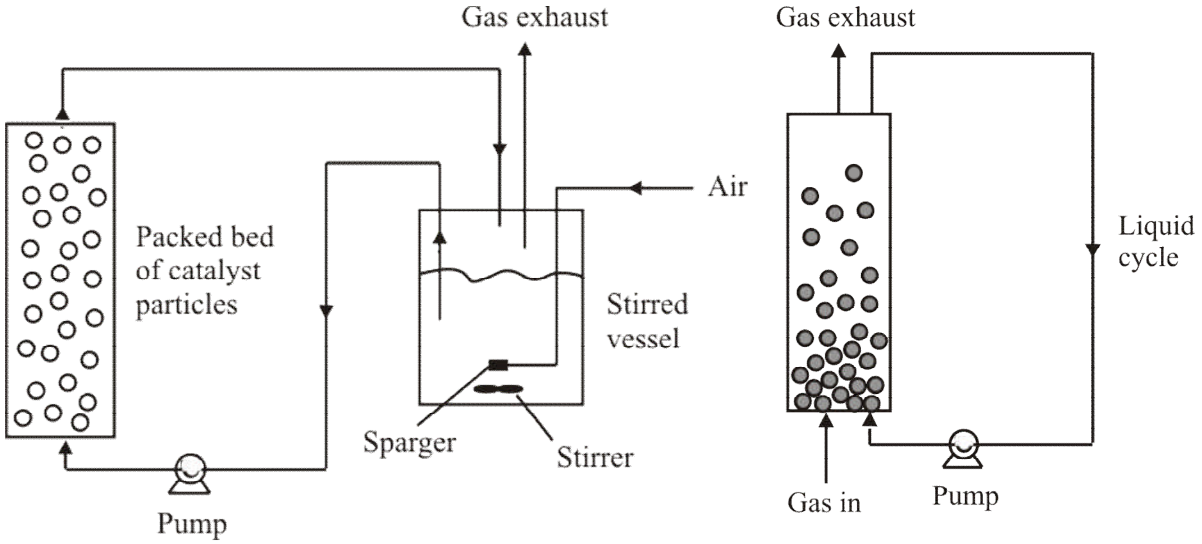


Figure 1.5. Schematic diagrams of the different types of bio-reactors: packed-bed and fluidized-bed reactors (adapted from [25]).

A fermentor is a stirred tank reactor with gas sparger, controllers of pH, flow and temperature, and a cooling water jacket if needed. Agitation and aeration are achieved by mechanical stirring, whereas gas sparging is used as the means of mixing in the column and the airlift reactors. Mechanical stirring provides better heat and mass transfer but can damage cells and has higher energy consumption compared to the air-agitation systems. Gas flow rates, sparger design, vessel construction, medium properties can be optimized to get better mixing.

Airlift bioreactors are widely used for Baker's yeast, glutamic acid, thuringiensin, and chitosan production [26]. Batch systems can be operated in a continuous mode (fed-batch reactor) with either free or immobilized biocatalysts. In the cases of packed and fluidized bed reactors only immobilized cells or enzymes can be used. In this case liquid nutrient broth is circulated through a tube filled with catalyst particles. Packed-bed reactors are applied for the production of aspartate and fumarate, conversion of penicillin to 6-aminopenicillanic acid and resolution of amino acid isomers [25].

Fermentation processes need to be monitored for a large number of parameters, such as temperature, pH, pressure, liquid and foam level, agitator speed, gas and medium flow rate, culture viscosity, broth composition, gas and biomass concentration [27].

In conclusion one can say that biocatalysis occupies a leading place in the food industry, pharmaceuticals and fine chemicals industries. There is a common tendency to replace traditional chemical catalysts for synthesis of chemicals and fuels with biocatalysts. Yet, most bioprocesses are based on using bacteria in batch systems, where productivity is low

(compared to isolated/immobilised enzymes or continuous fermentation), and the problem of product extraction from the multi-component broths exists. In spite of these drawbacks, biocatalysis offers environmentally friendly and economic way of synthesis of useful chemicals. Moreover, a feasibility of using bio-catalysts in organic solvents or organic/ aqueous mixtures and in tandem with the chemical catalysts may considerably broaden the range of their applications.

1.2 Biocatalysts in non-aqueous medium

Within the last two decades investigation of biocatalytic activity in the non-aqueous solvents has been being carried out intensively [28, 29]. The feasibility of using biocatalysts for transformation of water-insoluble compounds attracts more and more attention both in science and industry. The tolerance of enzymes to organic solvents is determined by the influence of solvents on the conformational mobility and stability of the protein structure, polarity of the enzyme's microenvironment and the presence of water residues on the enzyme's surface [28]. Several methods are used to enhance the stability of enzymes, such as lyophilization with inert excipients (inorganic or organic salts, crown ethers, cyclodextrines), mutagenic, chemical modification, immobilization, encapsulation by surfactant additives [29, 30].

Compared to enzymes, whole cells are more complex systems and solvent toxicity is dependent mainly on the stability of the cytoplasmic membrane. In the absence of water, van der Waals interactions between the hydrocarbon chains of the phospholipids in membranes increase and dry lipids turn into a gel phase at room temperature [31]. When such dry lipids are rehydrated, they become unstable. Moreover, dehydration leads to an increase in the free-

radical attack on phospholipids, DNA and proteins [32]. It has been proven that an organic solvent accumulation in the membrane depends on the concentration of solvent in the aqueous phase [33]. That is why solvents with high polarity and log P values below 4 (**Figure 1.6**) are considered extremely toxic to cells, as their degree of partitioning into the aqueous layer (which contains cells) and from there into the lipid membrane bilayer is high [31]. The trend shown in **Figure 1.6** is correct for enzymes too, because organic solvents with low log P values dissolve in the aqueous layer that covers enzyme globules and may cause protein denaturation.

Non-organic solvents, which might be less toxic for bio-catalysts, are attractive alternatives to organic solvents. Ionic liquids are known to be non-volatile and stable solvents with tunable properties [34-37]. Supercritical CO_2 and other supercritical solvents are considered to be suitable for enzyme-catalysed reactions [38, 39]. Another way to increase the tolerance of bacteria and enzymes to solvents is their immobilization onto solid surfaces (*e.g.* cellulosic materials, clays, glass), pore matrices (agar, chitosan, gelatin, collagen) or polymer fibers [40].

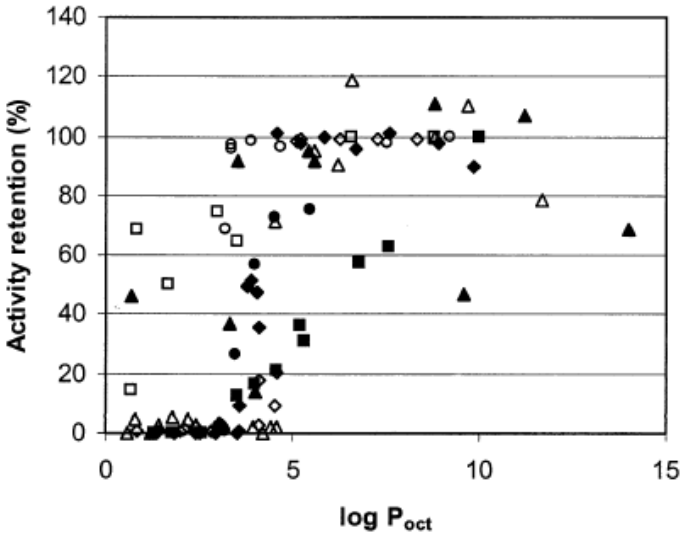


Figure 1.6. Dependence of the microbial activity in organic solvents on log P [41].

In conclusion one can say that after the revolutionary papers of A. Klibanov [42, 43] on performance of enzymes in organic solvents it has become clearer how organic solvents can influence activity of biocatalysts. In the case of enzymes, their protein conformation is mostly affected by organic solvents, whereas the cytoplasmic membrane prevents cells from dying in non-aqueous media.

1.3 Biphasic reaction systems

Biphasic reaction systems have many beneficial properties for reactions involving poorly soluble, unstable or inhibitory substrates and/or toxic products. An increase in productivity could be achieved *via* separation of a catalyst from a substrate or a product (by-product) in different phases, thus providing simple product recovery and avoiding inhibitory or other unwanted interactions. Hydrolysis may be suppressed and the overall reaction equilibrium may be shifted towards the desired products.

The choice of solvents is driven by their functionality. Thus, hydrophobic solvents like *n*-hexane, *n*-heptane, *n*-dodecane, benzene, and cyclohexane allow the water content to be decreased to ppm levels. However, an additional consideration is toxicity, and ethers, esters and alcohols are more frequently used due to their low toxicity [44, 45]. For example, butyl alcohol, ethyl acetate, diethyl, diisopropyl ether could be used in biocatalysis [45].

Two-phase bioreactors were developed for the degradation of xenobiotics such as phenol, toluene and benzene, which are toxic for cells even in small concentrations [46]. An example of a successful application of a biphasic water/organic solvent system is the reduction of 2-octanone by *Baker's yeast FD-12* [47]. Inhibition of the microorganism was avoided by using *n*-dodecane as an organic solvent to dissolve toxic 2-octanol. Moreover, this correlated with high hydrophobicity of *n*-dodecane (log P = 6.6). It is known that cell membrane would be easily destroyed by organic solvents with low log P values (high hydrophylicity) and cell activity is higher in the presence of more hydrophobic solvents compared to solvents with low log P [48, 49].

There are several other relevant examples of application of two-phase systems in biocatalysis. A water/silicon oil system was used for the oxidation of water-insoluble anthracene by the enzyme manganese peroxidase from the fungus *Bjerkandera sp. BOS55* [50]. Biphenyl degradation can be carried out by *Burkholderia xenovorans LB400* in a batch reactor with octadecene or bis(2-ethylhexyl)sebacate as water immiscible solvents [51]. An organic-aqueous system composed of isooctane and a buffer (1:5 (v/v)) can be used for enantiospecific hydrolysis of glycidyl phenyl ether, affording the yield of 44.5% and 100% *e.e.* for the

substrate concentration of 90 g L^{-1} in the isooctane phase [52]. A two-phase liquid system with the immobilized cells was used for the side-chain cleavage of β-sitosterol to 4-androstane-3,17-dione in a continuous flow and a packed-bed reactor [53]. Bis(2-ethylhexyl)phthalate as a biocompatible organic solvent was used to dissolve the initial substrate, the product and to disperse *Mycobacterium sp. NRRL B-3805* cells immobilized on an inexpensive high surface-area support Chrysotile. A stable reaction system was developed affording a constant yield of 50 % during 1 month of operation in a continuous mode [53].

A number of problems arise during organic/aqueous fermentation and the consequent work-up procedures. One of them is the formation of emulsions, which complicates product recovery. To separate the two phases mixed in an emulsion, centrifugation, decantation, steam distillation or membrane filtration can be used [46]. Another method is to use de-emulsifying hydrolases which reduce separation time from 1 week to 30 min [54]. Another problem is inhibition of enzymes due to their unfolding, occurring at the interface of the two phases followed by aggregation and precipitation [55].

Overall, we can say that the use of two-phase systems improves bioprocesses mostly *via* isolation of toxic substrates and/or products in the different phases. In some cases an organic solvent is used to dissolve a water insoluble substrate or to shift equilibrium by extraction of the product. Two-phase systems should be optimized in terms of efficient partitioning coefficients, non-toxicity and engineering design.

1.4 Immobilized biocatalysts

There are several techniques for the immobilization of bacteria, yeasts and fungi, such as: 1) attachment or adsorption onto solid carrier surfaces, 2) entrapment within a porous matrix, 3) self-aggregation (natural or with crosslinking agents - artificially induced) [40].

Among all the immobilization techniques for living cells, entrapment and adsorption are the most popular and widely used. The former method is carried out by mixing cells or spores with components of a soluble gel under sterile conditions, followed by gelation. The cell growth within gel beads exposed to broth solution depends on the diffusion of nutrient components and later on the impact of the accumulating biomass [56]. Diffusion affects distribution of cells within the polymer particles: generally, high concentration of microorganisms is restricted to the surface layer of a bead, this can lead to the release of cells into bulk solution. The cells growth also decreases when the density of cells per unit volume is high and cells compression increases [57].

One of the main difficulties of the immobilization techniques is to select a suitable material which should be 1) chemically inert, biocompatible, and durable, 2) thermally and mechanically stable, and 3) highly porous to facilitate cell immobilization as well as mass transfer into the deeper layers of the matrix. The overall growth pattern, activity and stability of the immobilized cells depend on the cell strain, the nature of the polymer material (alginate, carrageenan, agar, agarose, silica gel, acrylamide, chitosan), polymerization and inoculation conditions [58].

Another type of immobilization is to grow a biofilm onto the surface of a porous material (*e.g.* cellulose, wood, sawdust, polygorskite, montmorilonite, hydromica, porous porcelain, porous glass, *etc*.) [40]. Immobilization *via* adsorption begins mostly with a sterilized support being inoculated with the cell or spore suspension. The biofilm, subsequently developed upon exposure to growth medium, consists of multiple cell layers located in direct contact with the bulk medium.

Enzymes can also be immobilized by: 1) ionic binding to ion-exchange supports (*e.g.* cellulose and carboxylmethyl cellulose), 2) adsorption through van der Waals interactions to hydrophobic supports (*e.g.* polypropylene and teflon), 3) covalent binding between the amino or carboxyl groups of amino acids and the support membrane [59].

Despite significant mass-transfer problems, immobilized systems offer considerable advantages in comparison with conventional free biocatalysts: 1) prolonged stability of the biocatalyst, increased tolerance to high substrate concentrations and reduced end-product inhibition; 2) high biocatalyst density per unit of a bioreactor volume, which leads to high volumetric productivity and short reaction times; 3) easy product recovery without separation and filtration, thus reduction in the cost of equipment and energy; 4) regeneration and reuse of the biocatalysts for extended periods of time.

1.5 Membrane bioreactors

In the cases when initial substrate, intermediates or the final product could be hydrolized or inhibitory for biocatalysts, membranes can be used to avoid these problems. Moreover,

selective removal of products from the reaction site increases conversion in the product-inhibited or thermodynamically unfavourable reactions.

The main applications of biocatalytic membrane reactors in the agro-food sector are in: 1) reducing the viscosity of juices by hydrolyzing pectins; 2) reducing the lactose content in milk and whey by its conversion into digestible sugar; 3) the treatment of musts and wines by the conversion of polyphenolic compounds and anthocyanes, and 4) the removal of peroxides from dairy products [59]. The use of biocatalytic membrane reactors in the pharmaceutical industry is focused mainly on the production of amino acids, antibiotics, anti-inflammatories, anticancer drugs, vitamins. One of the largest applications of membrane bioreactors is in water treatment.

Biocatalysts (enzymes or microorganisms) can be used within membrane reactors being suspended in solution and compartmentalized by a membrane in the reaction vessel or being immobilized within the membrane matrix itself (**Figure 1.7**).

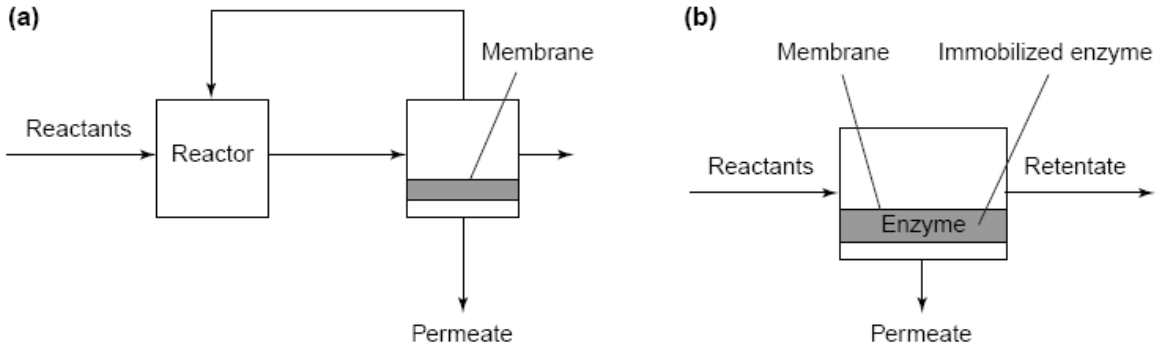


Figure 1.7. Two types of membranes: (a) as a separation unit, (b) as a support and separation unit simultaneously [59].

In the first method the system is a stirred tank reactor combined with a membrane that works as a separation unit to isolate the main product from the broth solution. In the second method, the membrane acts as a support for the catalyst and as a separation unit at the same time. In this case, a biocatalyst could be immobilized onto the surface of a membrane or entrapped inside of its matrix. Depending on the flow regime and the size of membranes, there are different types of membrane modules: flat-sheets, assembled in a plate-and-frame module, spiral-wound module, or tubular membranes, assembled in tube-and-shell modules (**Figure 1.8**) [59]. The biocatalyst can be flown along a membrane module, segregated within it, or immobilized onto the membrane by entrapment, gelification, physical adsorption, ionic binding, covalent binding or cross-linking [59].

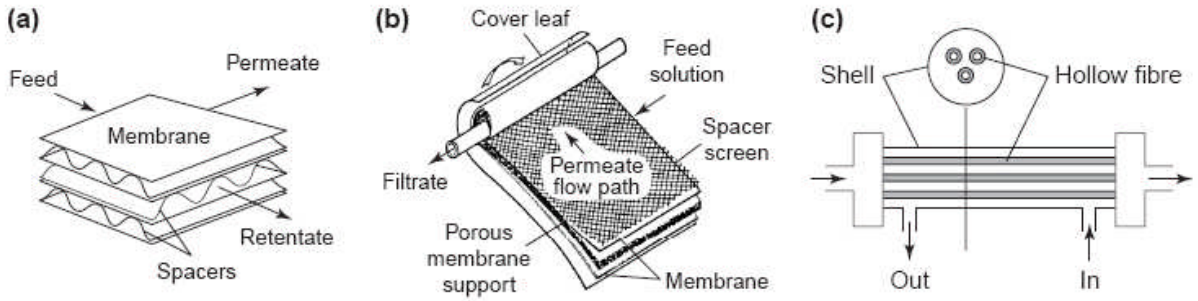


Figure 1.8. Different types of membranes: (a) flat sheet, (b) spiral wound and (c) hollow fiber [59].

High transfer coefficient, selectivity toward the desired products, low toxicity for biocatalyst, stability and tolerance to broth solution are important parameters to consider when searching for a suitable membrane for a specific separation.

A specific type of membrane reactor, based on a two-phase system separated by membrane with supported catalyst, is called the phase contactor. The main feature of the phase contactor

reactor is that reactions takes place within the boundary layer located within the contactor structure. Several parameters, such as trans-membrane pressure, flow rates of the two phases and the position of a catalyst play very important roles in the activity and stability of the system as was shown by Giorno *et al* for the hydrolysis of oleic oil catalysed by *C. rugosa* lipase [60].

The group of Oda with co-workers developed an interface system, based on microorganisms (yeasts) spread on an agar layer and covered by a decane solution of racemic citronellol, which was oxidized to *S*-citronellic acid at 30 °C with the yield of 70-85 % (**Figure 1.9**) [61, 62].

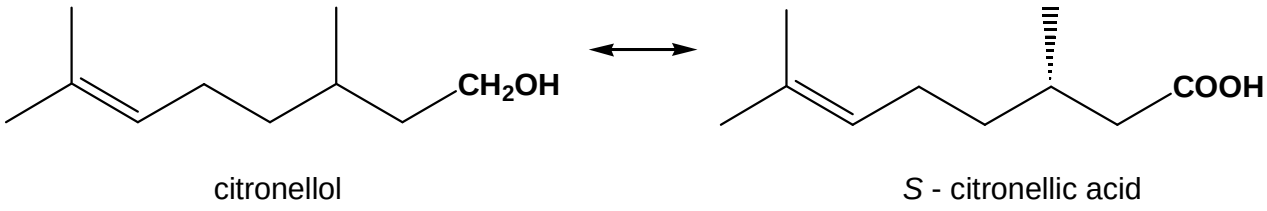


Figure 1.9. Oxidation of citronellol to citronellic acid.

The authors explained that the high activity and non-toxicity of the initial reagents could be achieved only in the case when sufficiently thick microbiological film was formed between the hydrophilic broth medium and the hydrophobic decane solution. At this point pre-cultivation period determines the effectiveness of the interface reactor. Also, the authors concluded that viscous solvents are not suitable due to the hampered diffusion of the substrate and oxygen. Scaling up of this system was achieved by soaking the agar-coated filter pads placed within a stainless-steel tank reactor with the yeast broth. However, this system was characterized by poor aeration and agitation between the pads that led to a long inactive period

and small amount of citronellal, the intermediate product [61]. A large volume reactor (88 L inner volume) was constructed of the stacked agar plates interface bioreactors, connected to one overflow line and a diaphragm pump [62]. This system was used for desulfurization of dibenzothiophene with *Rhodococcus erythropolis ATCC 53968* [63], synthesis of methyl ursodeoxycholate *via* microbial reduction of methyl 7-ketolithocholate with *Eubacterium aerofaciens JCM 7790* [64], hydrolysis of neat 2-ethylhexyl acetate [65], and the production of acetate esters of primary alcohols [66].

A two-phase reactor combined with a microporous nylon membrane was used for stereoselective hydrolysis of menthyl acetate by *Bacillus subtilis* [67]. Nylon membrane with cells supported on one side (prepared by filtration) was placed between two 9.25 mL volume units through which water and neat racemic menthyl acetate were circulated with a velocity of 1 mL min^{-1}. The total activity was not much better compared to the emulsion reactor, but specific activity (per unit mass) was five times higher. The reaction rate per unit area as 16 μmol s^{-1} m^{-1} was achieved but the system was unstable.

Another example of combining two-phase synthesis with membranes was demonstrated by Husken [68], when octanol with the dissolved substrate - toluene - was separated from an aqueous solution containing cells by a hollow-fiber polypropylene membrane module. The product obtained, 3-methylcatechol, was extracted back into octanol.

Membranes are characterized by their affinity to organic or aqueous liquids, their pore size and dominating functional groups. All features should be taken into account when a two-phase

membrane reactor is designed. The breakthrough pressure - the pressure at which a liquid wetting the pores is displaced by another, immiscible, non-wetting liquid - plays an important role in the membrane's stability. Vaidya *et al* postulated that the breakthrough pressure depends on the pore size, its geometry, changes of wetting characteristics of the membrane as the reaction progresses [69]. It was also shown that only hydrophilic, highly retentive, amphiphilic ultrafiltration membranes (Cuprophen, BM5) are suitable for two-phase membrane reactors, but amphiphilic microfiltration (Celgard2400, Nylaflo) and Teflon membranes are a poor choice because they have a low breakthrough resistance [70].

In general, one can say that membranes are not only used to separate products, but also can combine properties of specific permeability and active support for immobilization of biocatalysts to achieve better productivity. The use of membranes in a bi-phasic mode is beneficial in the cases when good stability of biocatalysts and preferable solubility of a substrate or product are required.

To summarise all written above we can say that biocatalysts (whole cells or enzymes) play a significant role in chemical industry, providing new routes for synthesis of fine chemicals and transformation of feedstocks. Some disadvantages of biosynthesis, such as sensitivity to contamination and the need for careful handling, can be compensated by the economy and effectiveness of bioprocesses. Although batch systems are more widespread, fixed-bed reactors with immobilized cells (or enzymes) are characterized by higher productivity and stability. To circumvent high toxicity or insolubility of substrates, processes with cells-friendly non-aqueous solvents are being developed. Membranes are being used to separate

inhibitory products from the broth medium. Moreover, complex biphasic systems with membranes as supports for biocatalysts began to attract attention. They demonstrate high activity, selectivity and stability.

1.6 Biocatalysis in tandem reactions

With the increased interest in mixed, renewable and waste feedstocks it became obvious that such materials are most frequently highly oxygenized and are not easily converted by conventional chemical processes. Biocatalysts, being adaptive to new, often non-purified substrates, appear to be more suitable for such transformations yielding a small number of chemicals that can be further converted to useful products by traditional chemical catalysis. Here, a new concept of combining bio- and chemical catalysts within one system emerged as a possible way to develop a chain of feedstocks transformations to value-added products through multi-step reactions. Such systems also allow to reduce waste and to intensify chemical processes by avoiding extraction and purification of the intermediates.

As early as in 1985 a combined bio-chemical conversion of *D*-glucose/*D*-fructose mixtures into *D*-mannitol (62-66 % yield) was offered by Makkee *et al* [71]. The reaction was carried out in an autoclave at 70 °C under H_2 atmosphere with the substrates dissolved in an aqueous buffer and mixed with both an isomerase and a copper catalyst immobilized onto silica.

A consecutive tandem bio- and chemical catalysis for hydroquinone synthesis from *D*-glucose was reported by Frost *et al* (**Figure 1.10**) [72].

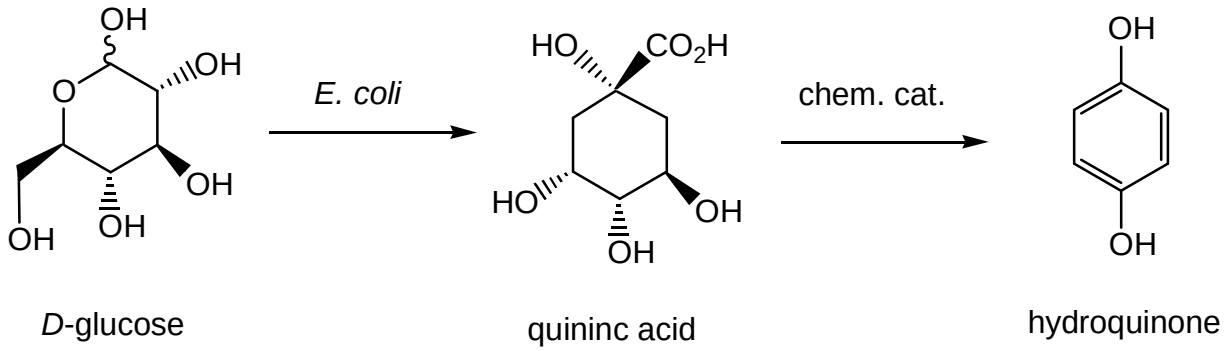


Figure 1.10. Tandem bio-chemical synthesis of hydroquinone.

The first step of the glucose transformation to quinic acid catalysed by genetically modified *E.coli AB2848,* was carried out in a continuous fermentor with free cells and monitoring of pH, temperature, oxygen and substrate flow. Before the second step of the quinic acid transformation to hydroquinone by a chemical catalyst ($(NH_4)_2Ce(SO_4)_3$, V_2O_5, $K_2S_2O_8$ /Ag_3PO_4 were tested), the cells were removed by centrifugation, then the proteins were precipitated and the broth solution was decolorized by stirring with charcoal and, finally, purified by passing through an H^+-exchanged resin column to remove ammonium. In spite of the time and material consuming procedure of purification, 85-91 % yield of the product was reached. The same research group developed the process of benzene-free synthesis of catechol (**Figure 1.11**) with the overall yield of 43 %. In order to attain this high yield, a combination of biotransformation of glucose to intermediate protocatechuate (**Figure 1.11**) with *in situ* resin-based extraction and thermal decarboxylation of the obtained precursor was used [73].

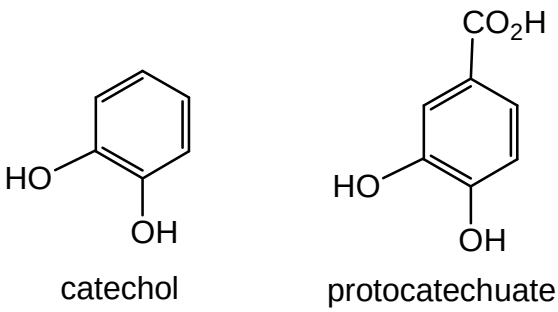


Figure 1.11. Structures of catechol and protocatechuate.

Walker *et al* showed a good example of consecutive biological and chemical catalysis in 1-(3-hydroxypropyl)-3-methylimidazolium glycolate IL as a medium for an enzyme and bis-(acetyl acetonato)-Co catalyst [74]. This hydrophilic IL dissolves codeinone, dehydrogenase, cofactors, and Co complex. The first step is fermentative codeinone-neopinone isomerization followed by conversion to oxycodone *via* formation of a complex with Co catalyst.

Tandem bio-chemical systems are used for resolution of racemic mixtures of alcohols and aminoacids [75, 76].

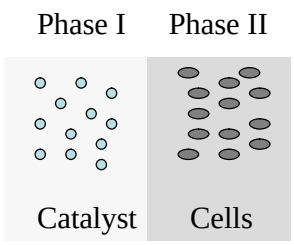
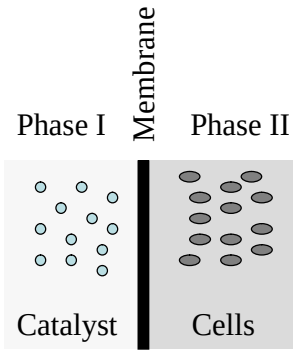
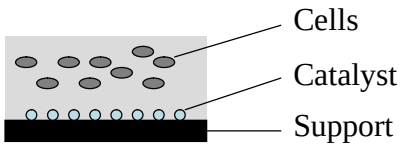
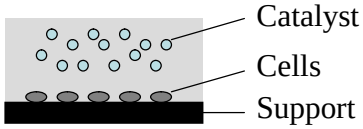
An example of extractive fermentation was shown by Oliveira *et al*, where glucose fermentation was facilitated by a simultaneous esterification of the product, ethanol, by *R. miehei* lipase in a biphasic broth/oleic acid system [77]. Better productivity was achieved because of the simultaneous extraction of the inhibitory ethanol from the fermentation medium. Mu *et al* combined enzymatic transesterification of oils for biodiesel production with the glycerol fermentation by *Klebsiella pneumoniae* to 1,3-propanediol. The total yield of 84 % for biodiesel and 1.7 g L^{-1} h^{-1} productivity of 1,3-propanediol were achieved, though some methanol penetrated into the fermentation broth and inhibited cells growth [78].

The few examples described above show that one-pot systems were developed mostly in a sequential mode. This means that the second step of a tandem transformation was carried out after the intermediate product had been isolated. Biocatalysts are rarely compatible with chemical catalysts. Therefore, optimization of a one-pot bio-chemical catalytic system is a challenging task. Bio-systems need careful, clean, non-toxic handling and they operate at low temperatures and pressures, whereas chemical catalysts can work in the extreme for cells and enzymes conditions. Therefore, development of a combined bio-chemical system faces some difficulties, such as toxicity of chemicals (products, by-products, solvents or chemical catalysts) for bio-systems, low catalyst's activity due to low temperature (pressure), low stability and selectivity, *etc*.

1.7 How to combine bacteria and chemical catalysts in one system

Several approaches can be proposed to carry out multistep bio-chemical transformation within a single reaction system (**Table 1.1**). All of them are based on the principles of spatial separation of bio- and chemical parts by using two immiscible solvents (with or without a membrane junction) or supported catalysts or cells.

Table 1.1. Different methods of combining bio- and chemocatalysts.

Schematic representation	Description
Bi-phasic	
	Cells and catalyst are located in different immiscible solvents, such as aqueous nutrient medium for cells and organic solvent for catalyst
	Membrane between two immiscible phases is used
Mono-phasic	
	Chemical catalyst is immobilised onto support, whereas cells are free in water solution
	The reverse to the previous: cells are immobilised and solution of catalyst is used

1) Two-phase system

A two-phase system makes it possible to carry out bio- and chemical reactions in two different phases because of their immiscibility.

In general, when a suitable solvent needs to be chosen several aspects should be considered in detail such as its “greenness”, physicochemical properties, the ability to dissolve reactants and products, feasibility of extraction or evaporation of the products and re-use of solvents. Water, supercritical liquids and fluorous solvents are considered to be more environmentally friendly solvents compared to organic solvents [79]. ILs are believed to be promising solvents with tuneable properties and low volatility, but there is a scanty knowledge about their toxicity and biodegradability. Among 26 organic solvents most frequently used only methanol, ethanol, diethyl ether, heptane and hexane are preferable solvents compared to, for example, dioxane, acetonitrile, acids, tetrahydrofuran, based on the environmental assessment considering environmental, health and safety issues during the production, operation, potential recycling and disposal of the solvents [44].

Biocatalysts work mainly in aqueous solutions. Therefore, water-immiscible solvents should be used to form a biphasic medium. Among different hydrocarbons tetradecane is a biocompatible and water-immiscible solvent, but it is also characterised by poor solubility of many organic and inorganic compounds.

Several aspects should be considered when choosing a suitable solvent for tandem bio-chemical reactions: 1) the organic solvent used should be non-toxic for cells; if used, inhibitory for cells compounds should not diffuse into aqueous phase; 2) both phases should be pre-equilibrated to avoid undesirable extraction.

In batch reactors intensive mixing of two phases has to be used to avoid mass transfer limitations. In this case emulsion formation also increases the contact area between phases, but results in the difficult product separation process. Another way to increase the surface contact area is to use micro-channels in which two liquids can form small droplets, as shown in **Figure 1.12**. This system mimics micro-channel reactors with segmented flow, which are known to have better mass and heat transfer than conventional gas-liquid and liquid-liquid two-phase reactors [80].

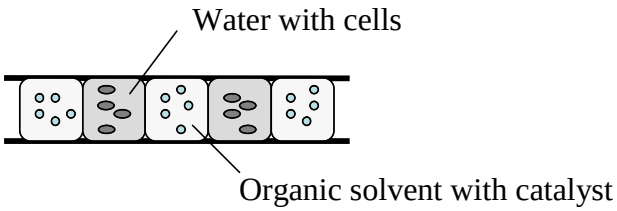


Figure 1.12. A microfluidic channel with segmented flow.

A membrane between the two phases can separate a toxic organic solvent from the solution of cells, or be selective for some reactants to prevent or enhance their diffusion from one phase to another.

2) Supported catalysts

Immobilization of a catalyst (bio- or chemo-) can resolve the problem of incompatibility of bio- and chemocatalysis. An immobilised chemical catalyst should be non-toxic for cells, active and stable in the presence of water, salts, and organic acids.

From another point of view, cells can be used as heterogeneous catalysts. There are two options to implement this approach: using beads for entrapment of cells or creating a thin biofilm on the surface of a suitable material (**Figure 1.13**).

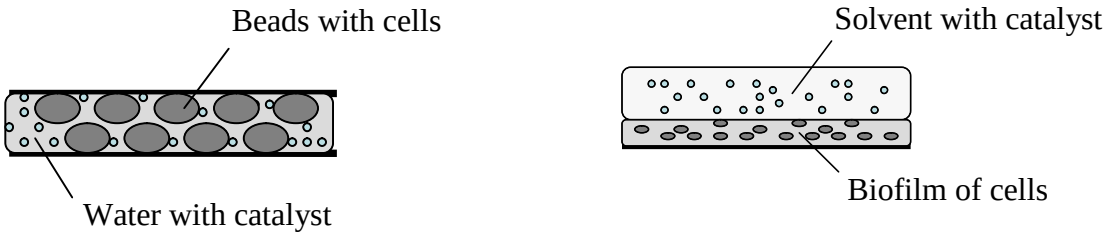


Figure 1.13. Tandem systems with cells immobilized in beads and as biofilm.

Entrapment of cells within gel beads or biofilm drastically increases their stability, although diffusion limitations can arise. Also, using beads in a continuous reactor can lead to high pressure drops, thus complicating engineering of the reactor. Biofilms are also difficult to stabilize and monitor.

Considering supported cells we come to a new aspect: using monophasic aqueous solution with both catalysts in it. Although this system seems easy to develop (one solvent instead of two), the chemical catalyst used has to be selective and stable in the complex aqueous solution. An aqueous biofilm of cells, which is in contact with a water-immiscible solution of catalyst, is a more appropriate choice. In this case the cells should be maintained by a nutrient medium, which can be flown along the other side of the support material, permeable for all components of the broth solution (**Figure 1.14**):

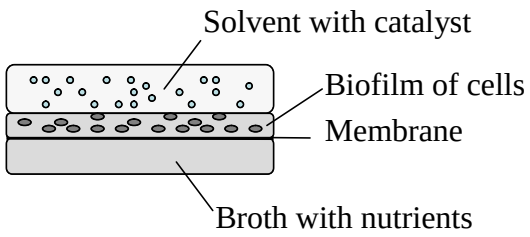


Figure 1.14. Tandem biphasic system with a biofilm of cells.

To maintain stability of biofilm, which tends to rarefy, a thin membrane can be used to separate the aqueous solution from the biofilm. In this case the biofilm can be grown on a membrane, or cells can be immobilized within a porous matrix exposed to the broth solution on one side and to the organic solvent with a catalyst on another.

Overall, several options could be considered to design tandem reactions involving bio and chemical catalysis. Every system described above has its own features. But the key factors are extraction, solubility, compatibility and immobilization.

1.8 Glycerol as a new type of feedstock

Glycerol is formed as the main by-product when vegetable oils undergo transesterification with methanol or hydrolysis reactions in the process of biodiesel production. The overall yield of glycerol in this transformation is 10 % (w). The amount of raw glycerol increases constantly and this triol has become an attractive feedstock material. A special project, named "The Glycerol Challenge", was launched in April 2007. The main aim of this project was to manufacture value-added chemicals from glycerol. However, there is a concern among the

companies that target glycerol as suitable feedstock about its unpredictable price, which depends on speculations on the market, high prices for biodiesel feedstock and variations in the demand for purified glycerol [81]. Current price of raw glycerol is 21-23 cents/lb in Europe and 27-31 cents/lb in the the United States compared to 2-4 cents/lb in 2006 [82].

Among the biggest consumers of glycerol – Europe, US, Japan – Japan needs to import half of its domestic demand, whereas Europe and the United States produce glycerol mostly for internal use [83]. Glycerol is used mainly in oral care, cosmetics as filler, in food industry as sweetener, in tobacco industry as moisturizer, in pharmacy and polymer synthesis (**Figure 1.15**).

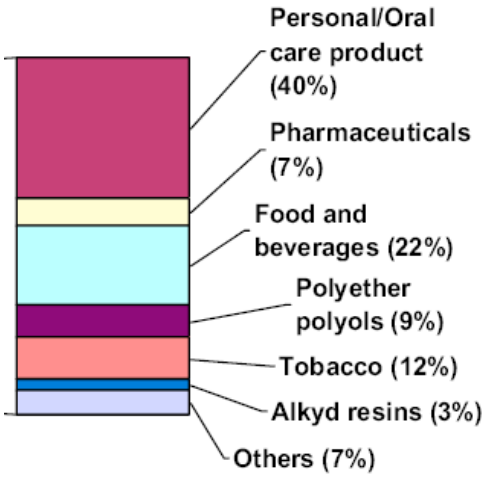


Figure 1.15. Application of glycerol in different industrial sectors [83].

Raw glycerol obtained as a by-product of biodiesel production is contaminated with catalysts, methanol, fatty acid salts (**Table 1.2**). After neutralization of an alkaline catalyst and removal of water and methanol, crude glycerol has a purity of 80-88 %. Its further distillation yields glycerol of 99 % or higher purity for cosmetics and food industry.

Table 1.2. Residues in raw glycerol obtained as a by-product of biodiesel production [84].

Contamination	%
Na and K salts	4-5
methanol	3
heavy metals and lignin	1
other organic materials	0.5
Water	26

Glycerol from biodiesel is considered as a cheap reagent in chemistry and biotechnology for synthesis of value-added products (**Figure 1.16**). Glycerol can be fermented to syngas that can be used for the Fisher-Tropsch or methanol synthesis, or reformed to H_2 or CH_4 [85]. Dihydroxyacetone, a chemical which is used as a tanning agent in cosmetics, can be obtained both biochemically using bacteria and by oxidation over Pd, Pt, Au catalysts [86]. Succinic acid obtained mainly *via* fermentation of glycerol is used as an intermediate in organic synthesis and as a monomer in polymer production [87]. 1,3-Propanediol, propylene glycol, acrolein and glycerol carbonate are used for synthesis of biodegradable polymers [1]. Glycerol ethers can replace fuel additives [88]. Many useful products like ethanol, butanol, lactic, propionic, acetic and butyric acids can be obtained *via* biological fermentation of glycerol [89].

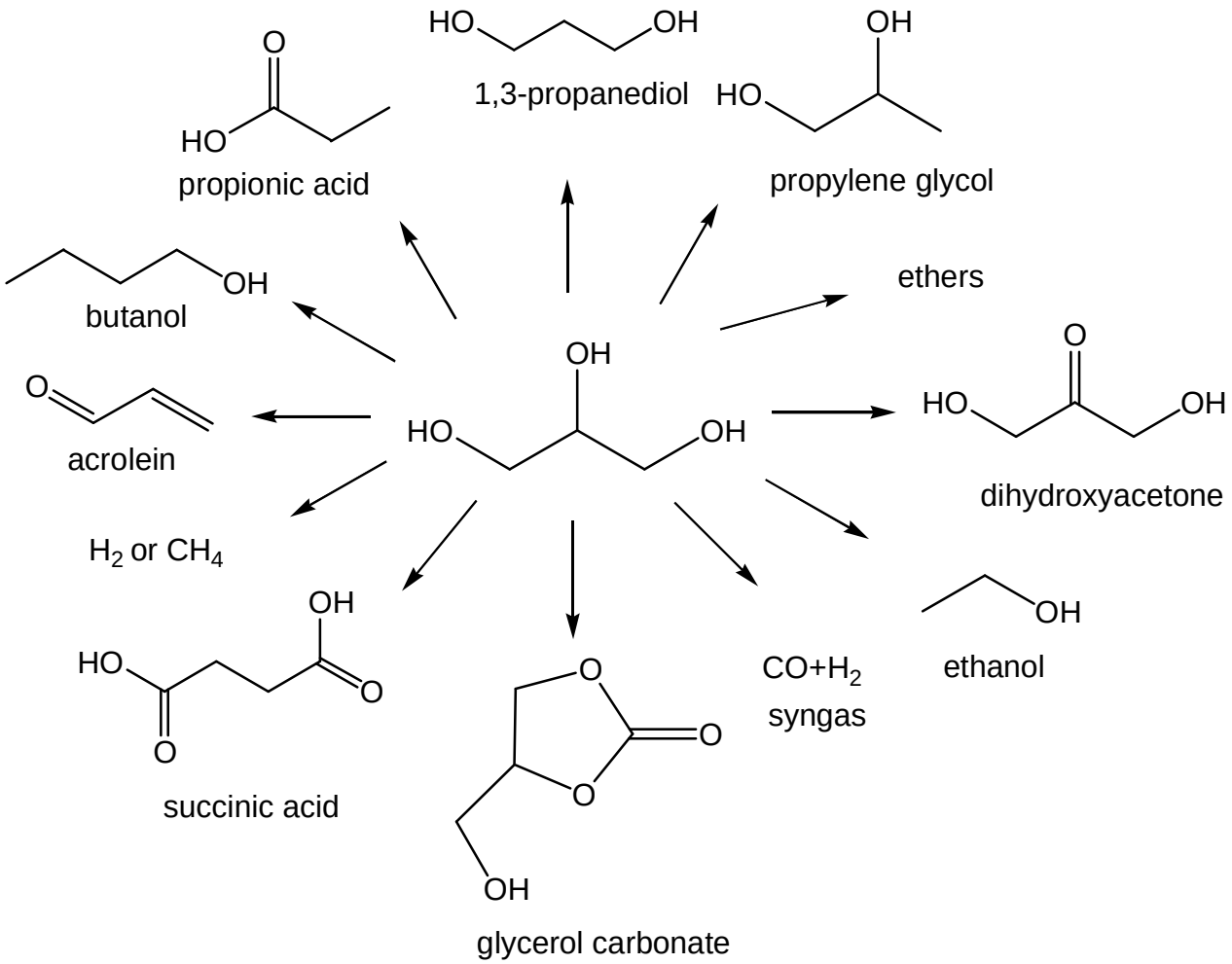


Figure 1.16. Possible useful products from glycerol [1, 85-89].

To conclude one can say that the increased production of glycerol as a by-product provoked a huge interest in its utilization for the synthesis of useful value-added products, gases and fuels. Biotechnological routes of glycerol transformation are dominating the proposed methods. However, only processes with low cost are commercially viable when the high risk of speculative price of crude glycerol is involved.

1.9 Bacterial fermentation of glycerol

Fermentation is one of the most convenient methods of conversion of glycerol. In the last few years glycerol conversion to 1,3-propanediol by anaerobic bacteria (**Figure 1.17**) was studied intensively due to the potential use of the product diol in the production of polymers with tunable properties [1].

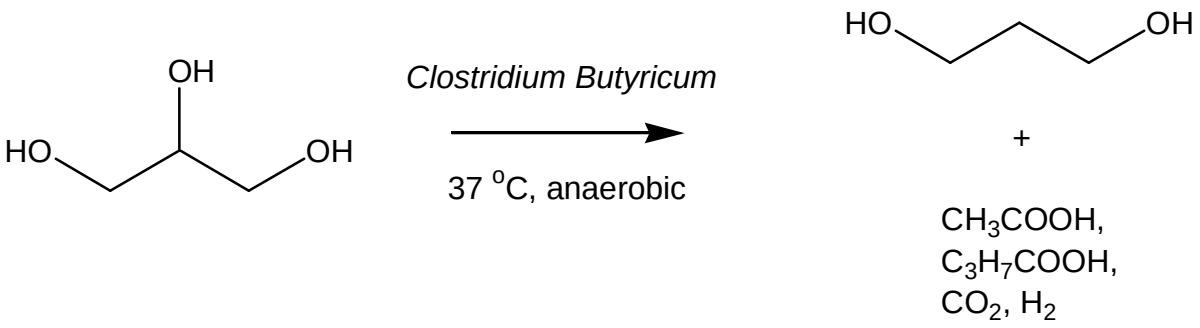


Figure 1.17. Anaerobic whole cell biotransformation of glycerol.

The fermentative transformation of glycerol to 1,3-propanediol by *C. butyricum* bacteria attracts much attention compared to other strains (*Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter agglomerans*), because it allows to obtain high yield of the product, close 1 M [90]. It is also more stable, since the key enzyme, glycerol dehydratase, is vitamin B_{12}-independent, not deactivated by glycerol, and the toxic intermediate - 3-hydroxypropionaldehyde - does not accumulate in this process [90]. In addition, the product streams are cleaner, since the only side products are H_2, CO_2 and some organic acids.

Figure 1.18 shows the metabolic pathway of glycerol transformation by *Clostridium* bacteria:

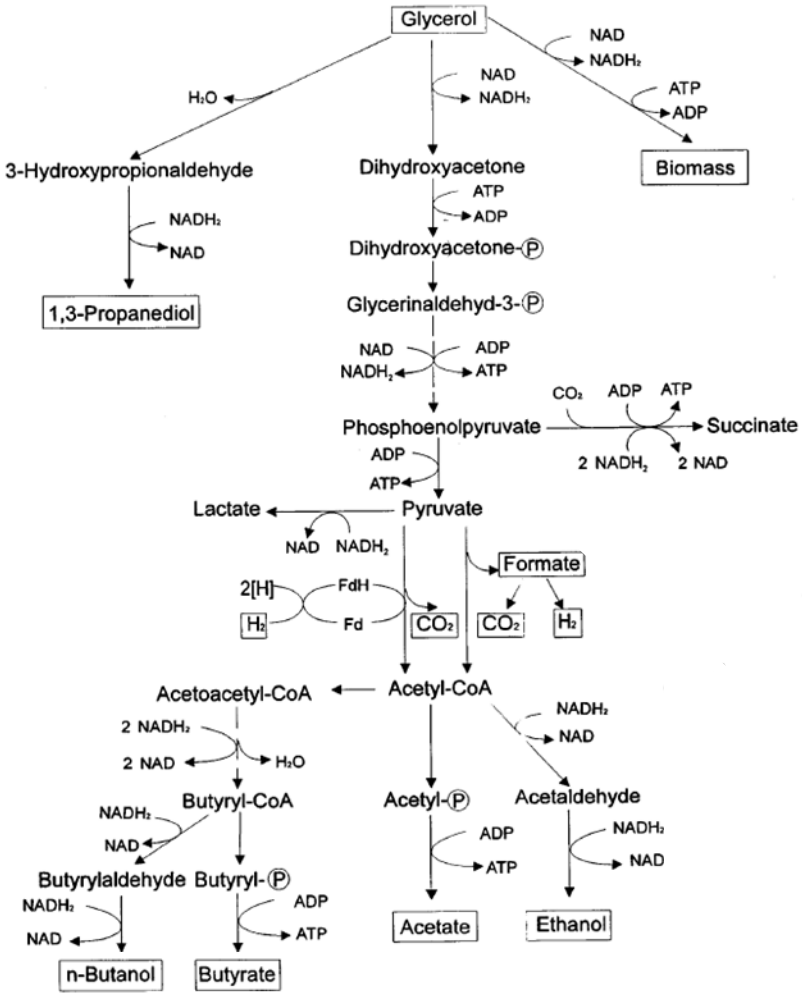


Figure 1.18. Metabolic pathways of glycerol fermentation by *Clostridium* species [91].

1,3-Propanediol is formed *via* reduction of 3-hydroxypropionaldehyde [92]. Some glycerol is converted to pyruvate, utilization of which leads to lactic, acetic and butyric acids. The theoretical yield of 1,3-propanediol is 64 or 50 %, if acetic or butyric acid is formed as the main by-product. Usually microorganisms use all the pathways simultaneously, therefore the total yield of 1,3-propanediol can vary [91]. **Table 1.3** shows that for *Clostridium* species the yield of 1,3-propanediol production is between 61-66 % (mol/mol).

Table 1.3. Comparison of glycerol fermentation by *Clostridia* in batch/fed-batch reactors.

Ref.	Strain	$V_{reactor}$, L, type	C^0_{Gly}, g L^{-1}	$Y_{PD,}$ mol mol^{-1}	Y_{AA}, mol mol^{-1}	Y_{BA}, mol mol^{-1}	Q_{PD}, g L^{-1} h^{-1}	C_{PD}, g L^{-1}
[93]	F2b	2, B	40[a]	0.66	0.05	0.10	1.2	23
[90]	CNCM 1211	1, B	20	0.62	0.12	0.09	1.1	10
[90]	CNCM 1211	1, B	70	0.64	0.07	0.10	1.3	37
[94]	CNCM 1211	1, B	129	0.63	0.08	0.07	-	67
[95]	DSM 5431	1, B	62	0.61	0.23	0.05	1.2	31
[95]	DSM 5431	1, FB	87	0.62	0.26	0.07	0.6	45
[95]	DSM 5431 2/2	1, FB	130	0.66	0.13	0.10	0.8	71

a - industrial glycerol was used.

It is still unclear whether high concentration of 1,3-propanediol inhibits the consumption of glycerol or not, but undoubtedly pH plays a significant role, leading to the decrease in 1,3-propanediol production at low pH values due to the formation of acetic and butyric acids. Fed-

batch fermentation allows a better final concentration of 1,3-propanediol (60-70 g L^{-1}) and volumetric productivity (~ 2.7 g L^{1} h^{-1}) to be attained compared to the batch systems [91]. A continuous reactor with or without cell recycling by a membrane module further improves the productivity, but the maximum product concentration cannot be significantly increased [96].

As it can be seen in **Table 1.3** high concentrations of glycerol up to 130 g L^{-1} can be fermented in batch cultures with *Clostridium* bacteria, leading to 70 g L^{-1} of 1,3-propanediol (0.9 M) in the fermentation broth. It is known that *Clostridium* bacteria do not accumulate the toxic metabolite 3-hydroxypropionaldehyde like *K. pneumoniae* and *C. freundii* enterobacteria [97]. Therefore, high concentrations of the substrate can be used for the fermentation [90]. Although it was shown in batch cultures that the initial glycerol concentration higher than 150 g L^{-1} inhibits bacterial growth, the combined tolerance of *Clostridium* species to relatively high concentration of the substrate and 1,3-propanediol allows to reach high concentration of the product up to 1 M in the fermentation broth [98]. It is important to mention that 1,3-propanediol at 84 g L^{-1} does suppress cell growth in batch cultures but, nevertheless, the total conversion of glycerol can be achieved [98]. No inhibition was detected in experiments with pulses of 40-80 g L^{-1} of 1,3-propanediol during a continuous fermentation, thus showing no product inhibition [93].

Industrial glycerol available directly from biodiesel production is frequently contaminated with salts and other residues. Despite the opinion that this glycerol is not suitable for biochemical processes, an example of using industrial glycerol for 1,3-propanediol production was demonstrated by Papanikolaou *et al* [93]. When technical glycerol (65 % w/w purity) was

used, a prolonged lag phase (10 h) was observed with no significant glycerol uptake, followed however by rapid growth and production of metabolites.

The volumetric productivity of 1,3-propanediol varies within 0.6-1.3 g L h^{-1} for batch/fed-batch cultures according to the data summarized in **Table 1.3**. One can notice that lower productivities are observed for the cultures harvested on high concentration of glycerol due to probably prolonged lag phase. Nevertheless, the high yield and concentration of the product were achieved. The difference in the yields of acetic and butyric acids shows that production of acidic metabolites is strongly dependent on the strain and fermentation conditions.

As some studies have shown [92, 93, 96] the volumetric productivity of 1,3-propanediol could be increased if the process of glycerol fermentation is carried out in a continuous mode. As it is shown in **Table 1.4**, the maximum productivity in a single-stage continuous reactor amounts to 5.5 g L h^{-1} (corresponding to 26 g L^{-1} of 1,3-propanediol), achieved at 0.21 h^{-1} dilution rate and grown on 90 g L^{-1} of glycerol. Recycling of cells by a membrane module led to the increase in productivity by a factor of 4, but the maximum product concentration cannot be significantly improved (23 g L^{-1} of 1,3-propanediol) [96]. More examples of glycerol fermentation by different *Clostridium* species are discussed by Biebl *et al* in [91].

Table 1.4. Comparison of glycerol fermentation by *Clostridi*a in continuous reactors.

N	Ref.	Strain	$V_{reactor}$, L, type	C^0_{Gly}, g L^{-1}	Y_{PD}, mol mol^{-1}	Q_{PD}^{max}, g L^{-1} h^{-1}	D, h^{-1}
1	[93]	F2b	2.0, single-stage	90	0.35	5.5	0.21
				60	0.44	3.3	0.15
				30	0.40	2.9	0.29
2	[96][a]	DSM 5431	0.5	56	0.49	16.0	0.70
				32	0.51	9.5	
3	[92][b]	VPI 3266	0.3	30	0.44	0.54	0.05

[a] with cells recycling by filtration, otherwise the productivity is 4 times lower.

[b] 18 mmol L^{-1} of glucose was added to the fermentation medium as co-substrate.

To conclude one can say that glycerol fermentation by *Clostridium* bacteria is a well-studied process. High concentrations of glycerol up to 150 g L^{-1} can cause prolonged lag phase and suppress bacteria growth, thus decreasing productivity. Nevertheless, total consumption of glycerol can be achieved. High concentrations of 1,3-propanediol up to 80 g L^{-1} are likely to be non-inhibitory. Volumetric productivity in batch/fed-batch cultures reaches 2.7 g L^{-1} h^{-1} with the final 1,3-propanediol concentration of 70 g L^{-1}. Carrying out fermentation in the continuous mode increases productivity but not the maximum product concentration.

1.10 1,3-Propanediol extraction from fermentation medium

1,3-Propanediol is a highly hydrophilic compound (log P = -1.1), that has a relatively high boiling temperature. Therefore, its recovery from complex aqueous fermentation broth solutions containing macromolecules, salts, remainining substrates and by-products, is a difficult task. First, microbial cells are removed by filtration or centrifugation. Then, 1,3-propanediol and by-products are distilled. This step requires a substantial energy input which accounts for high cost of the final product. At last, 1,3-propanediol can be purified by liquid chromatography after being desalinated. There are other methods of 1,3-propanediol recovery, based on multi-step extraction [99], pervaporation or formation of intermediate complexes followed by reverse reactions releasing 1,3-propanediol [100].

The UNIFAC group contribution method showed that short-chain aliphatic alcohols and aldehydes are better solvents for 1,3-propanediol extraction from aqueous solutions in the presence of glycerol compared to amines and acids, although all solvents tested appeared to be poor extractants with the highest distribution coefficient of 0.28 for 1,3-propanediol in hexanal/water mixture [99].

Li *et al* attempted to recover 1,3-propanediol by pervaporation through a X-type zeolite membrane from a model 1,3-propanediol/glycerol/water solution and broth solutions containing glucose [101]. The zeolite membrane (Si:Al ratio is 1.5) allows to extract about 33-55 % of 1,3-propanediol being at the same time more permeable for water as a smaller molecule (the kinetic diameters are 0.26 and 0.61 nm for water and 1,3-propanediol

respectively). Blockage of the membrane pores by components of the broth solution was detected, lowering the total flux to 57 %.

A simple extraction of 1,3-propanediol from mixture of diols, glucose and glycerol by ethylacetate was offered by Cho *et al* [102]. The maximum solubility of 1,3-propanediol was 40 g L^{-1} compared to 6 g L^{-1} for glycerol. 1,3-Propanediol can also be extracted from aqueous solutions *via* formation of alkyl-1,3-dioxane when an aldehyde is used as a solvent (**Figure 1.19**) [103]. But in this case pH of aqueous solution should be as low as 1.2-2.0 to catalyse the reaction of acetalization and the step of 1,3-propanediol recovery by hydrolysis is required. This method can be modified by using a H^+-exchange resin as a catalyst and *o*-xylene for 1,3-dioxane extraction [99].

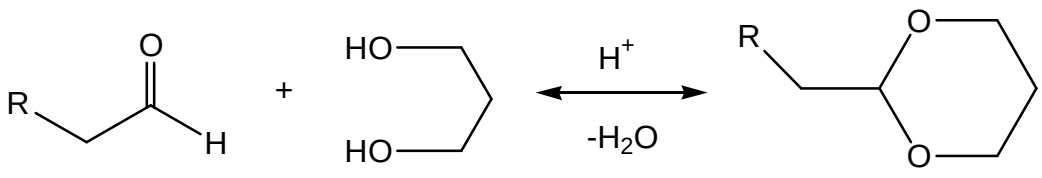


Figure 1.19. Acetalization of 1,3-propanediol.

Extraction wih boronic acids is a well known method for recognition and transportation of different diols [104, 105]. A diol is esterified with a boronic acid and the complex formed is dissolved in the organic phase. Then, hydrolysis of the ester releases the diol into a receiving phase (**Figure 1.20**).

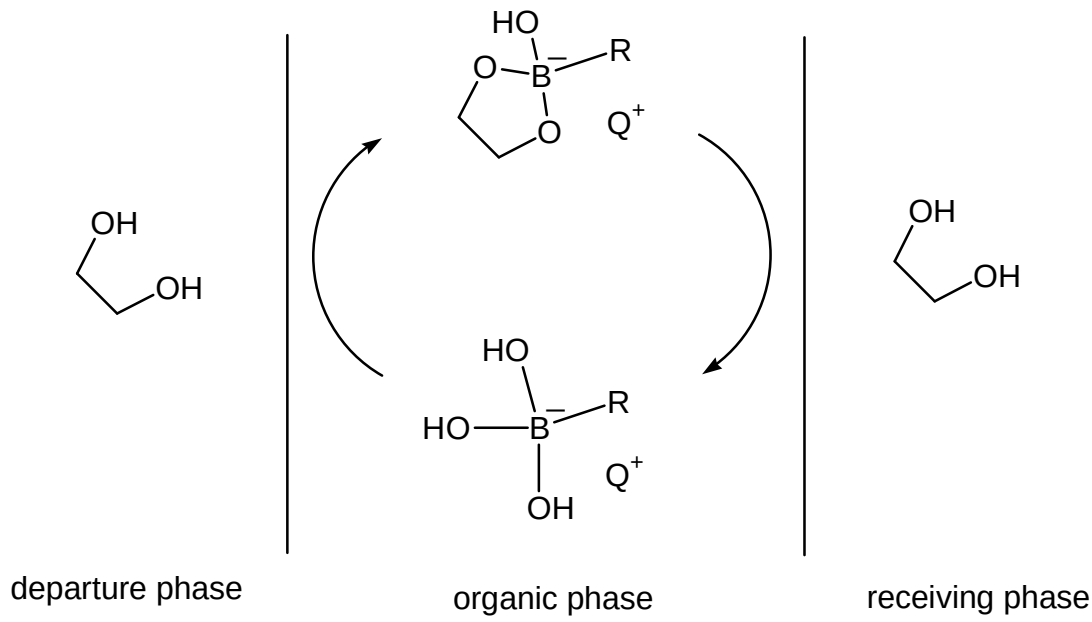


Figure 1.20. Diol transport through a liquid membrane by a boronic acid.

In general, the effectiveness of a liquid membrane depends on the correlation between pH and K_a parameters, which are defined by the composition of aqueous broth, the boron acid derivate used, the properties of the intermediate layer (organic or IL) and the membrane material itself. In order to facilitate the diol transport, quaternary hydrophobic ammonium ions are used [106]. Also, further elimination of the diol from the receiving phase can shift the equilibrium towards formation of borate complexes. Unfortunately, fluxes for, *e.g.*, Celgard 2400-based membranes at room temperature are very low, at $0.1\text{-}1\cdot10^{-3}$ mmol cm^{-2} min^{-1} [105].

Probably the easiest and the most effective method of 1,3-propanediol recovery is a two-phase aqueous extraction with ethanol (or methanol)/inorganic salts solution [100]. This approach showed the highest recovery of 93.7 % of 1,3-propanediol from fermentation broth by a mixture of ethanol and saturated $(NH_4)_2SO_4$ solution. Moreover, this method allowed to remove cells and proteins from the fermentation broth. In general, it was postulated that the

higher the polarity of the solvent used and the higher the valent charge of an anion, the better extraction of 1,3-propanediol can be achieved. However, 1,3-propanediol needs to be further separated from ethanol and purified.

As one can see, the problem of the down-stream processing of 1,3-propanediol obtained *via* microbial fermentation has not been solved effectively so far. Extraction is the most promising and studied method with the ethanol/inorganic salts solution being the most effective [100].

1.11 Industrial processes of 1,3-propanediol synthesis

As it was mentioned before 1,3-propanediol, like 1,4-butanediol, plays a significant role in the production of biodegradable polyesters with different properties [1]. Therefore, several methods of its synthesis were developed prior to the glycerol fermentation route. There are three main routes to 1,3-propanediol: acrolein based, ethylene oxide carbonilation and glucose fermentation.

The acrolein-based method was developed by Degussa and sold to DuPont in 1997 [107]. Firstly, acrolein is hydrated to 3-hydroxypropanal in aqueous solution at relatively low temperatures. Then, hydrogenation of the intermediate is carried out over Raney nickel catalyst at high temperature and pressure (**Figure 1.22**). A strict temperature control has to be maintained to increase selectivity and to avoid the formation of by-products. Also leaching of the catalyst leads to contamination of 1,3-propanediol.

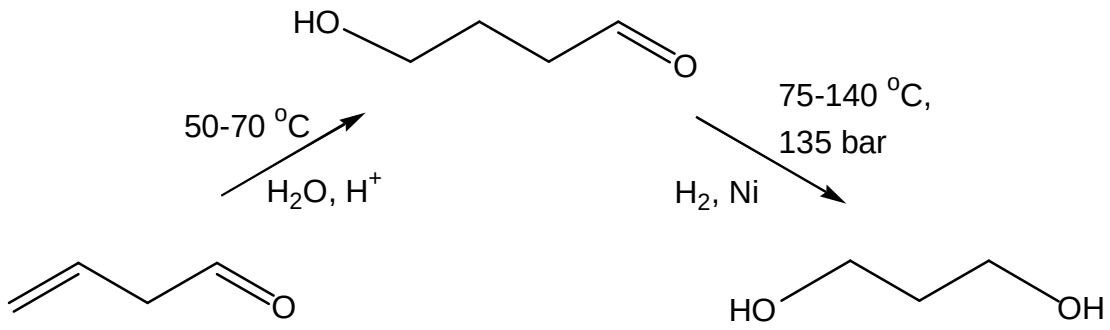


Figure 1.21. Synthesis of 1,3-propanediol from acrolein.

Ethylene oxide carbonilation was developed by Shell with promoted $Co_2(CO)_8$ as the main catalyst and MTBE as a solvent (**Figure 1.22**) [107]. Special precautions have to be made to avoid side- and consecutive reactions. Ethylene oxide can undergo isomerization to yield acetaldehyde and further ethanol, oligomerization and partial hydrogenation to 3-hydroxypropionaldehyde which can further dehydrate and condense (**Figure 1.23**) [107].

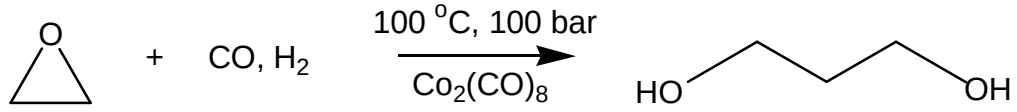


Figure 1.22. Synthesis of 1,3-propanediol from ethylene oxide.

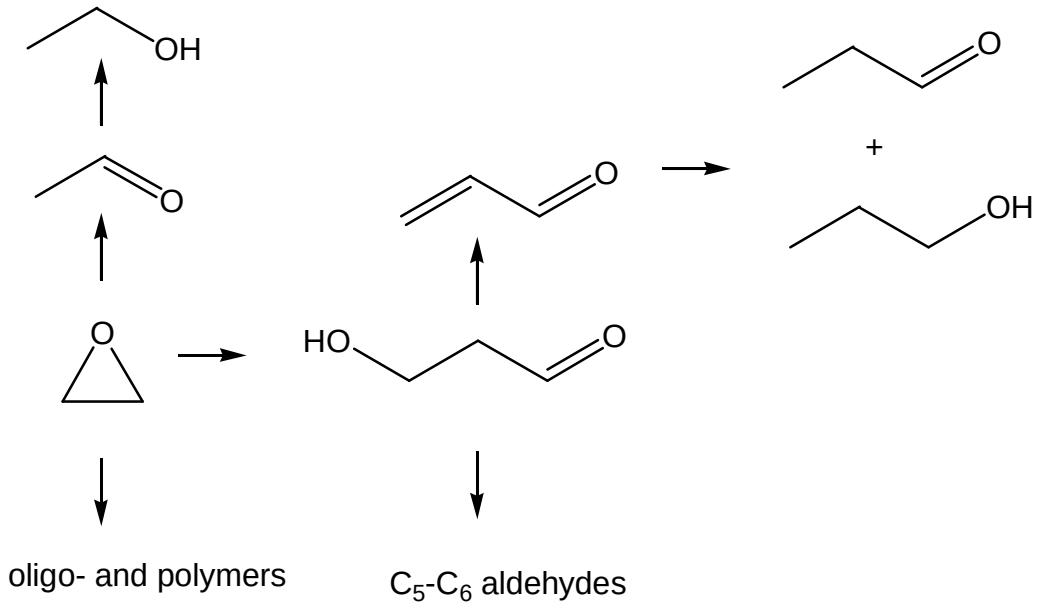


Figure 1.23. Side- and consecutive reactions of ethylene oxide synthesis.

Compared to the two oleochemical processes, glucose fermentation by recombinant *E.coli* is a biotechnological route to 1,3-propanediol (**Figure 1.24**) [107]. In this process glycerol from biodiesel production competes with glucose as a cheap substrate, although glucose is a highly demanded raw material for ethanol production. The problem of downstream product separation is common in both processes.

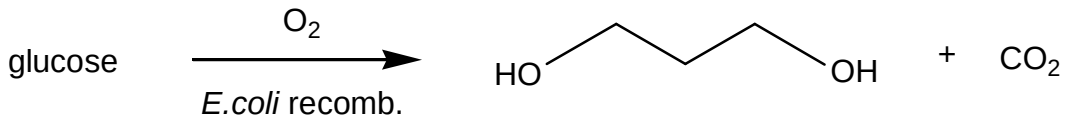


Figure 1.24. Fermentative production of 1,3-propanediol from glucose.

1.12 Chemical transformation of 1,3-propanediol

In order to convert 1,3-propanediol into commercially attractive simple organic molecules we can propose several reactions such as dehydration, oxidation, esterification and polymerization (**Figure 1.25**).

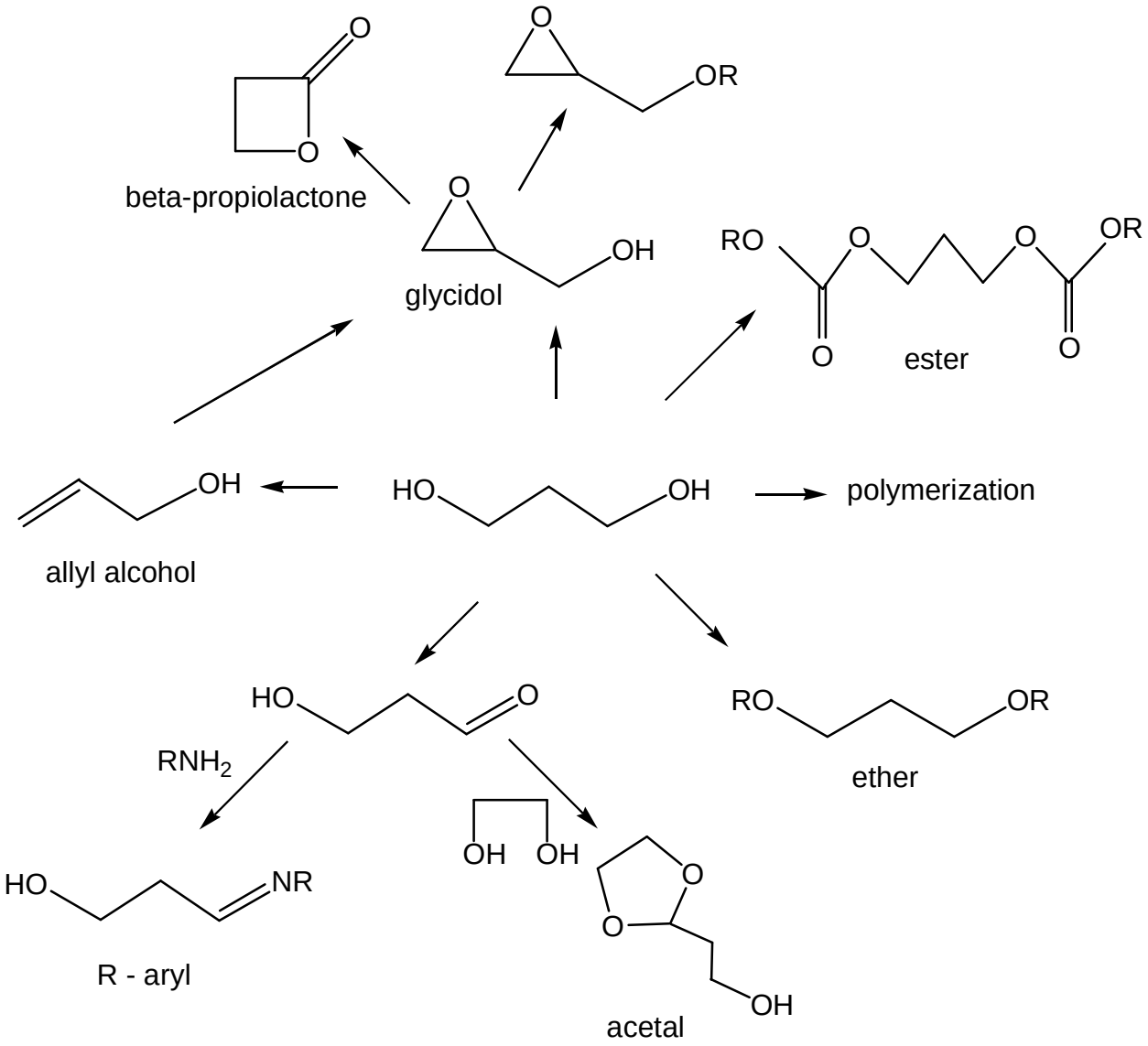


Figure 1.25. Potential routes for 1,3-propanediol transformation.

Possible chemical targets include beta-propiolactone, glycidol and allyl alcohol. Beta-propiolactone is used extensively in medicine as a sterilant of blood plasma, water and milk, and as a vapour-phase disinfectant. It acts as a sporicide against bacteria, fungi and viruses [108]. Glycidol is used in the synthesis of polymers [109, 110]. Allyl alcohol is consumed by the pharmaceutical, pesticide and perfume industries [111]. For example, it is used to synthesize epoxy chloropropane, 1,4-butanediol and allyl ketone, for plasticizers and plastics [112]. Its carbonate derivative is used in the synthesis of safety glass and display screens [112].

Polymerization with diacids is another possible route for 1,3-propanediol transformation. DuPont has already established the production of polymers based on 1,3-propanediol and terephtalic acid [1].

1.13 Esterification of 1,3-propanediol

1,3-Propanediol has two OH groups and shows properties of an alcohol. Therefore, reactions of esterification or polycondensation seem attractive with respect to *in situ* transformation following the fermentation process. These reactions can be induced by Brönsted or Lewis acidic catalysts. At the same time, production of water accompanies these reactions. Thus, water removal from the reaction zone is required to shift the reaction equilibrium towards the desired product.

Chlorides of Al, Zn and oxychlorides of Zr, Hf are known to be Lewis acids, that can be used for alcohol esterification [113]. Derivatives of CF_3SO_3H, which are stronger acids compared

to H_2SO_4, appeared to be very effective esterification catalysts [114]. A new emerging type of catalysts is ionic liquids with Lewis-acid properties, usually chloride-based or containing triflate as an anion [115].

In most cases the common features of esterification with inorganic catalysts are high temperature, organic solvents such as as toluene, heptane, *etc*., equimolar amount of reactants, removal of water either by distillation or with the help of drying agents. There is also a common problem of inactivation of catalysts in the presence of water.

Another type of acidic catalysts are ion-exchange resins, for example Nafion, Amberlyst, Amberlite, Dowex [116]. The main feature of these catalysts is the presence of the $–SO_3H$ acidic group which is active in the reactions of esterification. The reaction can be carried out at relatively low temperatures as 30-60 °C, but the water formed both deactivates the catalysts and lowers the conversion [116]. Also, selectivity towards monoester or monoether is observed for this type of catalysts.

Trifluoromethanesulfonate and trifluoromethane-sulfonimide catalysts, being strong Lewis acids, also show activity in polycondensation reactions. For example, $Sc(OTf)_3$ was used for the low temperature (35 °C) bulk melt polycondensation of 1,4-butanediol with methylsuccinic acid under reduced pressure, giving 94 % yield of the polymer with $M_n=1.2\cdot10^4$ [117]. 95 % catalyst recovery can be attained by its extraction into water from $CHCl_3$ solution of the reaction mixture. It is worth to note that the reaction lasts 4-4.5 days for room temperature polycondensation, and increasing temperature to 120 °C shortens the

reaction time to 5 h. When 1,3-propanediol was used, etherification side reaction occurred (9 %) and the resulting polymer was cooligo(ester-ether) [118]. Another example of the synthesis of copolyester of 1,3-propanediol with adipic and sebacic acids by polycondensation-transesterification is described by Umare *et al* [119]. The first step of the reaction was carried out at 170 °C for 5 h in the presence of $Ti(OBu)_4$ catalyst. In the second step, the reaction was carried out by heating the reaction mixture at 240 °C for 1 h under reduced pressure (<1 Torr) to remove water.

Overall, we can say that esterification and polymerization of 1,3-propanediol are suitable reactions for tandem bio-chemical processes because they lead to the formation of biocompatible products and do not involve strong oxidizing agents. At the same time strong acids, which are usually used for esterification, have to be avoided in tandem systems with bio-catalysts. Also, water plays a significant role in condensation, affecting the reaction equilibrium. Esterifications catalysed by inorganic catalysts are characterized by high temperatures, low stability of the catalysts in the presence of water and, therefore, need to be optimized if carried out simultaneously with the bio-chemical transformations. The reaction of esterification can be carried out at a low temperature and in the presence of water, but more active reagents as aldehydes have to be used [120].

1.14 Enzymatic esterification

Lipases have been intensely investigated in the last few decades in a wide range of different reactions of esterification, transesterification, hydrolysis and polycondensation [121-124]. Lipases offer some advantages compared to traditional inorganic or organic catalysts: aqueous

reaction medium, high selectivity, high yields of pure and improved-quality products. At the same time, being highly specific to substrate and sensitive to reaction conditions, lipases seem to show no common features, and need to be studied individually.

The active site of lipases consists of aspartate, histidine and serine residues, and the mechanism of esters hydrolysis catalysed by lipases can be written as in **Figure 1.26**. Firstly, serine residue is deprotonated *via* interaction with histidine and aspartate. Then the modified serine attacks the carbonyl group of an ester, forming an acyl-enzyme complex which releases the product after interaction with a nucleophile (*e.g.* H_2O), leading to the regeneration of the active centre. Lipases catalyse esterification in reverse direction to the described mechanism.

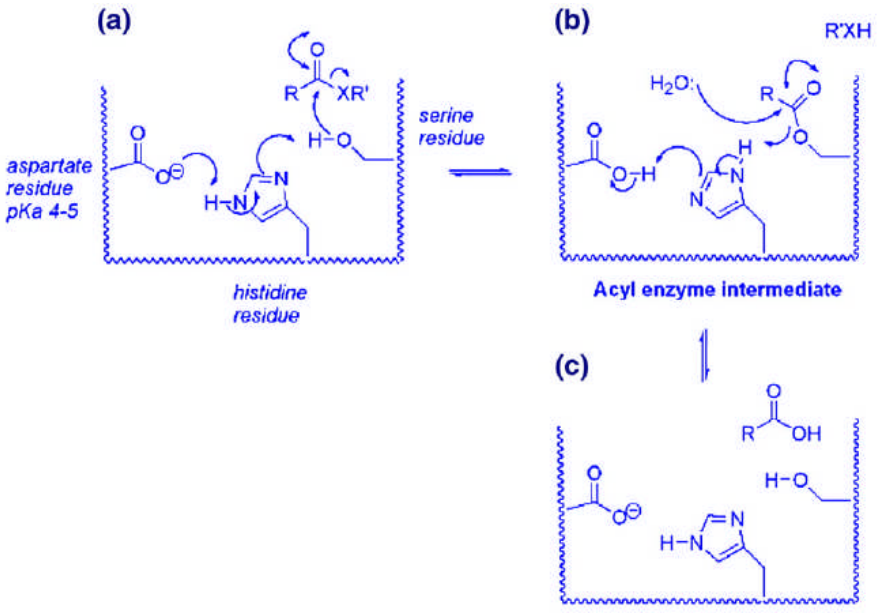


Figure 1.26. A scheme of the mechanism of lipase catalysis [125].

Discovery of lipases being active not only in the organic solvents but in the biphasic systems as well broadened the range of their application in catalytic processes [28, 46]. The problems

of poor solubility of a substrate or a product in water, their inhibitory effect on the lipases and equilibrium limitations can be overcome by using biphasic systems. Moreover, application of lipases in the biphasic catalysis opens various possibilities in green chemistry when extraction and purification can be avoided in such processes [78, 126-128].

The aqueous phase plays a significant role in the enzymatic biphasic esterifications. Water shifts the equilibrium towards the reverse reaction and lowers the total conversion. But high conversion can be achieved in the emulsions of organic hydrophobic substrates and an aqueous medium [129, 130].

Four levels of water influence on the esterification reactions catalysed by lipases could be distinguished: 1) water-free reactions (< 0.01 %), 2) microaqueous medium (0.01-3 %), 3) emulsions in which either the organic or water content is high, and 4) biphasic systems with > 3 % v/v water content. It is already well-established that most lipases show poor activity in dried organic solvents and this behaviour was explained by the necessity of the presence of water to stabilize the protein conformation in the organic solvents [28]. Molecules of water cover the surface of enzymes, thus creating a protective layer that retains enzyme catalytic activity in hydrophobic solvents. Hence, at low water content in a reaction medium, lipases work under conditions when protein molecules are perhaps partly coated in water and have active sites exposed to organic substrates. More hydrophilic solvents or substrates tend to strip the hydration layer from the enzyme, thus affecting its conformation [123]. The optimal water activity varies for different enzymes within the range of 0.01-3 %. At this point results from different sources should be compared carefully because, firstly, water activity does not

correspond to water concentration and, secondly, the optimal water activity depends on the initial substrates concentration as was shown by Wehtje *et al* [131].

The same requirement of water presence can be extended to the biphasic systems where this effect is named as interfacial activation of lipases. In this case protein takes favorable conformation at the boundary layer between water and the organic solvent. However, the concentration of water, its pH and saline composition, partitioning of substrates and products, their initial concentration and inhibitory effect, hydrophobicity and polarity of organic solvents should also be taken into account. This makes biphasic systems difficult to understand and optimise.

It seems that a higher than 3 % concentration of water shifts the equilibrium towards hydrolysis but, nevertheless, there are many examples when high conversion was achieved regardless of high water content in the reaction mixture. Even more incomprehensible seems the different activity of the same enzyme in similar biphasic systems. For example, esterification by *R. miehei* between oleic acid and 1-butanol in *n*-heptane/buffer medium (1:1 v/v) reaches 100 % conversion in 15 min despite the 1-butanol inhibitory effect on the lipase [132], whereas no more than 40 % conversion in 6 h was obtained in the reaction of esterification between oleic acid and ethanol in *n*-hexane/buffer medium (1:1 v/v) reported by Russell *et al* [129]. Foresti *et al* even postulated that water can act as a drain phase to extract water generated in the organic medium favouring ester synthesis, as was observed in the water/organic esterification of oleic acid and ethanol by CALB immobilized onto chitosan [133].

Some substrates (and/or products) inhibit enzymes and influence reaction rate, whereas their partitioning between the two phases defines the equilibrium conversion. Usually Bi-Bi Ping-Pong model is applied to esterifications but substrate inhibition, water concentration, pH and temperature are not always taken into account [134-136].

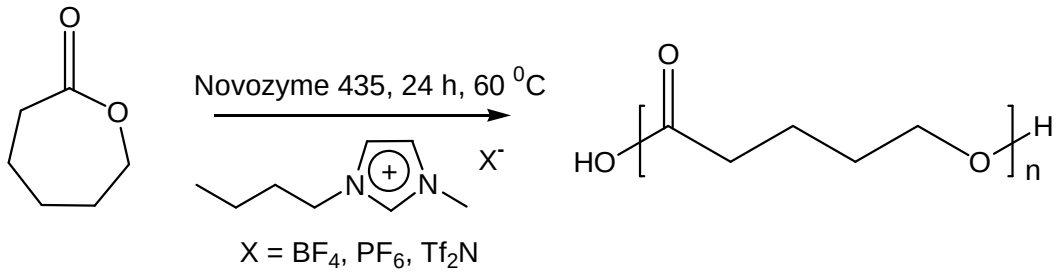
Lipases may be deactivated by hydrophilic alcohol or acidic substrates directly through protein denaturation or, as in the case of the reaction of biodiesel production, when lower alcohols adsorb onto the surface of the supported material causing blockage of the active sites for triglycerides. Chen *et al* offered a very simple method for Novozyme 435 pretreatment and regeneration through the immersion of the enzyme into 2-butanol and tert-butanol [137]. A protective "shield" can reduce inhibition of the enzyme. For example, different enzymes were modified with decanoyl chloride to make them soluble and stable in the organic solvents such as THF, chloroform, pyridine, acetone in the reaction of polymerization of ethyl lactate [138].

Another common way to increase an enzyme activity is to immobilize it onto a hydrophobic support [139]. Because some enzymes, like *R. miehei* lipase for example, have a "lid" that can be opened in contact with a hydrophobic solvent or ligands (hydrocarbon chains), the immobilization onto either hydrophobic or hydrophilic supports with hydrophobic ligands favors the enzyme activation. Hydrophilic carriers with conjugated hydrophobic ligands are found to be better carriers than purely hydrophobic supports for different lipases (*H. lanuginosa*, *C. antarctica* lipase B and *R. miehei*) in the reaction of *n*-butanol esterification with oleic acid in iso-octane with a variable water content (0.0–2.8%, v/v) [139]. Polymethacrylate polymers modified with hydrophobic octadecyl ligands showed the best

activity compared to styrene based supports. The authors attempted to explain the increased activity of the immobilized *R. miehei* and *H. lanuginosa* lipases by the different influences of the surface residues onto the catalytic site of the lipases, but the explanation was not clear.

In general, if in the case of microaqueous enzymatic esterification in organic solvents the effects of water, an organic solvent and substrates inhibition are more or less understood, the esterification in biphasic systems still needs to be carefully investigated and optimized.

Polymers are useful products, and lipases are widely used for polycondensation [123]. For example, immobilized Novozyme 435 was used for ring-opening polymerization of ε-caprolactone and polycondensation of 1,4-butandiol and dimethyl adipate in bis-(trifluoromethylsulfonyl)imide based ILs at 70°C (**Figure 1.27**) [140].



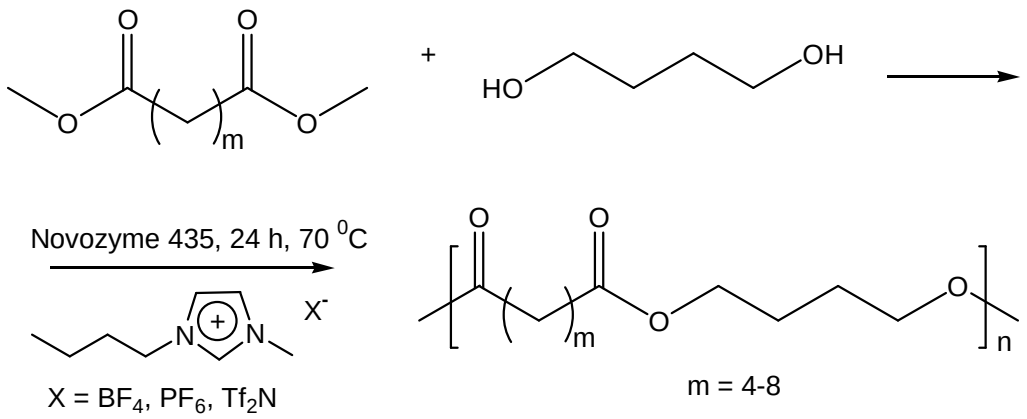


Figure 1.27. Enzymatic ring-opening polymerization and polycondensation [140].

Polymers with as high as 27,700 g mol^{-1} molecular weight were obtained *via* polycondensation of bis(2,2,2-trifluoroethyl) sebacate and 1,4-butanol in diphenyl ether as a solvent in 72 h at 37 °C by 36.4 % of *M. miehei* lipase, facilitated by vacuum evaporation of 2,2,2-trifluoroethanol [141]. If the alcohol was not removed during the reaction, only 7,800 g mol^{-1} of polyester was obtained. If the initial concentration of the substrate and the enzyme load were increased to 0.83 M and 73 %, then a linear polyester with M_w = 46,400 g mol^{-1} was obtained in 7 days. The authors also found that water had to be removed in the reaction between sebacic acid (unactivated substrate) and 1,4-butanol to obtain high molecular weight polyester [142]. It was also found that Novozyme 435 is sensitive to the relative position of the carboxylic groups on benzene ring, polymerizing only isophtalic acid but not the terephthalic counterpart, whereas *R. miehei* lipase showed no activity in the polycondensation of isophthalic acid with 1,6-hexanol [143].

To conclude the literature review we can say that biotechnology is experiencing a revivale of interest with the emergence of new type of substrates – waste and renewable feedstocks (*e.g.*,

glycerol, the amount of which increased due to the increased biodiesel production). Being adaptive and selective, bio-catalysts (bacteria, fungi, yeasts) are more suitable catalysts for transformation of such feedstocks. However, multi-step complex down-stream processing contributes significantly to the process emissions and cost of the biotechnological processes. A new approach was offered to combine fermentative transformation with chemical reactions within the same reactor to further convert intermediate chemicals to useful products. However, only a few examples of truly integrated bio-chemical systems are reported in the literature. Discovery of biocatalysts being active in organic, biphasic medium and development of immobilization techniques can help to overcome the main problem of incompatibility of bio- and chemocatalysts in a one-pot system. Our work is an example of verifying these methods for development of tandem bi-catalytic transformation of glycerol to industrially important products.

2 Experimental

2.1 Chemicals

Linoleic acid (≥ 99 %), decanoic acid (> 98 %), 4-phenylbutyric acid (99 %), 1,3-propanediol (≥ 99.6 %), glycerol (anhydrous, > 99.5 %), tetradecane (≥ 99 %), 1-butanol (99.4+ % ACS), heptane (99 %), eucalyptol (99 %), dibutyl ether (99.3 %), dihexyl ether (97 %), benzene (≥ 99.5 %), anisole (≥ 99 %), ethoxybenzene (99 %), veratrole (99 %), α – terpinene (90 %), α – pinene (97 %), acetic acid (> 99.7 %), butyric acid (> 99.5 %), 1-hexanol (> 98 %), 1-pentanol (> 99 %), heptaldehyde (95 %), poly(propylene glycol) methacrylate (M_n ~ 375), bis(2-ethyl-hexyl)sebacate (> 97 %), perfluoro-1,3-dimethyl-cyclohexane, poly-(hexafluoro-propylene oxide), 1-butyl-3-methylimidazolium hexafluorophosphate, tri-hexyltetradecylphosphonium dicyanamide, trioctylmethyl-ammonium chloride $CH_3N(C_8H_{17})_3Cl$ (Aliquat®336), lithium bis(trifluoromethylsulfonyl)amine $LiNTf_2$ (97 %) were purchased from Sigma – Aldrich, UK and used without further purification.

Methyl-trioctylammonium bis(trifluoromethyl-sulfonyl)imide $AmNTf_2$ was synthesized according to the literature procedure [144]: 40.6 g (0.1 mol) of trioctylmethylammonium chloride $CH_3N(C_8H_{17})_3Cl$ (Aliquat®336) was dissolved in 80 mL of acetone and added dropwise under stirring to acetone solution of 28.9 g (0.1 mol) of $LiNTf_2$. After 24 h white sediment LiCl appeared in the solution and was filtered through Celite plug, and acetone was evaporated under vacuum leaving yellow liquid. The former was washed with a copious amount of water and dried at 80 °C under vacuum for 48 h, affording 39.7 g (61.3%) of clear product. ^{1}H NMR (acetone-*d*6, 300 MHz, δ ppm/TMS): 3.3 (m, 6H), 3.0 (s, 3H), 1.7 (m, 6H),

1.3 (m, 12H), 1.2 (m, 27H), 0.7 (t, 9H). ^{19}F NMR (acetone-*d*6, 376.5 MHz, δ ppm/TMS): -79.7 (s, 6F).

Zr catalyst (1), Amberlyst-15, Novozyme 435 were kindly provided by Prof. M. Davidson (Department of Chemical Engineering, University of Bath). CLEA enzymes were purchased from CLEA Technology (The Netherlands). Nafion membrane was a standard DuPont membrane and was kindly provided by Dr. Dmitry Bavykin, University of Southampton. The *R. miehei* lipase (20,000 TBU g^{-1}) was purchased from Aldrich, UK. Novacarb membrane (522 m^2/g BET area, 17 nm micropore diameter, 0.78 μm mean macropore diameter) was obtained from MAST Carbon Ltd. Polyethersulfone (PES) membrane was kindly provided by Veolia International, Paris. Polypropylene (PP) hollow fibers were kindly provided by Professor Kang Li, Imperial College London.

2.2 Microorganism and growth medium

Clostridium butyricum strain DSM 10703 (DSMZ library) was isolated and provided by the Group of Biocatalysis (Manchester Interdisciplinary Biocentre, Manchester, UK, Professor Gill Stephens, Dr. M. Rebros). The microorganism was maintained on the 20 % glycerol medium.

The fermentation medium contained 20, 50 or 60 g of glycerol, 5 g KH_2PO_4, 5 g K_2HPO_4, 0.01 g $CoCl_2$, 3 g yeast extract, 0.2 g $MgSO_4$ and 0.01 g $FeSO_4 \cdot 7H_2O$ per litre of distilled water (the sulphates were added after sterilization to prevent precipitation) (modified from [145]). At the beginning of the fermentation pH was adjusted to 7.0 by addition of an

appropriate amount of NH_4OH. In most experiments an acetic buffer was used to maintain pH constant; for that 2 g of acetic acid was added to the fermentation medium. The medium was degassed and sterilized (120 °C) prior to fermentation.

The carbon sources used were: pure glycerol (99 %, Aldrich) and technical glycerol, obtained from the reaction of rapeseed oil transesterification with methanol. The reaction between 200 g of the oil and 100 mL of methanol was carried out at 65 $^{\circ}$C under reflux for 2 h, using 0.4 g of NaOH catalyst. After separation of biodiesel and glycerol/methanol phases, methanol was evaporated yielding crude glycerol, pH of which was adjusted to 7.0 by addition of H_3PO_4. Finally, crude glycerol was kept at 120 $^{\circ}$C for 20 min and separated from the fatty acids layer by decantation.

All fermentation experiments were carried out in a COY anaerobic chamber shown in **Figure 2.1**. Inoculum was grown from a single colony of *C. Butyricum* for 12-14 h at 37 °C. Batch and continuous cultures were carried out in 100 mL flasks with magnetic stirring, filled with 20 or 50 mL of glycerol medium, inoculated with 5 % (v/v) of the pre-culture, grown on the glycerol medium described above.

Figure 2.1. COY anaerobic chamber (10 % CO_2, 5 % H_2, 85 % N_2, 80 % humidity, 37 °C).

For the large-scale fermentation a 2.5 L fermentor with a 2 L working volume was used, which was equipped with temperature (35 ºC), stirring speed (200 rpm) and pH controllers (**Figure 2.2**). In this case 67 g L^{-1} glycerol medium was prepared without addition of acetic acid, and pH was maintained at 6.7 by automatic addition of 2 M KOH solution during the experiment. The fermenter was constantly degassed with N_2 (1 L min^{-1}) to mantain anaerobic atmosphere.

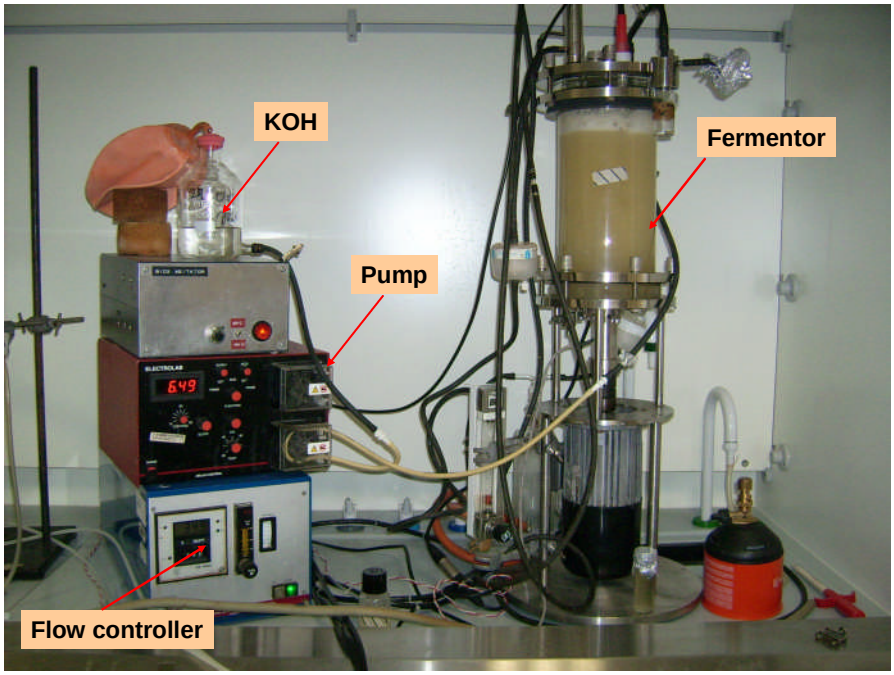


Figure 2.2. A 2.5 L fermentor, equipped with temperature, stirring speed and pH controllers.

To carry out continuous fermentation, a batch reactor was connected to the nutrient supply and waste bottles *via* a peristaltic pump (Ismatec, Cole-Parmer) (**Figure 2.3**). At the beginning batch cultures were grown until the end of the exponential phase, when production of the product is maximum, then continuous mode was applied. After the stationary phase was reached, continuous fermentation was kept until 4-5 volumes of the reactor have passed. Dilution rates were varied between 0.2-0.6 h^{-1}. No pH controller was used.

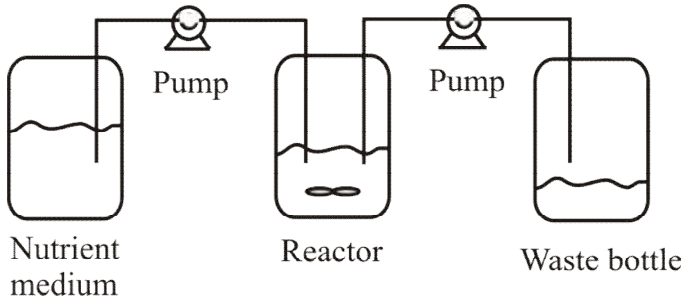


Figure 2.3. A schematic depiction of the continuous fermentation set-up.

During the experiments samples were taken every 1-2 hour to measure optical density, pH and concentration of fermentation products using HPLC method. A correlation between optical density and dry biomass weight was applied.

2.3 1,3-Propanediol esterification

The experiments of 1,3-propanediol esterification were carried out in a batch mode (total volume 6-10 mL) with magnetic stirring at 700 rpm. In a monophase system, 3 mL of tetradecane and 0.027 g of 1,3-propanediol (0.12 M_{org}) were mixed with 2.5 parts (0.3 M_{org}) of decanoic or linoleic acid (0.152 g or 0.248 g accordingly) in an open or closed vial submerged in an oil bath at 37 or 100 °C. Catalyst was added in the amount of 10 % (w) with respect to the amount of 1,3-propanediol.

A typical reaction mixture for the biphasic reaction consisted of two phases: 3 mL of an organic solvent (tetradecane in most experiments) with 0.252 g (0.3 M_{org}) of linoleic acid dissolved in it, and 3 mL of an aqueous solution of 0.027 g (0.12 M_{aq}) of 1,3-propanediol and an appropriate amount of a catalyst (usually 20 μL of the *R. miehei* solution) added. Organic and aqueous phases were always taken in 1:1 proportion unless otherwise stated. Linoleic acid/1,3-propanediol diol ratio was usually maintained at 2.5/1 unless otherwise indicated. Three different aqueous solutions were used: phosphate buffer 0.5 M (pH = 5.6), aqueous model solution containing the key components of the resulting fermentation broth with 9 g L^{-1} of 1,3-propanediol, 2.5 g L^{-1} of butyric acid, 1.5 g L^{-1} of acetic acid, 1 g L^{-1} of glycerol and 0.5 g L^{-1} of lactic acid (pH = 2.8), and actual fermentation medium obtained after a batch fermentation of glycerol by *C. butyricum* bacteria, filtered and contained different nutrient

components, such as: inorganic salts, yeast extract, ammonium hydroxide, (the medium contains 9 g L^{-1} of 1,3-propanediol and no residual glycerol as determined by HPLC prior to the esterification reactions). The biphasic reaction mixtures were mixed at 37 °C for 7-9 days and samples were withdrawn periodically from the organic phase for NMR analysis; at the end of the experiments aqueous phase was analyzed by HPLC, organic phase by Karl- Fisher titration if needed.

2.4 Determination of partition coefficients

Partition coefficients of 1,3-propanediol was determined according to the following procedure: 0.5 or 1 mL of an organic solvent was mixed at 700 rpm with 0.5 or 1 mL of fermentation broth for at least 24 h at 37 °C. Then the mixture was left for separation and aqueous solution was analyzed by HPLC. Experiments were done in triplicates. The amount of 1,3-propanediol extracted was determined by comparison of HPLC data obtained before and after mixing.

2.5 1,3-Propanediol esterification in reactors

Parameters of a segmented flow reactor established in a long Teflon tubing with two inlets and one outlet connected to a peristaltic pump (Ismatec) are shown in **Table 2.1**.

Table 2.1. Segmented flow reactor parameters.

Length, cm	Inner diameter, μm	Flow rate, μl min^{-1}	Residence time, h
612.3	500	4	20

The reactor was kept at 37 °C in the anaerobic chamber. Two phases, tetradecane with linoleic acid and fermentation broth, were pumped by a peristaltic pump into the reactor where segmented flow was formed. It is worth to point out that the segmented flow was unstable with disruption and deformation of the droplets of the both phases observed during the experiments. Hence, the obtained results correspond to the average behaviour of different bubble and slug lengths. 2.4 % (w/w_{PD}) CLEA *R. miehei* enzyme or 1 mL of free *R. miehei* were added as catalyst.

A hollow-fibre reactor was based on a 10 cm long, 0.5 cm internal diameter plastic tubing connected to a HPLC pump for organic phase and a peristaltic pump for aqueous phase (**Figure 2.4, 2.5**). Usually organic phase was pumped along the shell side of membranes and aqueous solution - through the lumen. Different types of membranes were tested in biphasic esterification of 1,3-propanediol. A summary of membranes used is shown in **Table 2.2**. In **Figures 2.6** and **2.7** FTIR spectra of both polymeric membranes are shown. The PP fibres membranes were glued together at the ends of the module in bunches of 30-50 fibres, as shown in a photographic image in **Figure 2.8**.

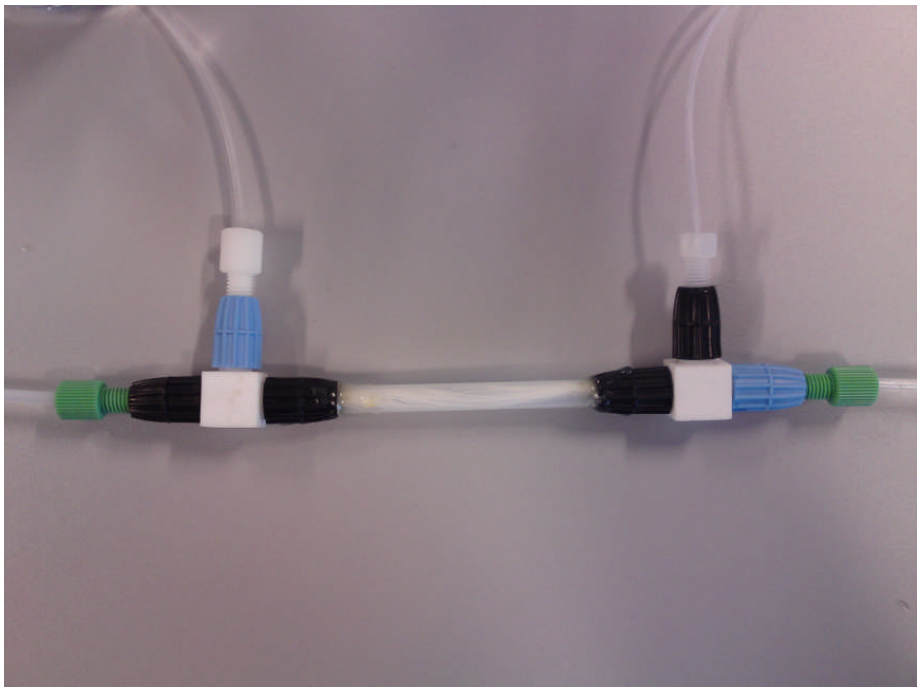


Figure 2.4. The hollow fiber membrane reactor with polypropylene membranes.

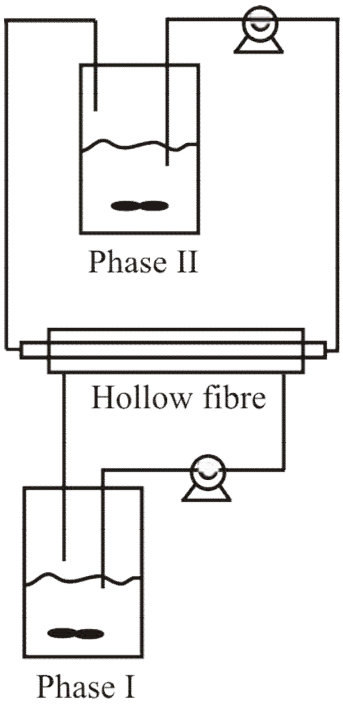
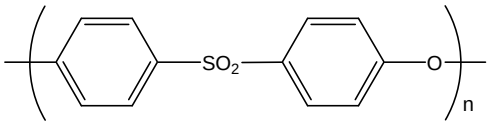
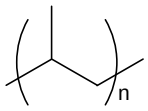


Figure 2.5. A scheme of the hollow fiber membrane biphasic reactor.

Table 2.2. Different types of membranes used in the hollow fiber reactor.

Membrane code	Structural unit	Int. diam., cm	Length, cm	Surface area, cm^2
PES (polyethersulfone)		0.20	24	15
Novacarb	carbon membrane	0.30	15	14
PP (polypropylene)		0.06	10-15	56-141

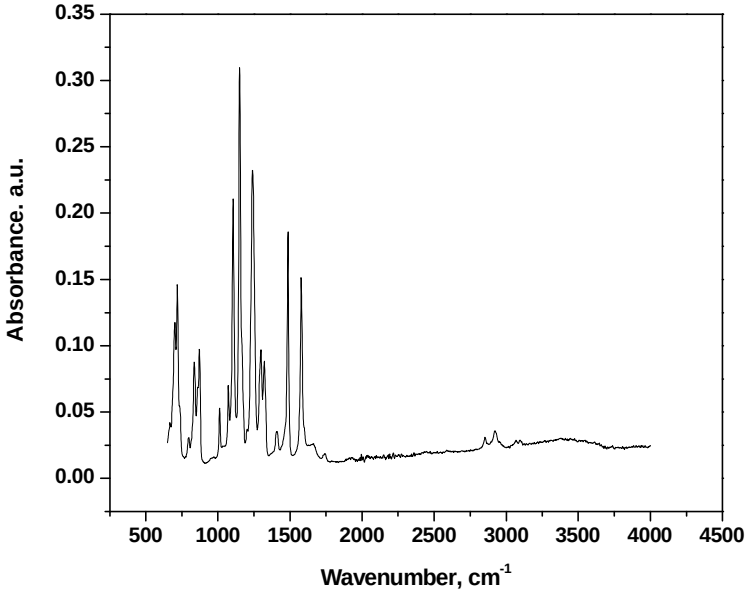


Figure 2.6. A FTIR spectrum of the PES membrane.

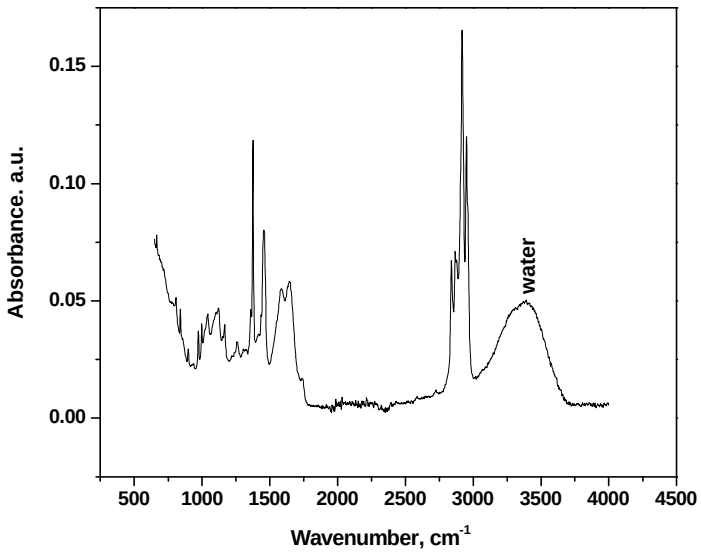


Figure 2.7. A FTIR spectrum of the PP membrane.

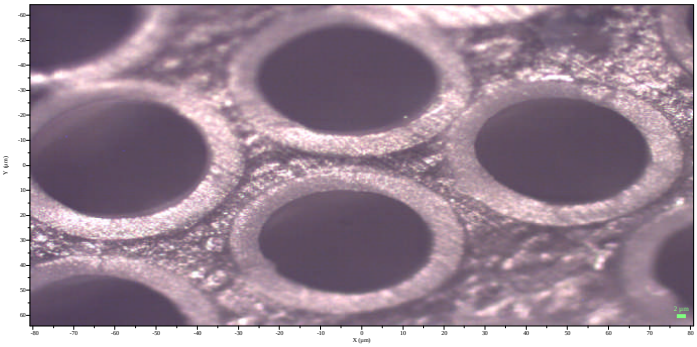


Figure 2.8. A cross section of PP membranes glued together.

Either fermentation broth or an aqueous solution of 0.12 M_{aq} of 1,3-propanediol in KPi buffer (0.5 M, pH = 5.6) were used as an aqueous phase. Pure linoleic acid (to maintain V_{org}/V_{aq} = 1 linoleic acid was taken in high excess so that LA/PD = 27/1 mol/mol) or its 0.3 M_{org} tetradecane solution (LA/PD = 2.5/1 mol/mol) were taken as an organic phase. 0.002 g (4 % w/w_{PD}) CLEA *R. miehei* powder *added to organic phase* was used as a catalyst for the

experiment with the PES membrane. In all other cases *R. miehei* enzyme was immobilized by physical adsorption or filtration.

Immobilization was carried out in the following way: 2-3 mL of free *R. miehei* enzyme solution was pumped for 4-24 h through the lumen side, followed by washing for 1 h with KPi buffer (0.5 M, pH = 5.6). The amount of the enzyme immobilized was evaluated by measuring the lipase activity of the filtrate before and after immobilization and expressed in % of the retained activity. Flow rates were varied between 0.1-0.8 mL min^{-1}. The reactor was kept at 37 °C in the anaerobic chamber.

2.6 Analytical methods

Optical density was measured at 660 nm with a Lambda 25 UV/VIS spectrophotometer (Perkin-Elmer instruments). After centrifugation and 1/10 dilution of the final fermentation broth, glycerol, 1,3-propanediol, acetic and butyric acids concentrations were analysed by HPLC method. A Rezex ROA-organic acid 300 x 7.8 mm column was used with 0.05N H_2SO_4 as eluent and a refractive index detector set up in a Shimadzu Prominence instrument. Retention times are: 15 min (lactic acid), 16 min (glycerol), 18 min (acetic acid), 20 min (1,3-propanediol), 27 min (butyric acid).

Organic samples were analyzed by NMR method. Standard ^1H NMR spectra were recorded on a Bruker Advance 300 NMR spectrometer. NMR samples were prepared by using 20 mg of sample dissolved in deuterated chloroform and chemical shifts were expressed in ppm with respect to the appropriate residual solvent peak (at 7.23 ppm for CH_3Cl). Conversion was

determined according to NMR peaks ratio between free linoleic acid (2.3 ppm, t, 4H) and the diester (4.1 ppm, t, 4H).

Water content in organic solvents was determined by Karl Fisher titration using a Mettler Toledo DL37 KF Coulometer.

Polymer weight mass spectrometry analysis was carried out by the EPSRC National Mass Spectrometry Service Centre at the University of Wales, Swansea. Ionic liquid mass spectrometry analysis was done at the Bath Mass Spectrometry Unit on a microTOF (ESI-TOF) instrument.

Lipase activity was assayed by a spectrophotometric method using *p*-nitrophenyl decanoate as a substrate. A 160 mmol L^{-1} ethanol solution of *p*-nitrophenyl decanoate was diluted 50 fold with KPi buffer (50 mM, pH = 7.2) prior to use. Enzyme activity was monitored within 2 min at 410 nm after addition of 5 µl of lipase solution to 300 µl of freshly prepared solution of *p*-nitrophenyl decanoate.

Calculations of solubility of water and 1,3-propanediol in organic solvents were done in COSMOtherm (X_C21_0108 by COSMOLogic GmbH).

3 Results and discussion

The main work is divided into three parts:

1) investigation and optimization of the glycerol bacterial fermentation,
2) development of a catalytic system for 1,3-propanediol transformation, and
3) design of a reactor for the combined tandem process.

The first part is within the area of biotechnology and was fulfilled with the help of Dr. Martin Rebros (Manchester University). The second part was the most challenging and involved screening of a suitable organic solvent/catalyst pair for biphasic transformation of 1,3-propanediol dissolved in the fermentation broth. The last part was devoted to combining the two processes together in a reactor system. Segmented two-phase flow in a pipe and hollow fiber contactors were tested. The hollow fiber reactor with and without immobilized into a porous membrane enzyme was further optimized for the aqueous/organic esterification of 1,3-propanediol.

3.1 Glycerol fermentation by C. butyricum: process optimization

Different parameters of glycerol fermentation by *C. Butyricum*, such as substrate and product inhibition, pH influence, by-products toxicity, *etc*. were investigated to determine optimal fermentation conditions required for the combined system. The key fermentation parameters, such as product yield and final concentration, optimal substrate concentration and productivity were evaluated.

Carrying out fermentation in the batch cultures is the simplest and quickest way for optimization of a bacterial fermentation. Continuous fermentation demands careful operation as contamination of the feeding medium, unstable flow rates and blocking of tubing could occur, leading to fluctuations in the results. At the same time continuous cultures allow to increase productivity of the process. In this chapter investigation of glycerol fermentation in batch cultures with different substrate concentrations and purity is presented. A 2 L fermentation is also considered and compared to the small-scale batch cultures. Further study of a continuous fermentation is shown.

3.1.1 Batch fermentation studies

The glycerol fermentation by *C. butyricum* in a small-scale volume (200 mL flask) batch system was carried out in an anaerobic chamber. Preliminary work on the optimization of both the medium composition and the inoculation process was carried out for *C. butyricum* growth (see Appendix, p. 181-182). As a result of the optimization, a single inoculation in the glycerol medium instead of a two-step inoculation to reinforce *C. butyricum* and glycerol-rich medium was performed in the fermentation experiments. A 5 % inoculum grown on the glycerol medium during 8-12 h was used for the fermentation processes.

Firstly, batch fermentation of pure and crude glycerol was studied. Different initial concentrations of pure glycerol, namely 20, 50, 60 g L^{-1} were investigated. The maximum growth rate μ_{max} evaluated at the exponential phase of the fermentations was 0.32, 0.38, 0.28 h^{-1} for 20, 50 g L^{-1} (with high concentration of yeast extract) and 60 g L^{-1} of glycerol in the fermentation media. **Figure 3.1** shows that increase in the substrate concentration from 20 to

50 g L^{-1} does not affect *C. butyricum* growth, as in both cases the biomass production reached 1.5 g L^{-1}. If at the same time the nitrogen source (yeast extract) was increased, a slight increase in the biomass production to 1.7 g L^{-1} could be detected. Further increase in the glycerol feed concentration to 60 g L^{-1} led to a decrease in the biomass production to 1.3 g L^{-1}. If the lower μ_{max} is also taken into account one can suggest a glycerol inhibitory affect on the bacterial growth. Furthermore, as one can see in **Figure 3.1**, bacteria need more time to adapt in the carbon- and nitrogen-rich mediums, therefore longer lag phases were observed.

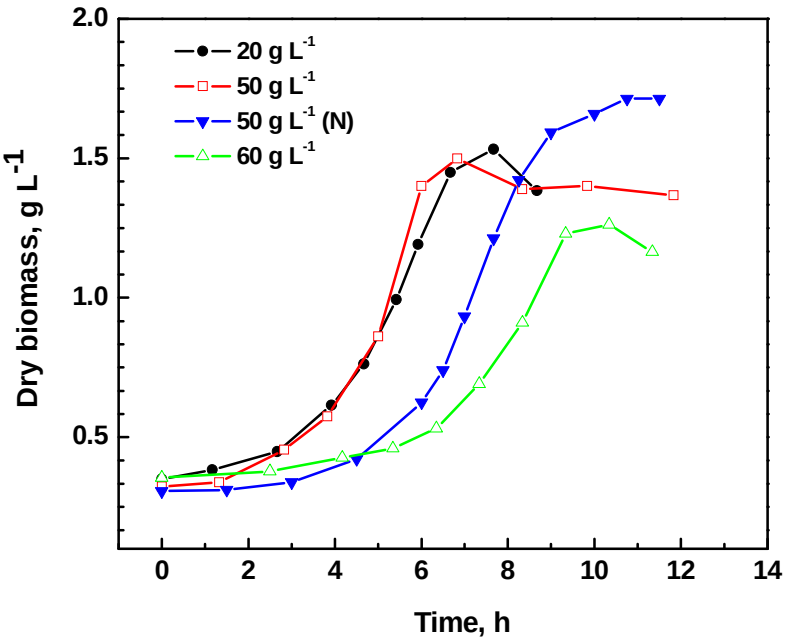


Figure 3.1. Biomass production in batch cultures with different initial concentration of glycerol: 20, 50, 60 g L^{-1}, (50 (N) g L^{-1} corresponds to fermentation medium with 7.5 g L^{-1} of yeast extract instead of 3 g L^{-1}).

Production of 1,3-propanediol could not be enhanced significantly by increasing the initial concentration of glycerol in the fermentation medium as it can be seen in **Figure 3.2**.

Regardless of the initial concentration of glycerol, the final concentration of 1,3-propanediol formed during the batch cultures was not exceeding 10.8 g L^{-1}. This could be improved to 14.6 g L^{-1} with a higher initial concentration of yeast extract. When the initial concentration of glycerol was increased from 20 g L^{-1} to 50 g L^{-1}, half of the glycerol remained unconsumed in the fermentation broth, thus the yield of 1,3-propanediol production lowered from 0.53 to 0.27 mol mol^{-1}. The shortage of the nitrogen source, yeast extract in this case, cannot be a reason for the 50 % of glycerol remained in the fermentation medium, as there was no significant difference detected when the yeast extract concentration was increased proportionaly to the higher concentration of the carbon source (**Figure 3.3**). Indeed, Himmi *et al* postulated that only if the C/N ratio exceeds 81/1, the lower concentration of nitrogen source begins to limit glycerol fermentation by *Clostridium* species [94].

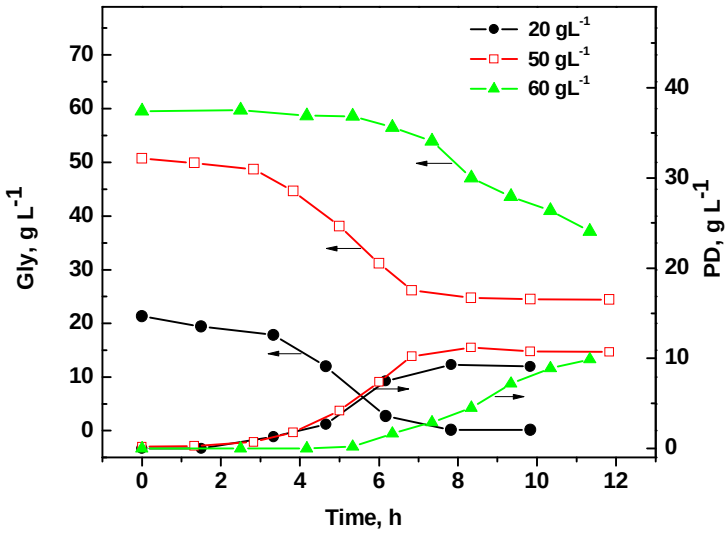


Figure 3.2. Glycerol consumption and 1,3-propanediol production in batch cultures with different initial concentration of glycerol: 20, 50, 60 g L^{-1}.

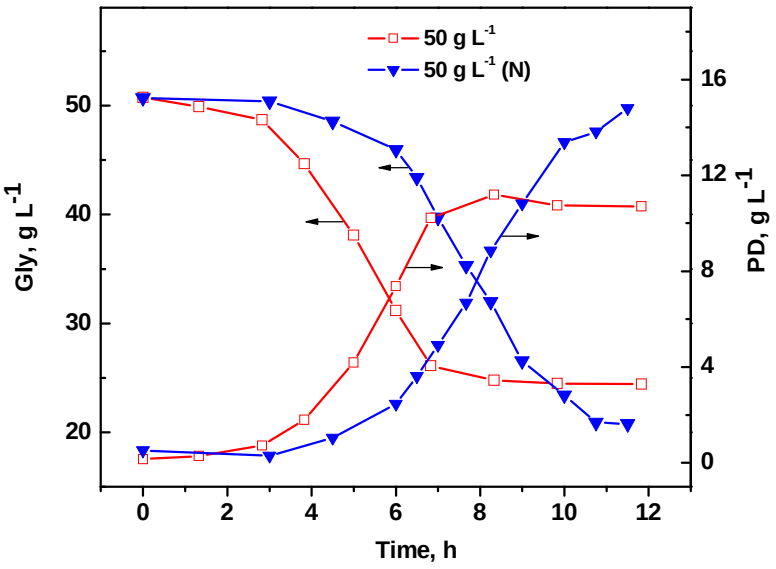


Figure 3.3. Glycerol consumption and 1,3-propanediol production in batch cultures with 50 gL^{-1} of glycerol and different concentration of yeast extract: 3 and 7.5 g L^{-1} (50 g L^{-1} (N)).

In order to understand what suppresses production of 1,3-propanediol, one can compare formation of acidic by-products with the variation in pH during the fermentation. **Figure 3.4** shows a reverse correlation between the concentration of butyric acid and pH during the fermentation. Dissociation of butyric acid leads to the decrease in pH which cannot be compensated by the acetic acid buffer, because butyric and acetic acids have close pKa (pK_a = 4.78). Terefore, acetic acid buffer appeared to be a weak buffer for the fermentation of glycerol by *C. butyricum*.

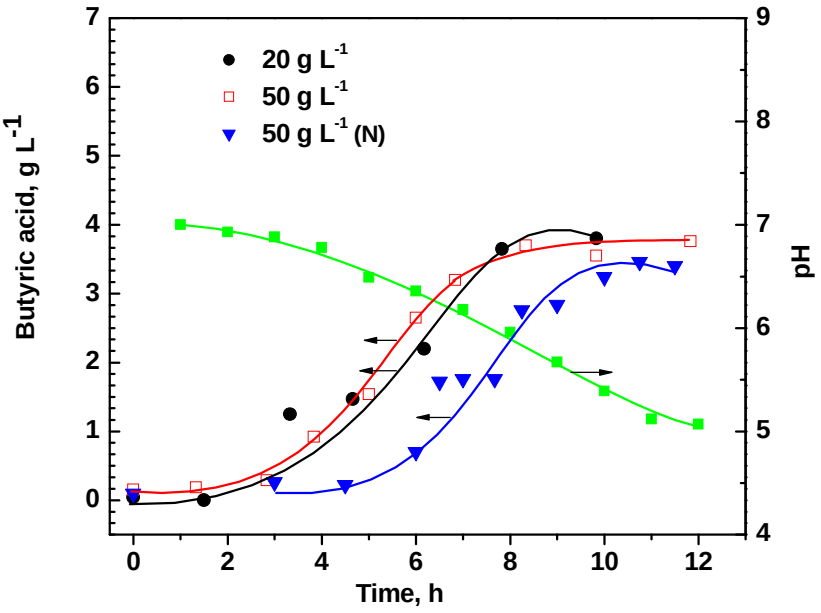


Figure 3.4. Butyric acid production and pH decreasing in batch cultures with different initial concentration of glycerol: 20, 50, 50 (N) gL^{-1} (7.5 g L^{-1} of yeast extract).

If pH is maintained constant during the fermentation by automatic addition of KOH solution, the maximum concentration of 31 g L^{-1} (0.56 mol mol^{-1}) of 1,3-propanediol could be reached in the batch culture even with 70 g L^{-1} of glycerol, as shown in **Figure 3.5**. However, production of acetic acid increases in this case, compared to the uncontrolled system, suggesting a feasibility of diverting glycerol transformation towards the acetic acid metabolic pathway when a pH-controlled fermentor is used.

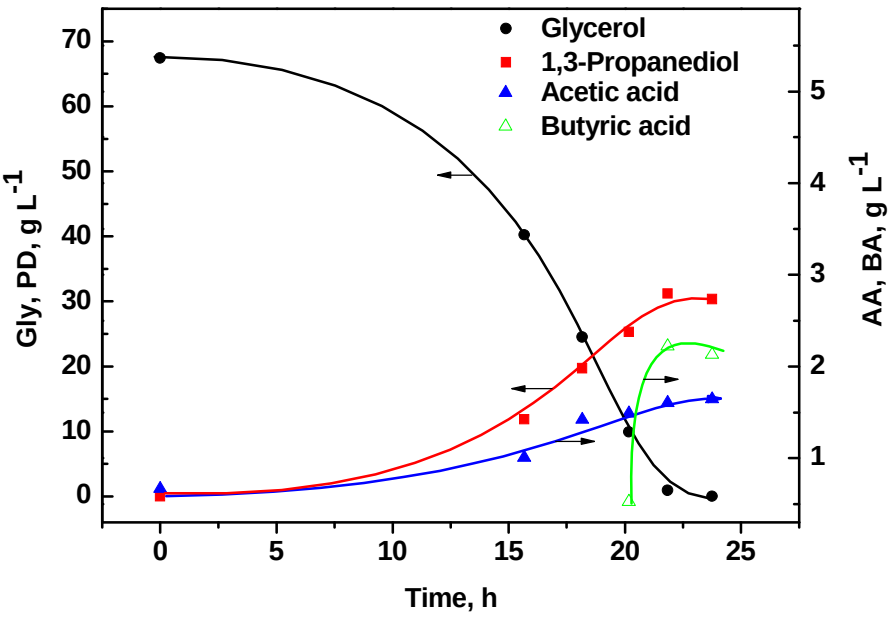


Figure 3.5. Glycerol consumption and 1,3-propanediol, acetic and butyric acids production in 2.5 L batch bioreactor with 67 g L^{-1} of glycerol.

Crude glycerol as a carbon source

In order to study feasibility of using crude glycerol from biodiesel production, we carried out reaction of rapeseed oil transesterification with methanol catalysed by NaOH at 65 °C. Excess of methanol was evaporated after completion of the reaction and the raw glycerol was used for batch fermentation. Already at the inoculation state it was noticed that the cells tended to agglomerate, perhaps to tolerate toxic residues in the crude glycerol. The most probable inhibitors are salts of fatty acids. No growth and 1,3-propanediol production was detected if unpurified glycerol, taken straight after rapeseed oil transesterification, was used as a substrate.

It is known, that by adding phosphoric acid (or other acid) to raw glycerol all soap formed during the transesterification reaction can be hydrolyzed to free fatty acids and easily separated from glycerol along with the precipitated sodium phosphates. After addition of H_3PO_4 glycerol was autoclaved and cleaned of fatty acids. The glycerol purified in this manner can be used as a substrate without any further purification, as it was shown for batch fermentation with 14 g L^{-1} of crude glycerol, when all glycerol was consumed and 6.3 g L^{-1} of 1,3-propanediol was formed (**Figure 3.6**).

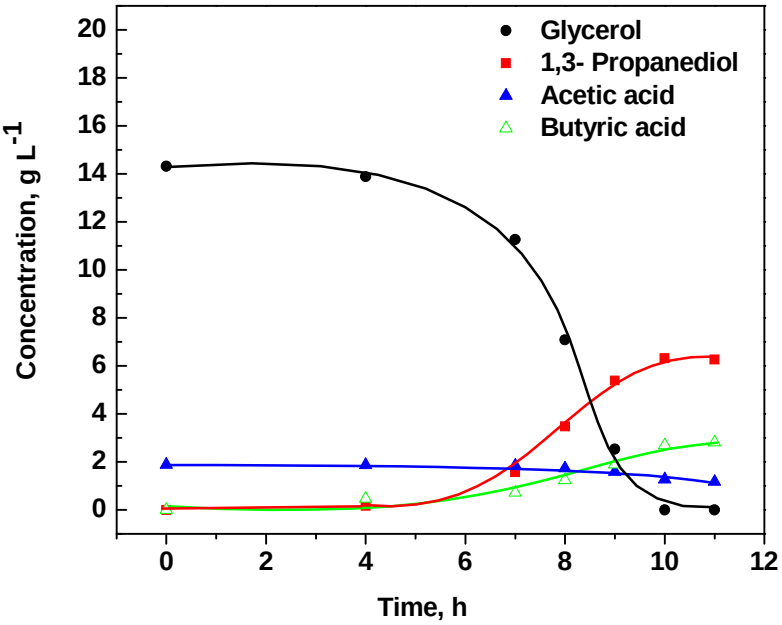


Figure 3.6. Glycerol consumption and 1,3-propanediol, acetic and butyric acids production in batch culture with 14 g L^{-1} of crude glycerol.

As one can see in **Figure 3.6**, a prolonged lag phase for the fermentation on crude glycerol was observed, suggesting a lower bacteria viability compared to the batch cultures harvested on the pure glycerol.

All results obtained for batch cultures with various concentrations of carbon and nitrogen sources are summarised in **Table 3.1**.

Table 3.1. A summary of the yield, productivity and final concentration of 1,3-propanediol and butyric acid obtained in batch cultures with different glycerol concentrations.

	C^0_{Gly}, g L^{-1}	Y_{PD}, mol mol^{-1}	Q_{PD}, g L^{-1} h^{-1}	C_{PD}, g L^{-1}	C_{BA}, g L^{-1}	Y_{BA}, mol mol^{-1}
1	20	0.53	1.2	9.3	3.8	0.19
2	50	0.27	1.3	11.2	3.8	0.07
3	50[a]	0.36	1.3	14.8	3.6	0.07
4	60	0.22	0.9	10.5	-	-
5	67[b]	0.56	1.4	31.2	2.2	0.03
6	14[c]	0.53	0.6	6.3	2.8	0.20

[a] 7.5 g L^{-1} yeast extract was used instead of 3 g L^{-1}

[b] fermentation was carried out at constant pH = 6.7 in 2.5 L fermenter equipped with a pH controller

[c] purified raw glycerol was used.

In the 12 h of the fermentation process, 20 g L^{-1} of glycerol was totally consumed, leading to the production of 9.3 g L^{-1} of 1,3-propanediol, which corresponds to 53 % (mol mol^{-1}) yield. This result is somewhat lower than 62 % reported by Barbirato *et al* [90]. The level of acetic acid does not change significantly during the fermentation, whereas the end concentration of

butyric acid reaches 3.8 g L^{-1} (19 %), which is the highest yield among reported [90, 94]. This points at the suppression of acetic acid production pathway (see 1.9 part of Literature review) in the presence of acetic acid in the broth solution. The concentration of acetic acid is constant during the fermentation. Therefore, the observed lowering of pH is caused by the butyric acid produced. **Table 3.1** shows that there was no difference in the yield of 1,3-propanediol (0.53 mol mol^{-1}) obtained in the batch cultures harvested on the chemically pure and the raw glycerol which undergone purification from fatty acids residues. However, the lower productivity was observed with the raw glycerol due to the prolonged lag phase. The same effect of the longer lag phase in the batch culture harvested on the crude glycerol was observed by others [93].

The much lower 1,3-propanediol yields were observed if a higher concentration of glycerol was added to the fermentation medium and the batch cultures were carried out without pH control. In these cases 50-60 % of glycerol remained in the fermentation medium. A higher concentration of yeast extract favoured the production of 1,3-propanediol (0.36 mol mol^{-1}yield of 1,3-propanediol comparing to 0.27 mol mol^{-1} if less yeast extract was used) but induced longer lag phase during bacterial growth, thus lowering total productivity of the product (1.3 g L^{-1} h^{-1}). The low level of glycerol consumption in the case of its high initial concentration cannot be attributed to its inhibitory effect, as the maximum growth rates and biomass production did not differ significantly. Indeed, the highest non-inhibitory glycerol concentration for *Clostridium* species was reported as high as 150 g L^{-1} [98]. The amount of butyric acid formed was not affected by different glycerol concentrations and, as it was shown above, butyric acid has strong inhibitory effect on the production of 1,3-propanediol. The

highest final concentration of 1,3-propanediol (14.8 g L^{-1}) was obtained when a rich in yeast extract medium was used in the fermentation process with acetic buffer as a pH stabilizer.

The highest obtained concentration of 1,3-propanediol - 31.2 g L^{-1} - was achieved when inhibition by acidic by-products was overcome by carrying out fermentation in a pH-controlled reactor. A lower 2.2 g L^{-1} final concentration of butyric acid was detected in this case.

3.1.2 Continuous fermentation studies

Continuous fermentation was studied in a CST reactor with a total volume of 20 mL. At the beginning the fermentation medium was inoculated with a 5 % of inoculum. **Figure 3.7** shows a typical sequence of batch and continuous modes in these experiments. Batch fermentation was allowed until the maximum rate of 1,3-propaneidol production was reached in the exponential phase of bacterial growth, after *ca* 7-8 h, region 1. Then continuous fermentation was started, shown as regions 2 and 3. Steady state (region 3) was monitored for at least 5 volumes of the fermentor have passed. Then, continuous mode was switched back to the batch culture mode, region 4, to check viability of the bacteria.

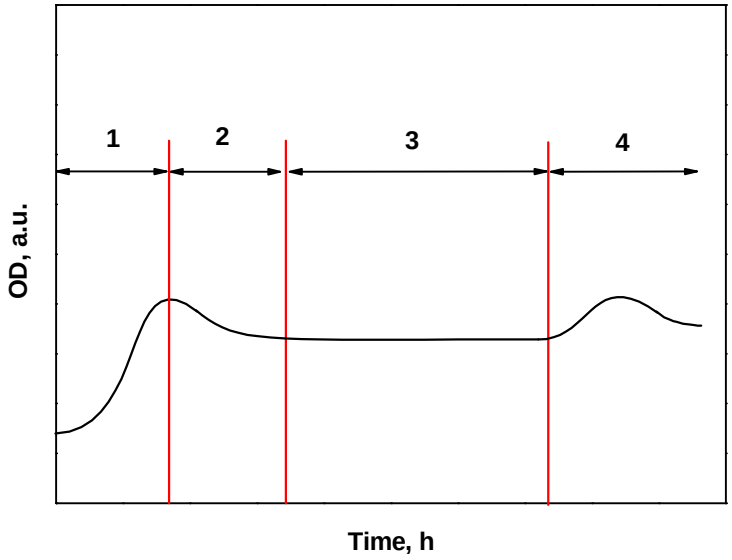


Figure 3.7. Time-line of a continuous fermentation process (described in the text).

Fermentation curves obtained during the continuous cultures are shown in **Figure 3.8**. The steady state was achieved in about 10-20 h. This time depends on the point at which the continuous fermentation was begun and also on the dilution rate used. When the maximum growth was exceeded or a very low dilution rate used, as for example for 0.2 h^{-1} (< μ_{max} = 0.32 h^{-1}), a longer period of time was required for bacteria to stabilize. When a dilution rate of 0.6 h^{-1} was used steady state was reached in the shortest period of 10 h.

A dependence of the production rate and the average concentration of 1,3-propanediol at the steady state on the dilution rate is shown in **Figure 3.9**. As one can see, an average concentration of 1,3-propanediol lowers from 8.5 g L^{-1} to 7.0 g L^{-1} between 0.2 h^{-1} and 0.4 h^{-1} dilution rates and then sharply decreases to 2.0 g L^{-1} at 0.5 h^{-1} dilution rate. The best productivity of 1,3-propanediol of 2.8 g L^{-1} h^{-1} was obtained at 0.4 h^{-1} dilution rate. This

productivity is twice higher compared to the batch cultures. It corresponds to 7 g L^{-1} of 1,3-propanediol and 3 g L^{-1} of glycerol (15 % from the initial concentration) in the fermentation broth. We can compare the result with the productivity reported by Reimann *et al* for the same *Clostridium* DSM 5431: 2.4 g L^{-1} h^{-1} for the continuous reactor with 32 g L^{-1} of initial glycerol and 0.7 h^{-1} dilution rate [96]. In our work a higher productivity of 1,3-propanediol of 2.8 g L^{-1} h^{-1} was obtained with a lower initial concentration of glycerol of 20 g L^{-1}. The average concentration of butyric acid reduces from 3 to 0.05 g L^{-1} when the dilution rate increases from 0.2 to 0.6 h^{-1} (**Figure 3.9**), whereas the concentration of acetic acid does not depend on the dilution rate and remaines 2 g L^{-1} in all experiments (not shown).

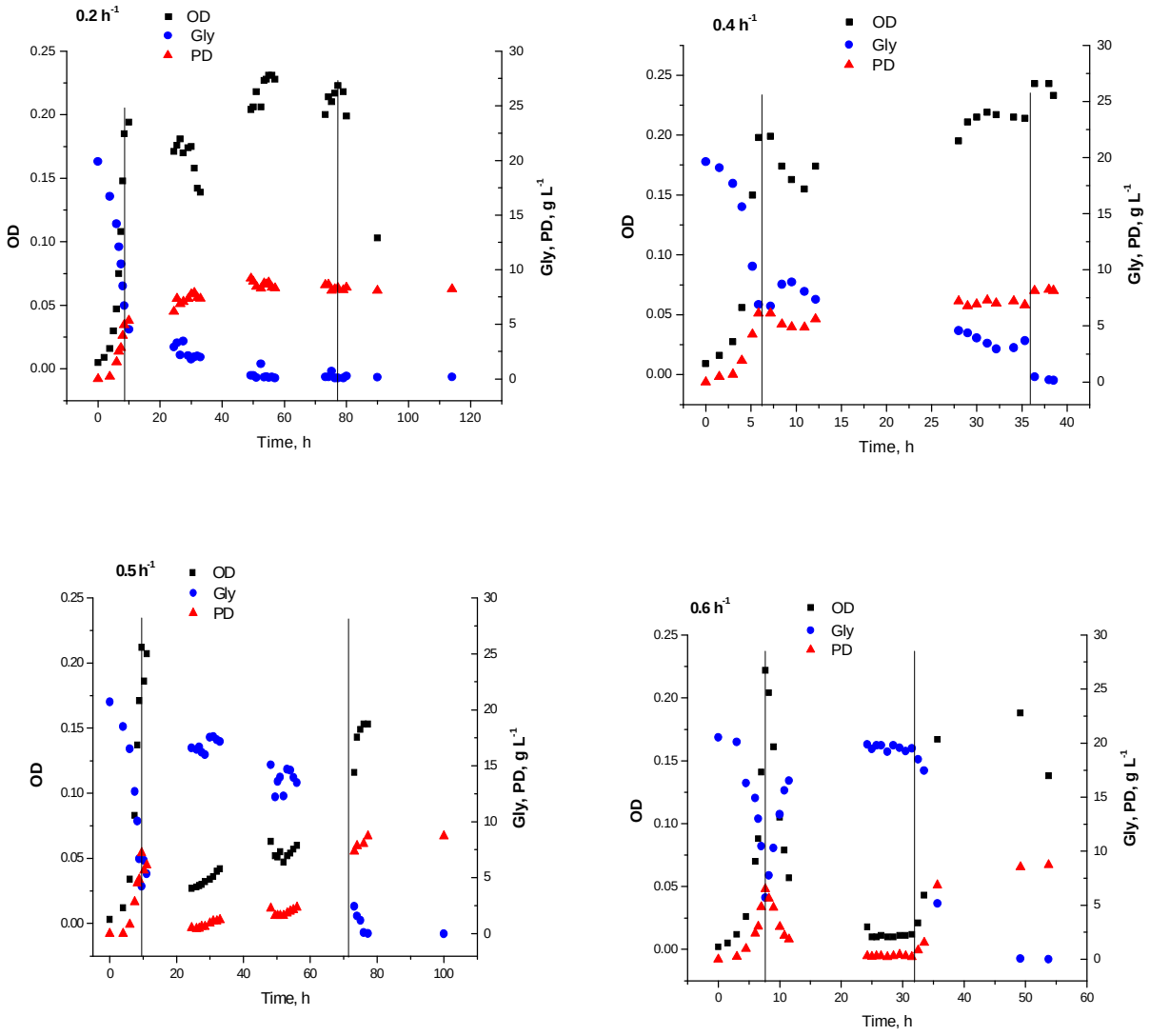


Figure 3.8. Variation of optical density, glycerol consumption and 1,3-propanediol production in continuous cultures with 20 g L^{-1} of glycerol and at different dilution rates (straight line shows when continuous mode was switched on and off).

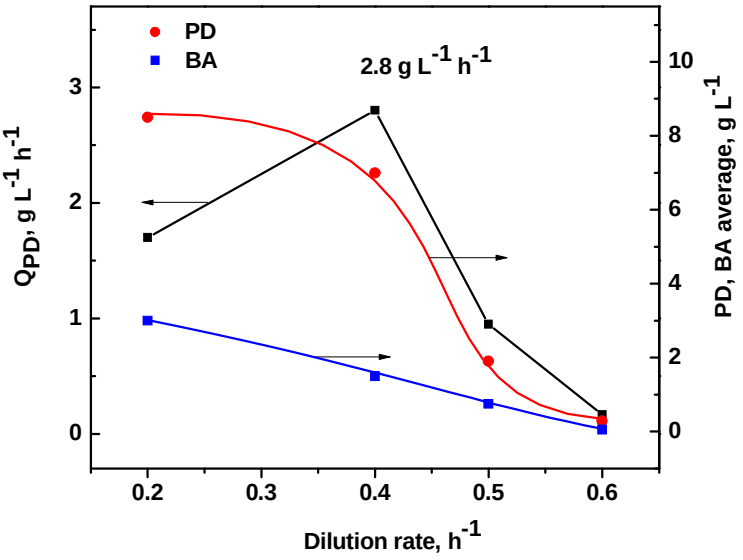


Figure 3.9. Dependence of volumetric productivity of 1,3-propanediol and an average concentration of 1,3-propanediol and butyric acid on dilution rate in continuous cultures with 20 g L^{-1} initial concentration of glycerol.

To conclude one can say that the substrate concentrations higher than 20 g L^{-1} affect growth of *Clostridium* bacteria, leading to a longer lag phase, slower exponential growth and a lower biomass production. At the same time this does not reflect on the resulting concentration of 1,3-propanediol, which remains approximately the same at 9-10 g L^{-1} if acetic buffer was used as a pH stabiliser during the fermentation process. This observation is in accordance with the conclusions made by Colin *et al* [98]. They found that the maximum growth rate decreases linearly with the increase in the glycerol concentration whereas the amount of 1,3-propanediol formed increases according to the rising glycerol uptake. Only if the amount of glycerol exceeds 150 g L^{-1} the total inhibition of 1,3-propanediol production was observed [98].

In our study 1,3-propanediol production increased with the increase in the initial concentration of glycerol only when pH was maintained at 6.7. In this case 31 g L^{-1} of 1,3-propanediol was achieved compared to 10.5 g L^{-1} obtained in the batch culture with acetic acid as a buffering agent. Lowering pH to 5 during batch fermentation process caused by the formation of butyric acid has s stronger inhibition effect on the 1,3-propanediol production compared to the inhibition by the substrate. To achieve total consumption of glycerol one should make sure that acidic by-products are neutralised and pH remains close to neutral, which is optimum pH for *C. butyricum*.

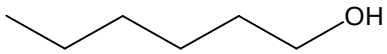
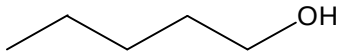
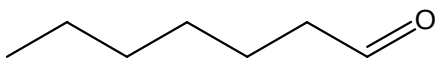
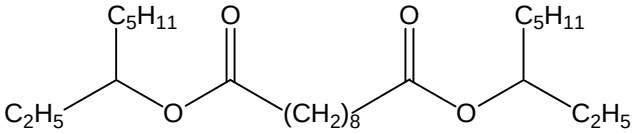
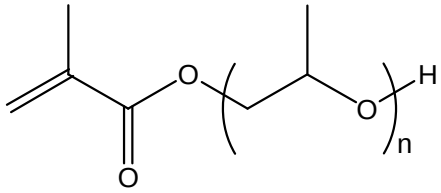
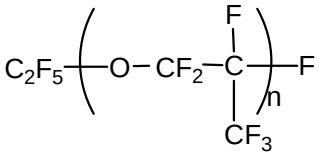
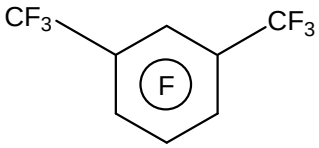
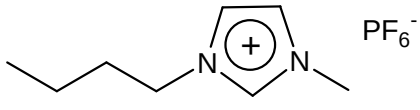
Although some authors presented results of fermentations performed with crude glycerol, our study showed that raw glycerol obtained straight after vegetable oil transesterification was not suitable for fermentation. Presumably residual fatty acids inhibit bacterial growth. Addition of phosphoric acid led to fatty acids salts to be converted to fatty acids, which could be easily separated yielding purified glycerol. Batch fermentation with such glycerol as a substrate had the same 1,3-propanediol yield (0.53 mol mol^{-1}) but a lower productivity of 0.63 g L^{-1} h^{-1} compared to the cultures harvested on pure glycerol due to the prolonged lag phase. This example showed the importance of the glycerol purification step in the downstream process of vegetable oil conversion to biodiesel and subsequent synthesis of value-added products.

The twice-higher productivity of 1,3-propanediol of 2.8 g L^{-1} h^{-1} was achieved if the fermentation was carried out in the continuous mode at 0.4 h^{-1} dilution rate. At the same time the maximum concentration of 1,3-propanediol did not exceed 7 g L^{-1}, and 15 % of initial glycerol remained in the fermentation medium unconsumed.

3.2 Extraction of 1,3-propanediol from the fermentation medium

The main approach, which we propose to use in a tandem or sequential transformation of glycerol, is separation of the two reactions in the immiscible solvents. The intermediate 1,3-propanediol is obtained as a result of the glycerol fermentation by *C. butyricum*. The final product filtered from cells solution obtained at the end of a fermentation process is a diluted aqueous mixture of nutrient salts, yeast extract, acetic and butyric acids, unconsumed glycerol and 1,3-propanediol. Instead of extraction of 1,3-propanediol from this medium we propose its further transformation by a catalyst dissolved in a hydrophobic organic solvent. It is obvious that using a solvent that extracts 1,3-propanediol from fermentation broth will enhance its further catalytic transformation. However, highly hydrophobic water-immiscible solvents will not extract highly hydrophilic 1,3-propanediol (log P = -1.1) effectively. There is also a problem of selectivity if some glycerol remaines in the fermentation broth. There are some methods for separation of glycerol and 1,3-propanediol, reviewed in the Literature section. None offer high separation efficiency, although a system of alcohol/salts is being used commercially [100, 101]. New solvents with specific properties – fluorous and ionic liquids – might be a solution to this problem. We tested several fluorous solvents and ionic liquids, as well as some hydrophobic alcohols, aldehydes and hydrophobic esters for “passive” extraction of 1,3-propanediol from fermentation broth (**Table 3.2**). The main criteria for all chosen solvents were hydrophobicity, availability and price. Extraction of glycerol, acetic and butyric acids was also studied.

Table 3.2. Different hydrophobic solvents tested for passive extraction of 1,3-propanediol from fermentation medium.

	Solvent	log P	Formula
$C_6H_{13}OH$	1-hexanol	2.0	
$C_5H_{11}OH$	1-pentanol	1.5	
$C_7H_{14}O$	heptaldehyde	~2.3	
BES	bis(2-ethyl-hexyl)sebacate	10.1	
PPGM	poly(propylene glycol) methacrylate, M = 375		
PhFPO	poly(hexafluoro-propylene oxide)		
$C_6F_{10}(CF_3)_2$	perfluoro-1,3-dimethylcyclohexane		
$BmimPF_6$	1-butyl-3-methylimidazolium hexafluorophosphate		

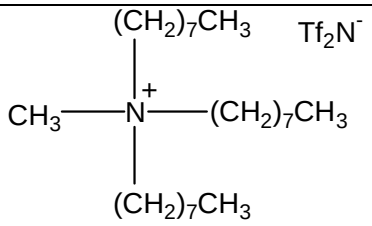
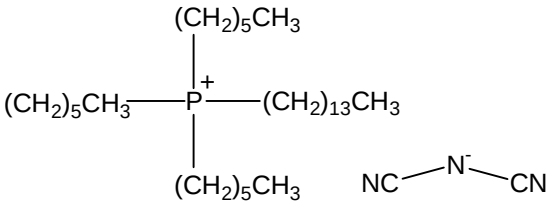
$AmNTf_2$	methyl-trioctylammonium bis(trifluoromethyl-sulfonyl)imide	
$PmN(CN)_2$	trihexyltetradecyl-phosphonium dicyanamide	

Figure 3.10 shows results obtained for the extraction of 1,3-propanediol, glycerol, acetic and butyric acids. Two-phase samples were mixed for 24 h and the aqueous solutions were analyzed by HPLC method before and after mixing.

As one can see in **Figure 3.10,** among all the solvents tested only $C_7H_{14}O$ and $PmN(CN)_2$ have different from zero partition coefficients for glycerol $P^{Gly}_{solv/aq}$ = 0.05 and 0.16 correspondingly. No glycerol extraction (detectable by HPLC method) was observed for the rest of the solvents. The best solvent for the extraction of 1,3-propanediol among all the solvents tested appeared to be $C_5H_{11}OH$ which has the highest $P^{PD}_{solv/aq}$ = 0.42 that corresponds to 30 % of 1,3-propanediol being extracted from the aqueous solution. PPGM, PhFPO, BES and $PmN(CN)_2$ have somewhat lower $P^{PD}_{solv/aq}$ varied in the range of 0.22-0.28. These solvents extract 18-22 % of 1,3-propanediol.

The acidic by-products of the fermentation appeared to be easily extracted by all the solvents tested, with the exception of the fluorous solvents, with $PmN(CN)_2$ being the best for the extraction of acetic acid and $C_5H_{11}OH$, $C_6H_{14}OH$ - for butyric acid. The alcohols and PPGM have $P^{AA}_{solv/aq} \sim 1$, therefore up to 50 % of acetic acid can be extracted by these solvents. The same, close to 1, $P^{BA}_{solv/aq}$ values were observed for $C_7H_{14}O$, BES, $BmimPF_6$ and $AmNTf_2$ solvents. $PmN(CN)_2$, PPGM, $C_6H_{14}OH$ and $C_5H_{11}OH$ extract 75-90 % of butyric acid from the aqueous solution.

If one takes into account that an active extraction (*i.e.* reactive extraction) can be facilitated by passive extraction, a solvent that extracts as much as possible 1,3-propanediol from the fermentation medium should be used as the second, hydrophobic, solvent in the biphasic system. However, our study showed that those solvents that extracted 1,3-propanediol best, presumably because of their low log P, namely heptaldehyde and pentanol, cannot be used due to their high reactivity. The esters appeared to undergo hydrolysis when enzymes were used as catalysts in 1,3-propanediol conversion. The fluorous solvents showed low solubility for fatty acids. ILs are not only poor solvents for 1,3-propanediol, but also have high density, must be carefully purified from Cl^- prior to use, and yield contaminated product: ILs have high boiling points and miscible with many organic solvents, therefore product recovery is problematic (see Appendix, p. 183-194).

In conclusion one can say that among the different solvents with high log P, such as aldehydes, long chain alcohols, hydrophobic esters, fluorous solvents and ILs, no one solvent

meets the requirements for effective extraction of 1,3-propanediol from the complex fermentation medium, mainly because of poor extractive properties.

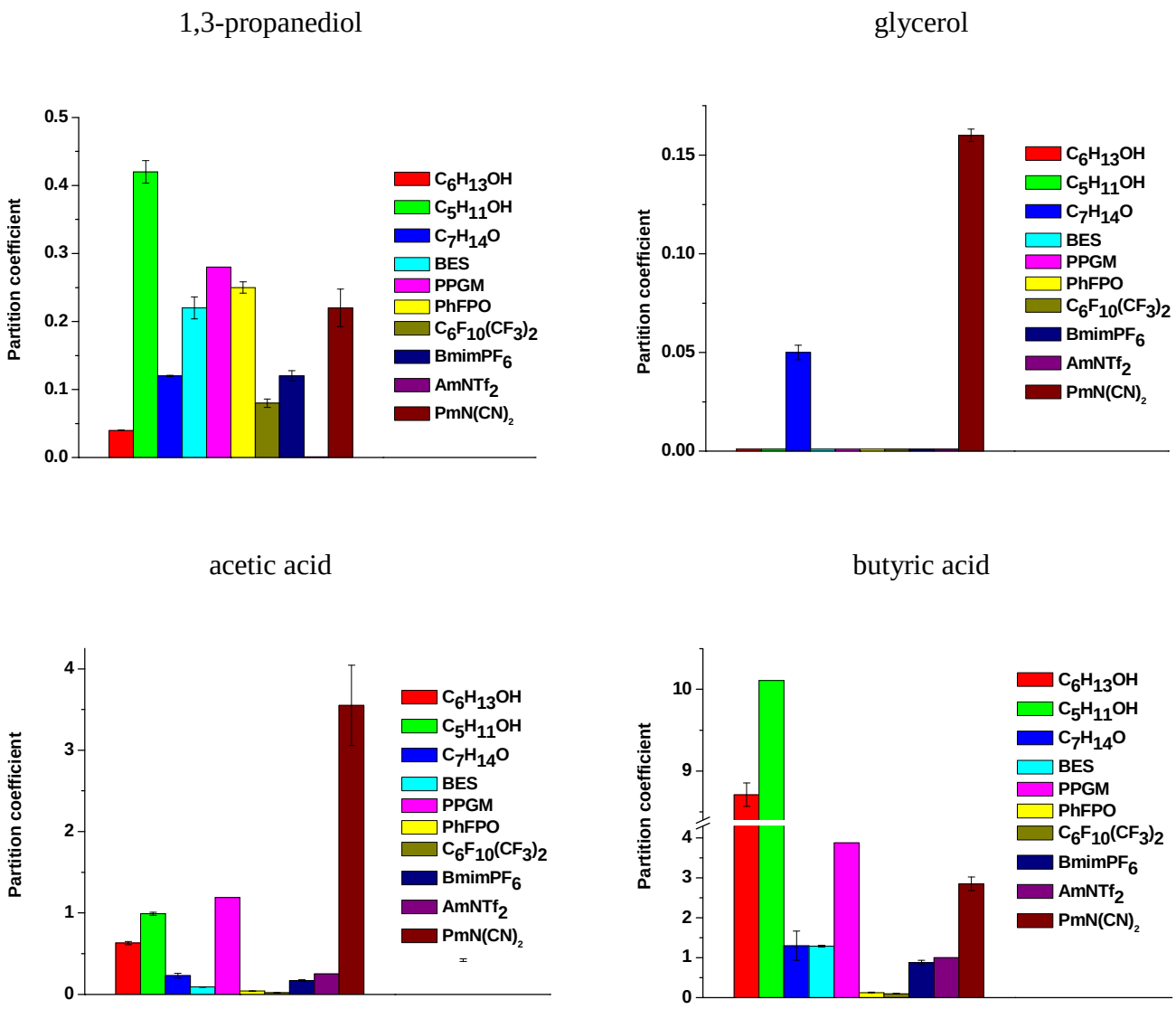


Figure 3.10. Partition coefficients of the main components of aqueous fermentation broth for different solvents.

3.3 1,3-Propanediol esterification

3.3.1 1,3-Propanediol esterification with decanoic acid in a monophasic system

In order to elicit the best catalytic/solvent pair for the active extraction of 1,3-propanediol from the fermentation broth several parameters were varied, such as initial acidic reagent, the nature of catalyst and solvent. As we pointed out before, a hydrophobic solvent should be used to obtain a two-phase system. Tetradecane was tested as an appropriate highly hydrophobic and non-toxic to bacterial cells solvent. The choice of an acid to be esterified was limited by a requirement for the acid to be liquid at 37 °C or soluble in tetradecane, non-toxic and hydrophobic. Catalysts, active in the esterification reactions between alcohols and acids are known to be Lewis or Brönsted acids. Moreover the acid chosen could catalyze the reaction of esterification itself.

Firstly, the reaction of decanoic acid esterification (**Figure 3.11**) was studied in the solvent-free conditions, in tetradecane and decanoic acid as solvents.

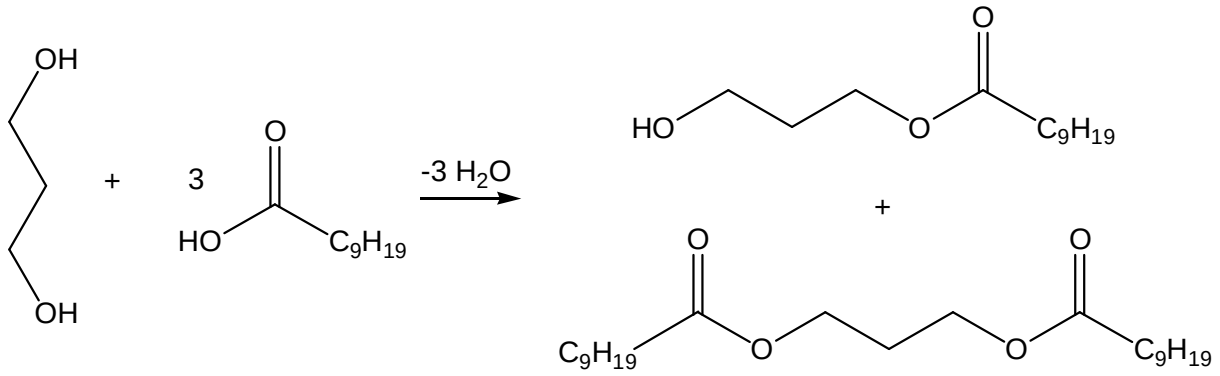


Figure 3.11. Decanoic acid and 1,3-propanediol esterification.

The reaction was monitored by NMR analysis. NMR spectra obtained during the reaction are shown in **Figure 3.12**. In order to calculate conversion the intensity of the peak at 4.1 ppm, corresponding to CH_2 protons of 1,3-propanediol diester, can be compared to the peak at 2.3 ppm, corresponding to the protons of the CH_2 group closest to the carboxylic groups of the acid. It could be clearly seen in **Figure 3.12** that at the beginning of the reaction there is only one peak at 3.9 ppm corresponding to CH_2 protons of the free 1,3-propanediol. The intensity of this peak decreases during the reaction, whereas the peaks corresponding to the monoester product appear at 3.7 and 4.2 ppm and finally, the peak of diester appears at 4.1 ppm and increases with time (see also Appendix, p. 195). Selectivity can be estimated based on peaks areas. The same approach was used by Decagny [146].

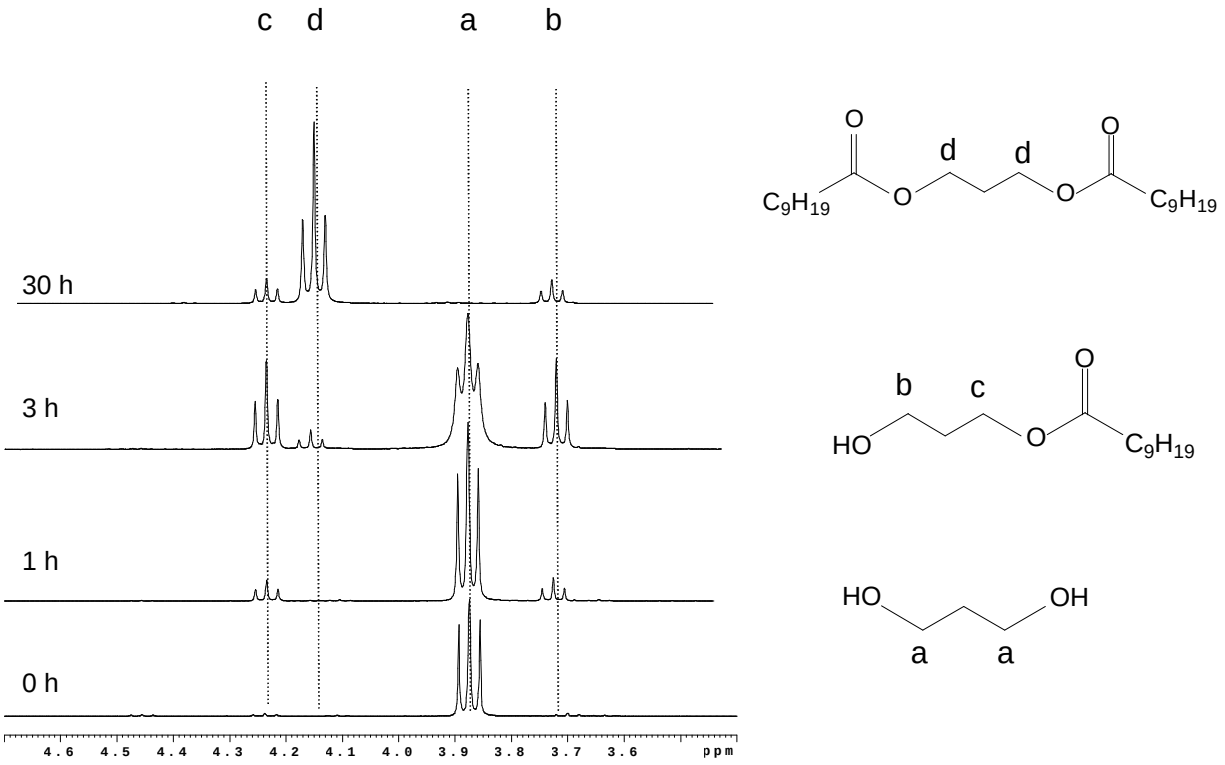


Figure 3.12. NMR spectra of 1,3-propanediol and decanoic acid esterification (DA/PD = 2.5/1 (mol), 100 °C, no solvent, no catalyst).

Figure 3.13 shows a typical behavior of the reaction mixture when the total conversion increases reaching the equilibrium, whereas the initially formed monoester concentration decreases with time leading to the formation of diester, suggesting a sequential mechanism of the esterification of 1,3-propanediol.

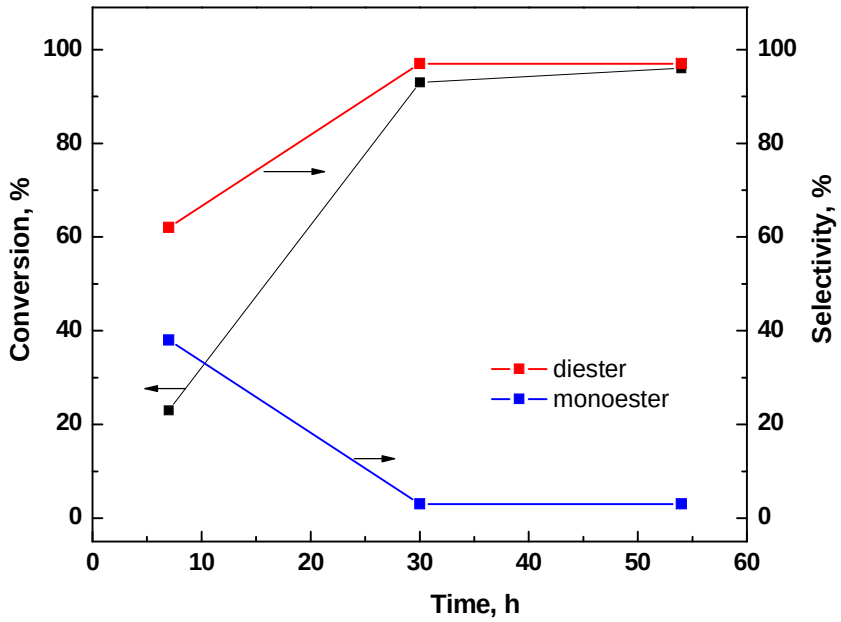


Figure 3.13. Conversion and selectivity as a function of time in esterification of decanoic acid with 1,3-propanediol at 100 °C (DA/PD = 2.5/1 (mol), no solvent, no catalyst).

Table 3.3 shows results of self-esterification of 1,3-propanediol and decanoic acid depending on the solvent and temperature. It can be seen that conversion of 96 % can be reached in two days at 100 °C when reacting the equimolar amounts of 1,3-propanediol and decanoic acid. In this case evaporation of water during the reaction facilitates esterification. If 0.12 M_{org} concentration of 1,3-propanediol (corresponding to the final concentration of 1,3-propanediol in fermentation broth) was taken initially in tetradecane as a solvent, only 17 % conversion

with a 55 % selectivity towards diester was obtained at 100 °C. As water was not removed during the reaction we can suggest that the equilibrium limitations, as well as low concentration of the initial reactants, suppress the formation of the esters. Moreover, when temperature was as low as 37 °C, reaction between 2.5 units of decanoic acid with 1 unit of 1,3-propanediol in tetradecane does not occur. Low reaction rate and low solubility of 1,3-propanediol in tetradecane are probably the main limiting factors. If decanoic acid was used as a solvent, *i.e.* taken in excess, conversion of 35 % was achieved in 7 days at 37 °C with 10 % of diester. In this case decanoic acid acts as an acidic catalyst (pKa = 4.88), catalysing the reaction of esterification.

Table 3.3. Results obtained in non-catalytic esterification of 1,3-propanediol with decanoic acid at 37 and 100 °C.

N	Solvent	T, ºC	Time, days	Conversion, %	Selectivity to diester, %
1[a]	No	100	2	**96**	97
2[b]	Tetradecane	100	4	**17**	55
3	Tetradecane	37	4	No product	
4[c]	Decanoic acid (10:1)	37	7	**35**	10

[a] Reaction vessel was open to allow water to evaporate

[b] Reaction vessel was closed so no water evaporation occurred

[c] 1,3-Propanediol is soluble in the acid

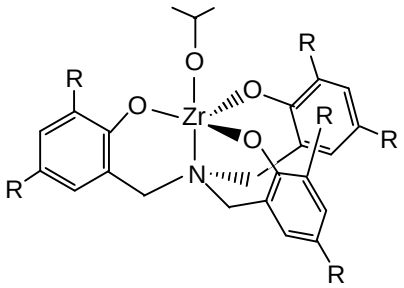
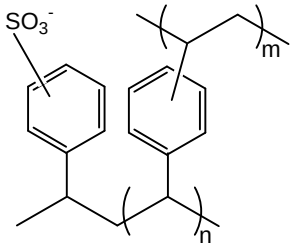
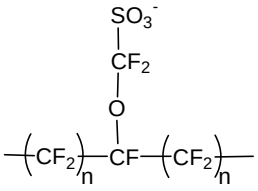
As a result, one can say that high conversion can be obtained when the reaction of esterification is carried out at high temperatures and between undiluted reactants. Contrary, at low temperatures and low concentrations of the reactants no satisfactory result can be achieved. Decanoic acid as a catalyst showed low activity in the reaction at 37 °C.

Catalytic esterification of 1,3-propanediol and decanoic acid was carried out with different catalysts. We chose Zr catalyst (1) as an example of organometallic catalyst with Lewis acidity, Nafion and Amberlyst-15 as commercially available acidic resins, and Novozyme 435 as the most common bio-catalyst for esterification processes. A brief description of the catalysts and their concentrations are shown in **Table 3.4**.

The Zr catalyst (1) is known to be active in the reaction of lactide ring-opening polymerization [147] (see Appendix p. 187-190 for an example of ROP of *L*-lactide). However, it showed no activity in the esterification of 1,3-propanediol and decanoic acid probably due to its deactivation by water or the acid.

Figure 3.14 shows the conversion profiles obtained with and without addition of the Zr catalyst (1). The initial conversion after 5 h comes to 8 % in the case of the Zr catalyst (1) compared to 16 % if the catalyst was not added. It should be noted that a 5-fold increase in the acid concentration did not enhance the reaction rate which was so slow at 37 °C that the equilibrium state was not reached in 7 days (168 h), according to the shape of the conversion curve in **Figure 3.14**.

Table 3.4. Catalysts tested in esterification of 1,3-propanediol with decanoic acid.

Catalyst	Description	Concentration, % w_{cat}/w_{PD}
Zr catalyst (1)  R-^{t}Bu	C3-symmetric aminotriphenolate ligands coordinate Zr atom	1
Amberlyst 15 	Styrene-divinylbenzene based acidic resin	4-6
Nafion 	PTFE based acidic resin	4.5
Novozyme 435	Lipase from *C. antarctica* recombinant, expressed in *Aspergillus oryzae* immobilized on acrylic resin, ≥10,000 U g^{-1}	3-10

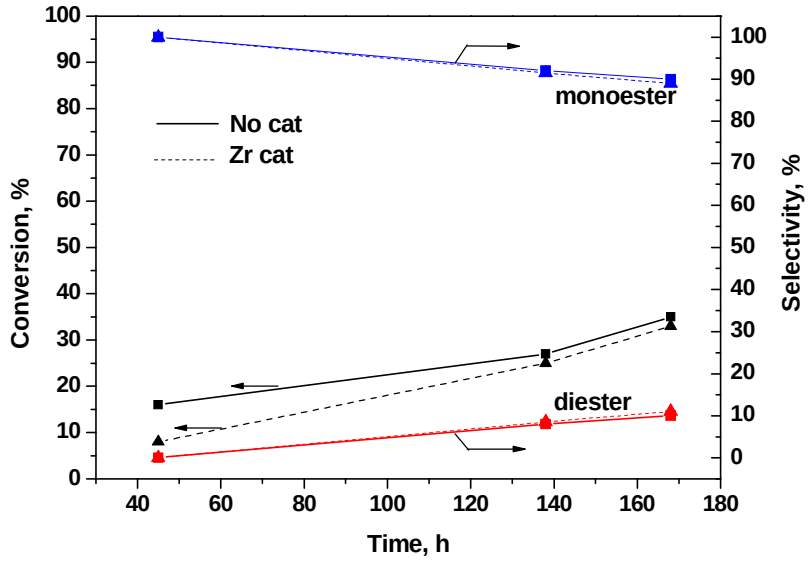


Figure 3.14. Conversion and selectivity profiles in the reaction of decanoic acid with 1,3-propanediol at 37 °C (DA/PD = 10/1, no solvent, no catalyst or 1.1 % Zr catalyst (1)).

Investigation of Amberlyst-15 acidic resin and Nafion catalyst in the reaction of 1,3-propanediol and decanoic acid esterification showed that the highest conversion of 69 % could be obtained with Amberlyst-15 as a catalyst and tetradecane as a solvent at 37 °C, but only monoester was detected (**Table 3.5**, row 3). Both catalysts showed low activity at 100 °C yielding 23-24 % conversion, though with high percentage of the diester (**Table 3.5**, row 2 and 5), compared to 17 % conversion obtained if no catalyst was used. Because the maximum temperature for the resins is about 120 °C, their thermal deactivation must have occurred, especially in the presence of water in the reaction medium that may cause hydrolysis of $-SO_3H$ active sites [148]. Comparative graphs for the experiments corresponding to rows 1, 3, 4, 6 in **Table 3.5** are shown in **Figure 3.15**.

Table 3.5 Results obtained in the reaction of 1,3-propanediol with decanoic acid in solvent-free conditions and in tetradecane, catalyzed by Amberlyst-15 and Nafion.

N	Solvent	Catalyst, % (w)	T, °C	Time, days	Conv., %	Select. diester, %
1[a]	No	4.2 % A-15	37	11	**50**	10
2[a]	Tetradecane	5.4 % A-15	100	4	**24**	68
3[a]	Tetradecane	5.7 % A-15	37	3	**69**	0
4	No	4.4 % Nafion	37	11	**38**	0
5[b]	Tetradecane	4.4 % Nafion	100	4	**23**	50
6	Tetradecane	4.9 % Nafion	37	11	**13**	0

[a] Resin is damaged during mixing at 250 rpm

[b] The catalyst changed color during reaction

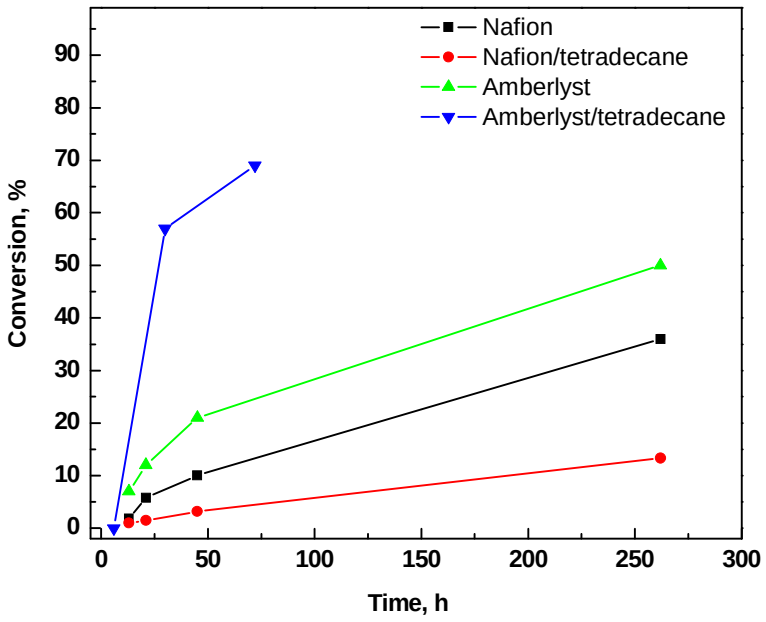


Figure 3.15. Conversion as a function of time in the reaction of decanoic acid with 1,3-propanediol at 37 °C (DA/PD = 2.5/1 with or without tetradecane as solvent; catalysts concentrations according to Table 3.6).

As one can see in **Figure 3.15**, Nafion appeared to be a poor catalyst in the reaction studied, yielding the lowest conversion of 13 % at 37 °C in tetradecane in 11 days. If the reaction was carried out in the solvent-free diffusion-improved conditions, 38 % conversion was obtained with Nafion compared to 50 % with Amberlyst-15. Surprisingly, if the reaction was carried out in tetradecane with Amberlyst-15 a higher conversion of 69 % was obtained in 72 h. Better activity of Amberlyst-15 resin compared to Nafion could be explained by higher concentration of acidic centers. Acidic capacity of Amberlyst catalysts is usually around 4.7 mmol g^{-1} compared to 1 mmol g^{-1} for Nafion [116]. The monoester product was formed preferably with these two catalysts. Selectivity towards mono-products was explained by the following

mechanism: 1,3-propanediol dissolves in the acidic aqueous layer formed on the surface of the resin, then esterification occurs and the mono-product migrates into the organic layer thus not undergoing further esterification [149].

Novozyme 435 lipase was tested next in the reaction of 1,3-propanediol esterification with decanoic acid. As shown in **Table 3.6**, the use of the lipase allows to achieve 100 % conversion in 30 h with predominantly diester product in decanoic acid as a solvent.

Table 3.6. Results obtained in the esterification of 1,3-propanediol with decanoic acid in tetradecane, catalyzed by Novozyme 435 at 37 °C in 30 h.

N	Solvent	Catalyst, % (w)	Conversion, %	Selectivity to diester, %
1[a]	Tetradecane	3.1	**83**	86
2[b]	Decanoic acid (10:1)	3.5	**100**	89

[a] 8 % (w) of water was added, the catalyst is damaged during mixing at 250 rpm

[b] 3.2 % (w) of water was added

A comparison of decanoic acid and tetradecane as solvents, see **Figure 3.16**, shows that the conversion in tetradecane amounts to 28 % after 10 h, which is twice lower than if decanoic acid is used. This is probably due to a lower solubility of 1,3-propanediol in the hydrophobic

tetradecane compared to that in decanoic acid. High amount of diester (90 %) at the beginning of the reaction in tetradecane reduces with time due to its hydrolysis catalysed by the lipase.

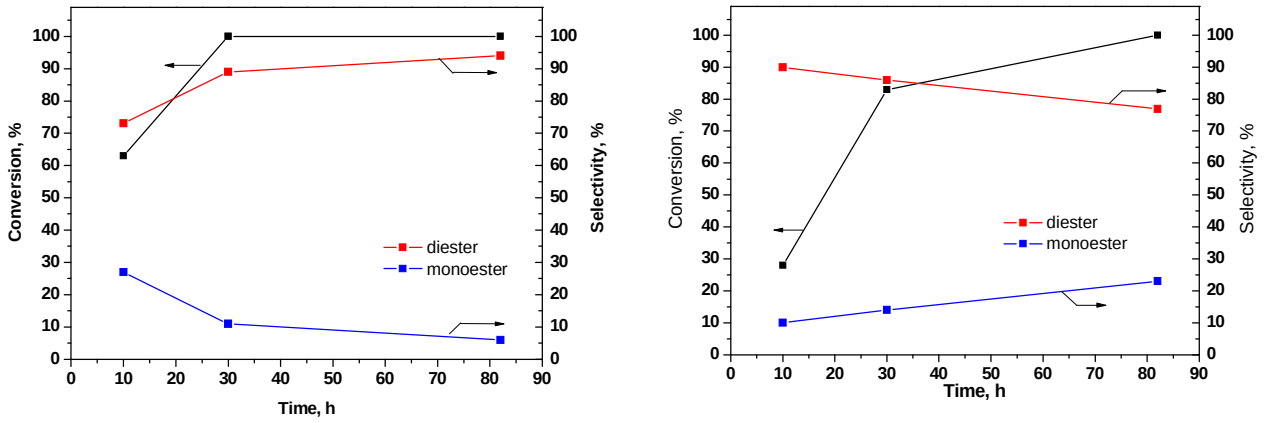


Figure 3.16. Conversion and selectivity as functions of time in the esterification of decanoic acid with 1,3-propanediol by Novozyme 435 at 37 °C (left – DA/PD = 10/1, no solvent, right – DA/PD = 2/1, tetradecane).

As a result we can conclude that self-condensation of 1,3-propanediol with decanoic acid is a very slow reaction at 37 °C (35 % conversion in 7 days) compared to 100 °C (96 % in 2 days). To sum up the results obtained in tetradecane as a solvent at **37 °C**, activity of all catalysts tested was compared in **Figure 3.17**. The Zr catalyst (1) does not show catalytic activity in this reaction. The reaction with Nafion yields only 2.2 % of monoester whereas Amberlyst-15 allows to obtain 57 % of monoester in 30 h. Novozyme 435 was found to be the best catalyst for the reaction of 1,3-propanediol esterification with decanoic acid in tetradecane at 37 °C giving 83 % conversion with diester being the main product.

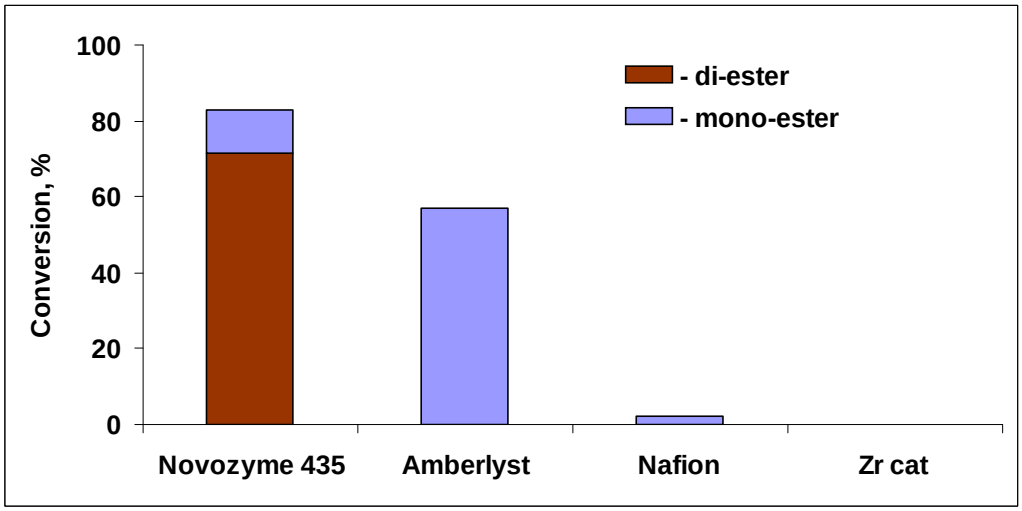


Figure 3.17. Activity of different catalysts in the esterification of decanoic acid with 1,3-propanediol in tetradecane at 37 °C (DA/PD= 2.5/1, 30 h).

3.3.2 1,3-Propanediol esterification with decanoic acid in biphasic system

Further investigation was carried out in a **biphasic** system, mimicking the aqueous solution/organic reaction medium. Amberlyst-15 and Novozyme 435 were studied in a two-phase system with tetradecane as a hydrophobic water immiscible solvent and a model aqueous solution of 1,3-propanediol, glycerol, acetic and butyric acids with concentrations close to those in the fermentative broth at the end of fermentation. The actual fermentation broth was also used in some reactions. Reactions were carried out in a flask under vigorous mixing. Results are summarized in **Table 3.7**.

Table 3.7. Results obtained in the esterification of 1,3-propanediol with decanoic acid in aqueous/tetradecane biphasic system, catalyzed by different catalysts at 37 °C.

N	System	Catalyst, % (w)	Time, h	Conv., %	Select. diester, %
1	Tetradecane + mixture	-	54	No product	
2[a]	Tetradecane + mixture	4 % A-15	72	No product	
3[b]	Tetradecane + mixture	3.2 % N-435	60	**4**	100
4[c]	Tetradecane + mixture	3 % N-435	60	**9**	91
5	Tetradecane + mixture	10 % N-435	54	**11**	100
6	Tetradecane + broth	10 % N-435	72	**11**	100
7	Tetradecane + (PD+Gly) solution	3 % N-435	54	**6**	100

[a] Amberlyst was damaged during mixing

[b] The lipase is on the boundary between phases, 400 rpm mixing

[c] Particles of acrylic resin were damaged during stirring at 750 rpm

Mixture: aqueous solution of 9 g L^{-1} PD, 1.5 g L^{-1} AA, 2.5 g L^{-1} BA, 1 g L^{-1} Gly, 0.5 g L^{-1} LacA. Broth: fermentative medium after fermentation was finished, containing 9.1 g L^{-1} PD,

1.7 g L^{-1} AA, 3.8 g L^{-1} BA, 0 g L^{-1} Gly, 0.04 g L^{-1} LacA determined by HPLC analysis. (PD + Gly) solution: aqueous solution of 5 g L^{-1} PD and 10 g L^{-1} Gly.

As one can see from **Table 3.7** the maximum conversion of 11 % was achieved with 100 % of diester when 10 % (w/w_{PD}) of Novozyem 435 lipase was used in the two-phase system under vigorous mixing at 37 °C. A lower 3 % load of the lipase leads to a lower conversion and selectivity. Total 4 % of conversion was obtained if only lower (aqueous) phase was stirred suggesting that diffusion limitations caused the decrease in conversion. Amberlyst-15 did not show any activity in the two-phase system, most likely due to its deactivation by water.

According to **Figure 3.18** a higher concentration of the lipase, 10 % (w/w_{PD}) compared to 3 %, enhances the initial rate of the reaction and leads to a higher conversion. Using the broth solution instead of the model solution shows no significant difference in the esterification reaction. If the aqueous solution of 1,3-propanediol and glycerol was used, 6 % conversion was achieved in 54 h time period and the lipase appeared to be selective towards 1,3-propanediol compared to glycerol.

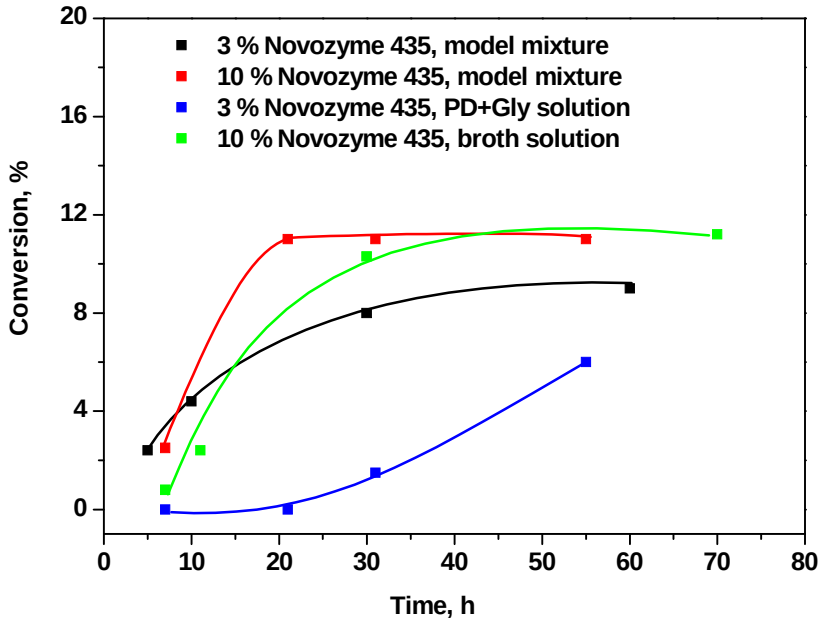


Figure 3.18. Conversion of decanoic acid and 1,3-propanediol condensation at 37 °C as a function of time for different aqueous solutions and concentrations of the lipase in tertradecane/aqueous biphasic medium.

Solubility of fatty acids depends on pH of aqueous solution: with an increase in pH the solubility of acids also increases. Therefore, when fermentation broth has pH close to 7 one would expect partial extraction of decanoic acid into aqueous solution, which was proven by mixing fermentation medium and a tetradecane solution of decanoic acid. Hence, more hydrophobic fatty acid should be used for 1,3-propandiol esterification to avoid acid extraction into the aqueous solution. Further in this work **linoleic acid** was investigated as the most suitable acidic reactant.

In conclusion one can say that the reaction of esterification of 1,3-propanediol with decanoic acid is a self-catalysed reaction and 66 % of conversion can be obtained at 100 °C in 5 h. A decrease in temperature from 100 °C to 37 °C leads to lowering the reaction rate significantly, with a maximum conversion of 35 % and 10 % diester selectivity in 7 days. Using decanoic acid as a solvent enhances the yield, probably due to the acidic properties of the acid itself and solubility of 1,3-propanediol in it, whereas using hydrophobic tetradecane does not allow to obtain any product at 37 °C without a catalyst. Among the catalysts tested, the Zr catalyst (1) did not show any activity in the reaction of esterification probably due to its deactivation. The total conversion of 69 % with 100 % of the monoester was obtained after 72 h if Amberlyst-15 was used as a catalyst in tetradecane at 37 °C. The highest activity was shown by the lipase Novozyme 435 supported onto acrylic resin.

In the two-phase system, only Novozyme 435 shows activity depending on the amount of the catalyst and a second aqueous phase, but no more than 11 % conversion of 1,3-propanediol could be obtained due to equilibrium limitation in the presence of water. The highest reaction rate was observed when 10 % (w/w_{PD}) of the lipase was used. Moreover, analysis of aqueous solution of 1,3-propanediol and glycerol showed 100 % selectivity of the lipase towards 1,3-propanediol esterification.

As a result it can be concluded that Novozyme 435 is the only active and selective catalyst among those tested in the esterification of 1,3-propanediol in a biphasic system at 37 °C.

3.3.3 CLEAs for esterification of 1,3-propanediol with linoleic acid

Linoleic acid was further used in the esterification of 1,3-propanediol instead of decanoic acid. Linoleic, C18 unsaturated, omega-6 fatty acid, being essential nutrient for all mammals, is non-toxic, liquid and soluble in many solvents. Reaction of linoleic acid esterification with 1,3-propanediol is shown in **Figure 3.19**.

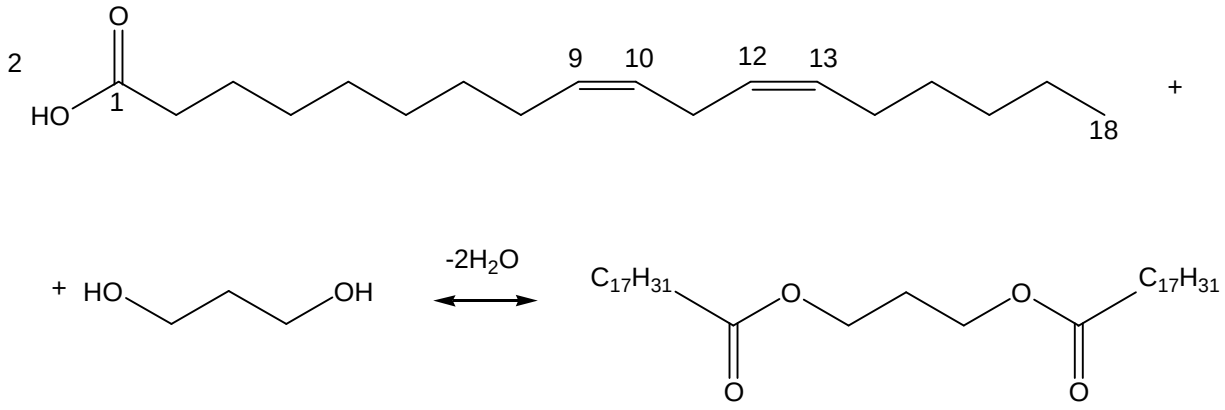


Figure 3.19. Esterification of linoleic acid and 1,3-propanediol. Monoester is not shown.

Novozyme 435 was found to be active in 1,3-propanediol esterification with decanoic acid in biphasic aqueous/tetradecane system, but during mixing significant damage of acrylic resin particles was observed due to their abrasion. CLEA enzymes are known to be stable over time [150, 151]. Moreover due to their specific structure they offer better activity in some processes [152]. Several CLEA enzymes (**Table 3.8)** were tested in the reaction of 1,3-propanediol esterification.

Table 3.8. Names and activities of the CLEA enzymes tested.

CLEA name	Activity, TBU g^{-1}
Rhizomucor miehei lipase	81,300
Candida antarctica lipase A	11,400
Alcalase	490 (AGEU g^{-1})
Candida antarctica lipase B	14,000
Thermomicea lanuginosus lipase	410,000

1 TBU catalyses the release of 1 μmol butyric acid per min from tributyrin (10 % v/v) in 25 mM phosphate buffer, pH = 7.5 and 40 °C

1 AGE unit catalyses the formation of 1 μmol N – acetylglycine per min

A comparison of conversions obtained with all CLEA enzymes studied is plotted in **Figure 3.20**. If the reaction was carried out in tetradecane with 0.12 M_{org} of 1,3-propanediol and 0.3 M_{org} of linoleic acid (2.5 times more than the diol) 88 % conversion of 1,3-propanediol could be obtained in 24 h catalysed by *R. miehei* and *T. lanuginosus* lipases. The reaction rate for *Lipase B* was slower, giving 91 % only in 100 h time. *Lipase A* was found to be less active yielding 15 % of the diester after 100 h. To compare CLEA enzymes with Novozyme 435 supported on acrylic resin, the result obtained for decanoic acid esterification with 1,3-propanediol in tetradecane at 37 °C was also plotted on the graph. As on can see, the profile of conversion obtained with Novozyme 435 lipase repeats the same one for *Lipase B* CLEA enzyme, resulting in a slightly higher conversion.

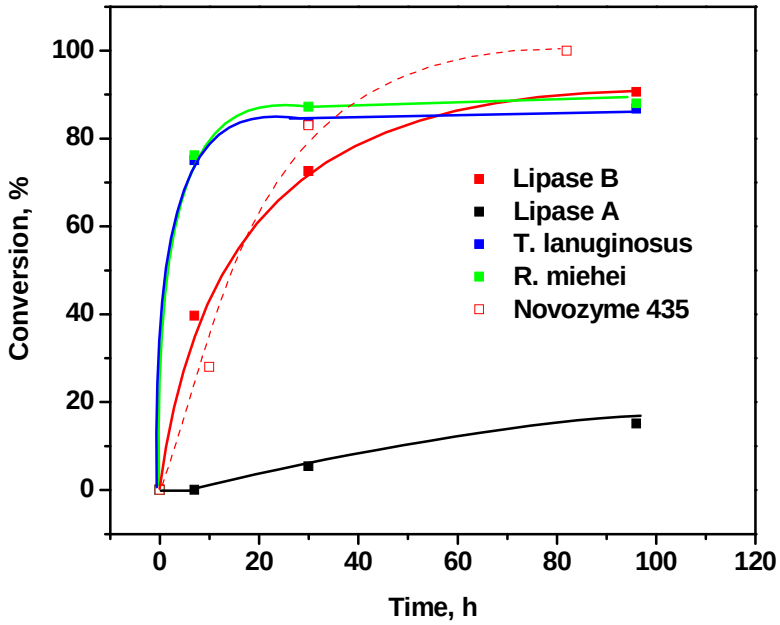


Figure 3.20. 1,3-Propanediol conversion in esterification with linoleic acid in tetradecane at 37 °C catalysed by different CLEA enzymes (filled squares) and Novozyme 435 (empty squares; for this reaction decanoic acid was used). Vial was charged with 0.027 g of PD, 10 % (w /w_{PD}) CLEA enzyme, 0.25 g linoleic acid and 3 mL tetradecane; LA/PD = 2.5/1.

To study the activity of CLEA enzymes in a two-phase system reactions were carried out between 1,3-propanediol dissolved in aqueous solution with glycerol, butyric and acetic acids and linoleic acid dissolved in tetradecane at 37 °C. As one can see in **Figure 3.21** *Lipase A* has the lowest activity, whereas for the rest of the enzymes conversion between 7.5-9 % can be achieved with *R. miehei* lipase being the most active. This is lower than the 11 % conversion obtained in the case of 1,3-propanediol esterification with decanoic acid catalysed by Novozyme 435. The shape of the curves shows clearly that the equilibrium was reached in 30 h from the beginning of the reaction. Probably, due to equilibrium limitations higher conversion cannot be obtained.

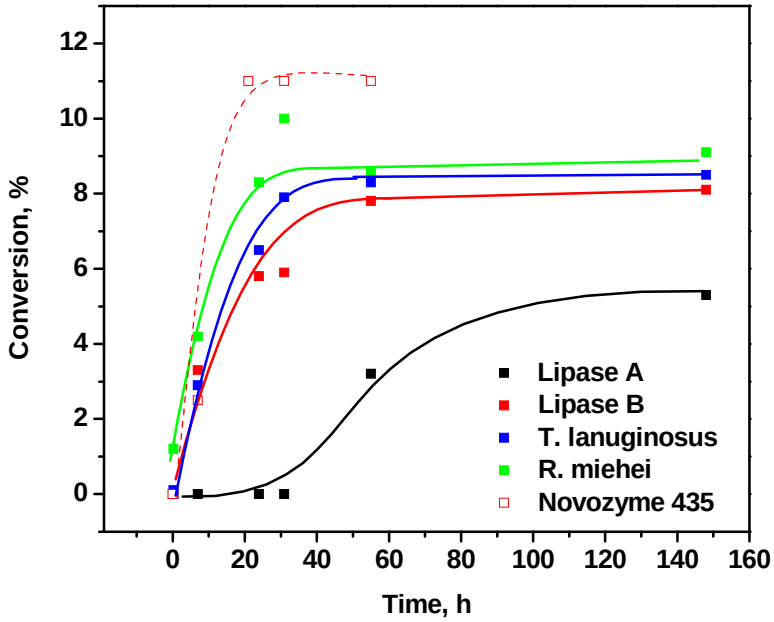


Figure 3.21. 1,3-Propanediol conversion in esterification with linoleic acid at 37 °C in a model aqueous mixture/tetradecane solution for different CLEA enzymes (filled squares) and Novozyme 435 (empty squares; for this reaction decanoic acid was used).

To conclude we can say that among all the CLEA enzymes tested in the reaction of 1,3-propanediol esterification with linoleic acid at 37 °C *Alcalase* showed no activity, *Lipase A* appeared to be a very poor catalyst, whereas *Lipase B*, *T. lanuginosus* and *R. miehei* allow to obtain high conversion of 1,3-propanediol in a single-phase system with the highest conversion of 88 % for *R. mihei* achieved in 24 h. In the two-phase system no more than 8-11 % of conversion can be obtained with either of enzymes due to equilibrium limitations in the presence of water. The highest reaction rate was observed for *R. miehei* lipase.

3.3.4 Enzymatic biphasic esterification: influence of different parameters

As it was shown in the previous chapter, among the different enzymes tested, namely *Alcalase*, *lipase A*, *lipase B*, *T. lanuginosus* and *R. miehei*, the last two were the most active in the two-phase system in the esterification of linoleic acid with 1,3-propanediol dissolved in aqueous solution, but no more than *ca* 10 % conversion was obtained. Different parameters, such as acid/diol ratio, their initial concentrations, temperature, different aqueous solutions, enzyme concentration and the nature of the solvent were varied to investigate the reaction of esterification to obtain the highest yield and reaction rate. In most experiments free *R. miehei* lipase (>20,000 U g^{-1}) was used.

Effect of temperature

The effect of reaction temperature on the activity of free *R. miehei* is shown in **Figure 3.22**. The reaction rates and conversions at 37 and 25 °C are only slightly different, whereas an increase in temperature to 50 °C seems to lead to the enzyme inactivation. Therefore only 2.5 % of the product was obtained, which is almost 3 times lower compared to that at 37 °C.

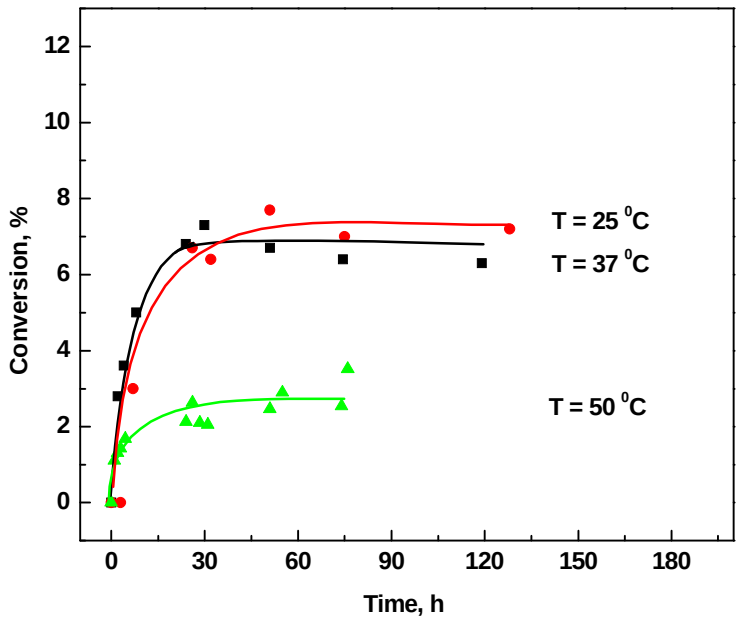


Figure 3.22. Esterification profiles obtained in the tetradecane/fermentation broth system at different temperatures (PD/LA = 1/2.5, V_{org} = V_{aq} = 3 ml, C_{PD} = 0.12 M_{aq}, 20 µl *R. miehei* free).

Lipases are usually used at temperatures 30-50 °C in the reactions of esterification [129, 153-155] and as a rule, an increase in reaction temperature leads to the increase in enzyme activity [156, 157]. In some cases maximum temperatures reported are as high as 65-70 °C [157], although Distel *et al* observed denaturation of commercial Novozyme 435 at 55 °C [138]. *R. miehei* in its immobilized form, Lypozim IM-20, was found to be stable up to 55 °C in the reaction of isoamyl alcohol and isovaleric acid esterification in *n*-hexane [158]. In the work by Vaysse *Mucor miehei* lipase was used in the esterification of fatty acids in a biphasic system at a temperature as high as 50 °C without any thermal inactivation of the enzyme [159]. Generally, in each specific case the maximum operating temperature should be investigated

individually because such parameters as enzyme form (native or immobilized), organic solvent, pH and composition of the aqueous phase could effect the lipase's thermal stability as was shown by Noel *et al* [160]. In the case reported in this study, the complex fermentation medium used as a second aqueous phase being saturated with different salts and nutrients could cause lowering the thermal stability of free *R. miehei* at 50 °C.

Effect of aqueous solution nature

Figure 3.23 shows that activity of *R. miehei* lipase depends on pH and composition of the aqueous solution used as a second phase in the two-phase system with tetradecane as an organic hydrophobic solvent. Both the high and low pH (6.4 or 2.8) of the aqueous solution suppress enzyme activity as the low reaction rates and conversions of 4 and 5.7 % were obtained in pure distilled water and the aqueous acidic solution of 1,3-propanediol, glycerol, acetic, butyric and lactic acids.

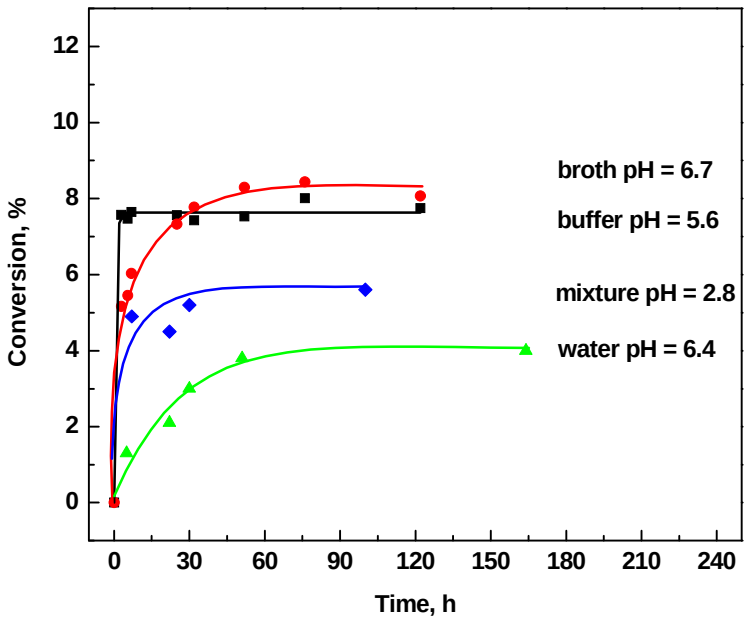


Figure 3.23. Esterification profiles obained in a two-phase system with different aqueous solutions: broth after completed fermentation, KPi buffer, aqueous mixture of 1,3-propanediol, glycerol, acetic and butyric acids and distilled water (PD/LA = 1/2, V_{org} = V_{aq} = 3 ml, C_{PD} = 0.12 M_{aq}, 20 µL *R. miehei* free, 37 °C).

The highest reaction rate was observed when the phosphate buffer with pH = 5.6 was used, suggesting an optimal pH for *R. miehei* lipase. Oliviera *et al* also reported the maximum activity of free *R. miehei* at pH = 5.6 of the buffer solution, when used in a biphasic system in the esterification of oleic acid and ethanol [77]. Similarly, the enzyme activity decreased with the increase in pH. One might expect that different optimal pH values would exist for the different lipases. It was found, for example, that *C. rugosa* lipase showed maximum activity in the reaction of esterification of fatty acids with ethanol at pH = 6.5 [161], whereas *R. delemar* was most active at pH = 6.0 in the reaction of oleic acid and octanol esterification [162]. In both cases the lower activities at both the higher and the lower than optimum pH values were

observed. This sharp change was explained by the influence of proton activity of aqueous solution on enzyme conformation, protonation or deprotonation of aminoacids in the active site of enzymes in particular. However, it was hypothesized that pKa of acidic substrate is also important as only protonated forms of acids are the actual susbtrates for enzymes [163].

Surprisingly, the use of the broth solution with a relatively high pH = 6.7, close to that of distilled water, showed a lower reaction rate but somewhat higher conversion of 8.4 % compared to that obtained in the KPi buffer.

It is possible that the enzyme conformation is effected not by pH of the aqueous solution, but also, and perhaps even more importantly, by its saline composition. In this case the fermentation broth saturated with different inorganic salts appeared to be less inhibitory for the enzyme than distilled water. This can be explained by the increased enzyme stability caused by salts dissolved in the buffer solution and broth used as a second aqueous phase. The same effect was shown by Krishna *et al* when an increased stability of the immobilized *R. miehei* towards substrate inhibition was observed when heptane was saturated with a buffer solution prior to use [164].

If we take into account that fermentation broth contains about 3.5 g L^{-1} of butyric acid, we can assume that the lipase can be active towards this acid. However, HPLC analysis showed that no esterification of butyric acid with 1,3-propanediol occurred. Indeed, it was shown in a study of esterification of the different length-chain fatty acids, that due to the solvation of short-chain fatty acids their reactivity is lowered [159].

T. lanuginosus lipase showed higher tolerance towards high pH of distilled water giving about 6 % conversion compared to the activity of *R. miehei*, which drastically decreases if distilled water was used as an aqueous phase (**Figure 3.24**).

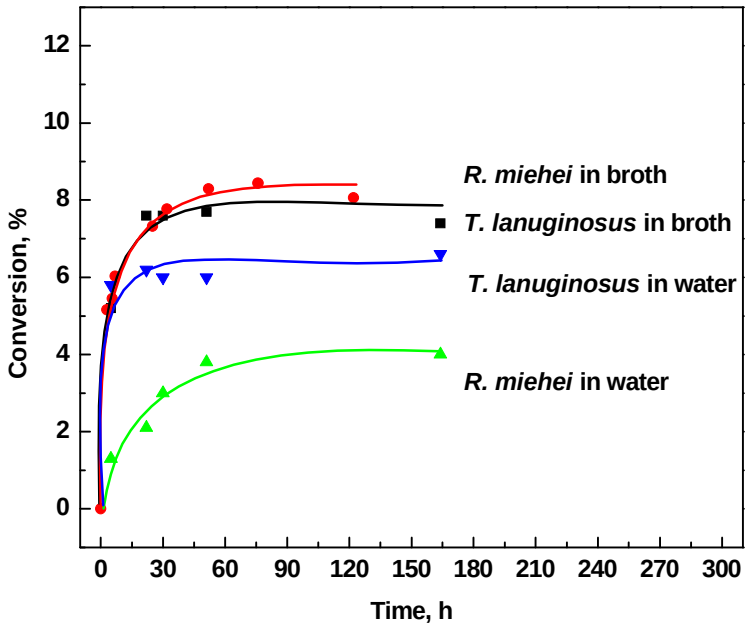


Figure 3.24. Esterification profiles obtained in the tetradecane/(fermentation broth or distilled water) system with *R. miehei* and *T. lanuginosus* lipases (PD/LA = 1/2, V_{org} = V_{aq} = 3 ml, C_{PD} = 0.12 M_{aq}, 20 µL of the free enzyme, 37 °C).

To study whether tetradecane or tetradecane/aqueous solutions inhibit *R. miehei* activity, the enzyme was pre-equilibrated for 36 h in different solutions prior to the reaction. As one can see in **Figure 3.25** that when the enzyme was pre-stabilized in tetradecane with linoleic acid or tetradecane/buffer solution of 1,3-propanediol for several hours, lower conversions were detected. Furthermore, in the case of 1,3-propanediol dissolved in the buffer solution, a much

lower reaction rate was also observed, proving strong inhibitory affect of 1,3-propanediol. The enzyme remains active after being pre-stabilized for 36 h in a two-phase tetradecane/buffer medium (without 1,3-propanediol). Thus, presumably, the enzyme conformation is not affected after prolonged mixing in the dispersed organic/water system and even seems to be better stabilized compared to the pure hydrophobic tetradecane/linoleic acid solution.

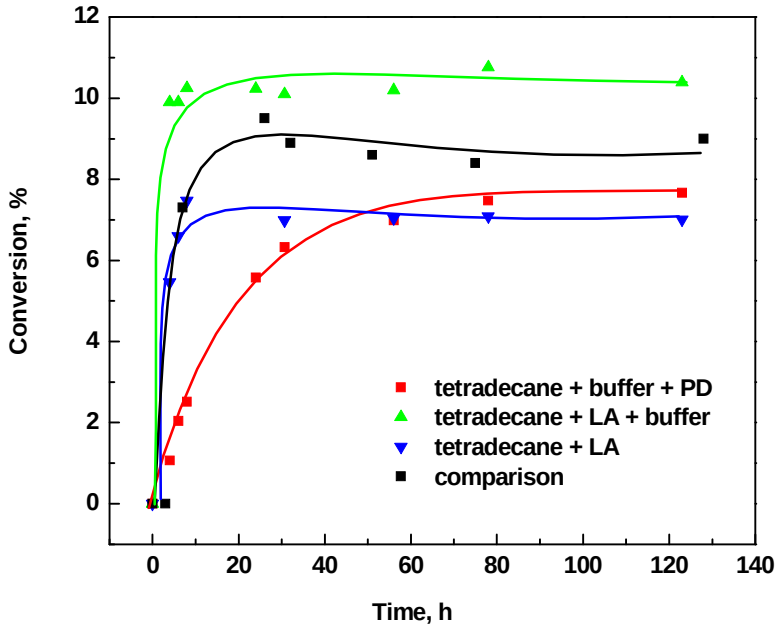


Figure 3.25. Esterification profiles obtained in the tetradecane/fermentation broth system after the enzyme was stabilized for 36 h in different mixtures prior to the reaction (PD/LA = 1/2.5, $V_{org} = V_{aq} = 3$ ml, $C_{PD} = 0.12$ M_{aq}, , 20 µL *R. miehei* free, 37 °C).

Effect of enzyme concentration

One of the most important parameters that needs to be examined is enzyme concentration. **Figure 3.26** shows that if a twice higher than 72,000 U L^{-1}_{aq} concentration of *R. miehei* lipase was taken, the higher conversion and reaction rate can be achieved. Instead of 9 % in 30 h in

the case of 72,000 U L^{-1}_{aq} the equilibrium can be reached in 15 h time with 10 % conversion. A further increase in the enzyme concentration does not enhance the reaction rate. The dependence of the initial reaction rate on the enzyme concentration is shown in **Figure 3.27**.

It is very difficult to compare the results obtained with others, reported previously in the literature, as enzyme concentration could be expressed in different units such as g L^{-1}, U L^{-1}, % wt, and the information of the initial enzyme activity (U g^{-1}) is not always given. Such differences in presentation of the results were pointed out by Foresti [165]. Nevertheless, the same effect of independence of the initial reaction rate on the enzyme concentration after some optimum level (**Figure 3.27**) was reported by different authors [130, 154, 155, 157, 166]. Enzyme agglomeration was proposed as a possible mechanism explaining this phenomenon, studied in the reaction of oleic acid with ethanol catalysed by Novozyme 435 with high enzyme concentration of 10 % wt, corresponding to 700,000 U L^{-1} [157].

The higher enzyme load cause an increase in the observed rate of the reverse reaction, see **Figure 3.26**. This is due to higher availability the enzyme active sites for all the substrates (esterification and hydrolysis). If we compare CLEA and the native *R. miehei* one can see that CLEA lipase showed the same activity with no visible effect on the reverse de-esterification reaction. As free enzyme cannot be reused in batch reactors, the reusable CLEA, which are known to have higher sability, being though more expensive [150, 152], can be considered as an appropriate choice for this reaction.

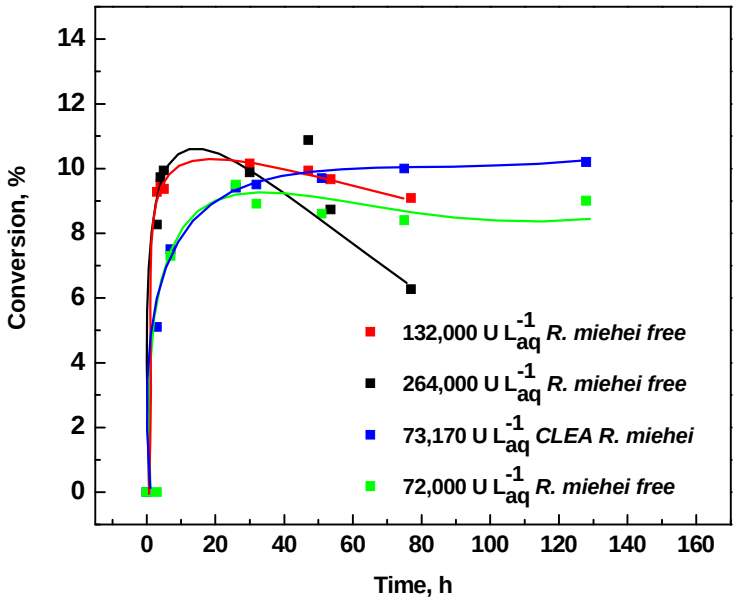


Figure 3.26. Esterification profiles obtained in the tetradecane/fermentation broth system with different concentrations of *R. miehei* (PD/LA = 1/2.5, V_{org} = V_{aq} = 3 ml, C_{PD} = 0.12 M_{aq}).

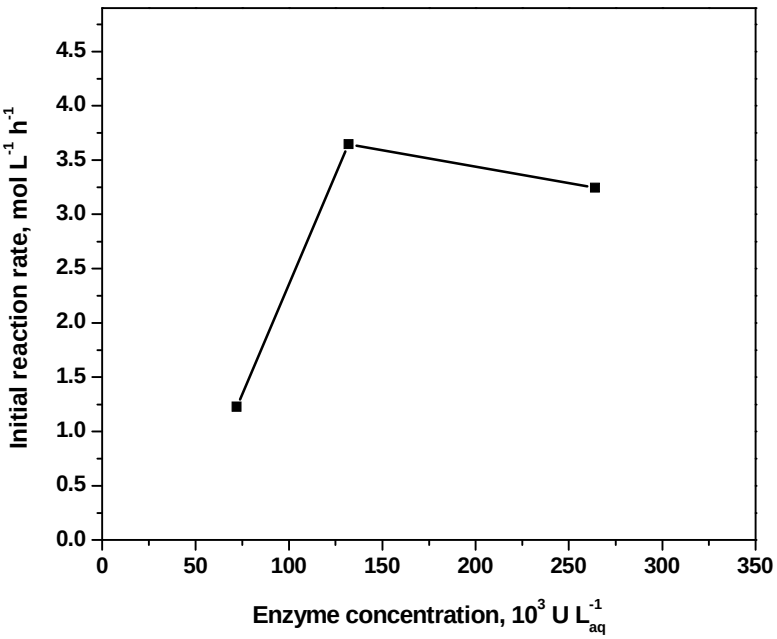


Figure 3.27. Dependence of the initial reaction rate on the enzyme concentration in biphasic esterification of 1,3-propanediol with linoleic acid.

Effect of the initial substrate concentration

Figure 3.28 shows that if a 10-fold higher initial concentration of the substrates was taken (1 M of 1,3-propanediol and 2 M of linoleic acid) then a 4 times higher conversion could be obtained (28 % compared to 7 %). To study how initial concentration of substrates influences the yield, the reaction of 1,3-propaneidol esterification with linoleic acid was carried out at different concentrations of one substrate when another substrate was fixed at the saturation level.

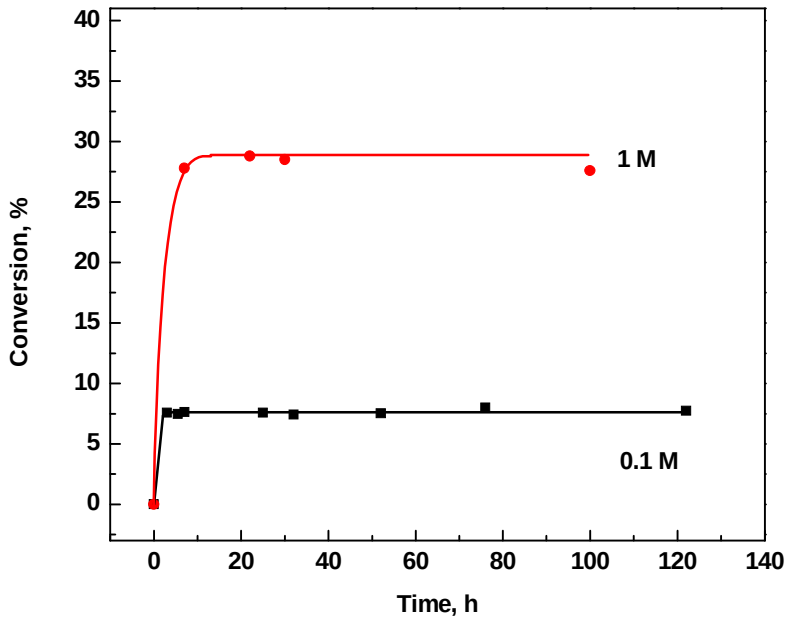


Figure 3.28. Esterification profiles obtained in the tetradecane/phosphate buffer system at 0.1 M_{aq} and 1 M_{aq} concentrations of 1,3-propanediol (PD/LA = 1/2, V_{org} = V_{aq} = 3 ml, 20 µl *R. miehei* free, 37 °C).

The linoleic acid/diol ratio was varied between ½ and 10 at the initial concentration of 1,3-propanediol fixed at 0.12 M. **Figure 3.29** shows that the higher ratio, and therefore higher initial concentration of linoleic acid, leads to the higher conversions: when a 10-fold excess of linoleic acid was taken, 20 % conversion was obtained, which is twice higher compared to the result obtained when the stoichiometric ratio was used.

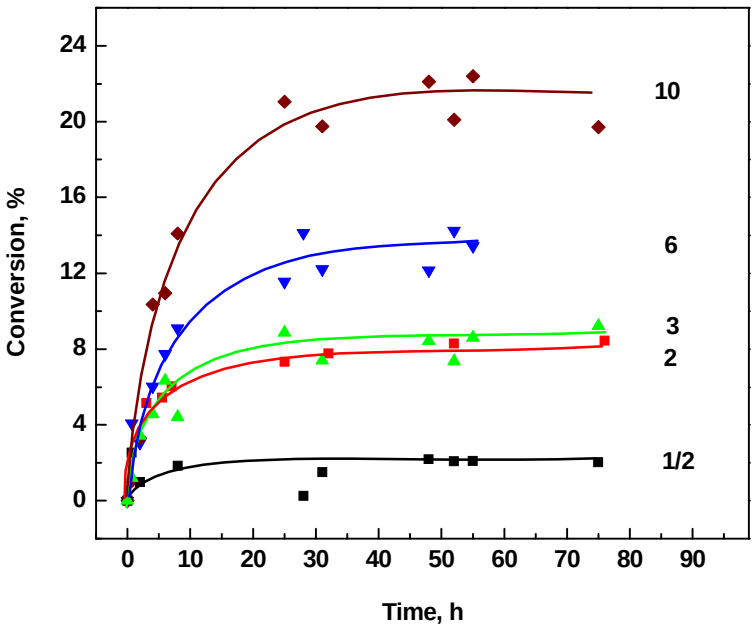


Figure 3.29. Esterification profiles obtained in the tetradecane/fermentation broth system at fixed C_{PD} = 0.12 M_{aq} and different LA/PD ratios: 1/2, 2, 3, 6, 10 (V_{org} = V_{aq} = 3 ml, 20 µL *R. miehei* free, 37 °C).

If the initial reaction rate is plotted against the initial concentration of linoleic acid, see **Figure 3.30**, the dependence exhibits an initial increase, leveling off at about 0.6 M and subsequently sharply increasing further at higher concentration of linoleic acid. This behaviour was also observed by Kraai *et al* for the case of esterification of oleic acid with 1-butanol in a biphasic system [132], and, to our opinion, can be explained by the changes in the physical properties of the reaction medium, which becomes similar to that of the solvent-less environment when concentration of linoleic acid increases. Also at high concentrations of linoleic acid, which is a good emulsifier, the interfacial area could increase, thus leading to higher reaction rates.

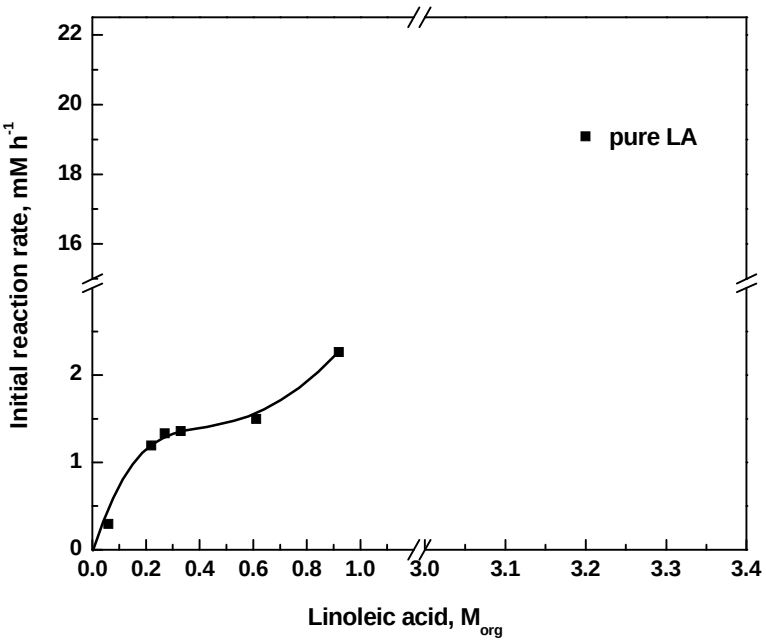


Figure 3.30. Dependence of the initial reaction rate on linoleic acid concentration in the tetradecane/fermentation broth system with *R. miehei*.

In our study linoleic acid was found to be a non-inhibitory substrate up to concentrations of 3.2 M. Oliveira *et al* reported inhibition of free *R. miehei* by oleic acid at concentrations above 1.25 M, although the reaction of its esterification with ethanol, which can be an inhibitor itself, was carried out in a biphasic system with pure oleic acid as a second phase [77]. Fatty acids are weak acids and have log P (log P = 5 for linoleic acid) higher than the boundary hydrophobicity (log P ~ 4) reported for enzymes to affect their activity. Janssen noticed inhibition of *C. rugosa* lipase by 0.5 M of butanoic acid and 1 M of octanoic and decanoic acids in the reaction of esterification with sulcatol in toluene, whereas no inhibitory effect was observed for the fatty acids with longer chains [167]. Janssen also found that K_m increases with the increasing of the fatty acid chain length, suggesting better binding of the short-chain

fatty acids to enzyme compared to the long-chain fatty acids. Contrary, Vaysse reported lower initial reaction rates for the short-chain fatty acids (C_8-C_{10}) compared to long-chain fatty acids (C_{12}-C_{18}) in the esterification with methanol [159].

A dependence of conversion on initial concentration of 1,3-propanediol in the aqueous phase is shown in **Figure 3.31**. An increase in 1,3-propanediol concentration from 0.13 M to 2 M leads to the decrease in the conversion from 10 % to 4 %, suggesting an inhibitory effect of the diol substrate.

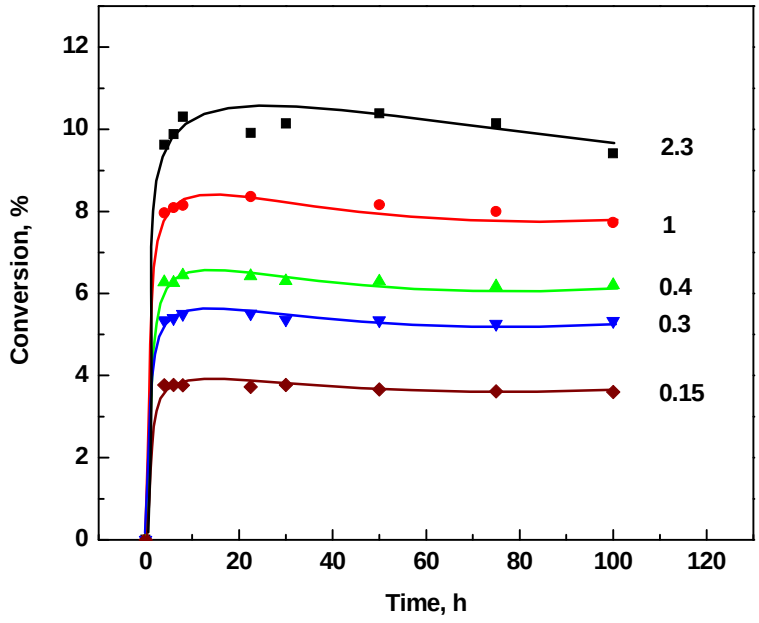


Figure 3.31. Esterification profiles in the tetradecane/fermentation broth system at fixed C_{LA} = 0.3 M_{org} and different LA/PD ratio: 0.15, 0.3, 0.4, 1, 2.3 (V_{org} = V_{aq} = 5 ml, 33 µL *R. miehei* free, 37 °C).

A dependence of the initial reaction rate on the initial concentration of 1,3-propanediol in the aqueous phase is shown in **Figure 3.32**. The observed reaction rate decreases drastically with the increase in the initial concentration of the diol substrate. The inhibitory effect of alcohols, caused by the formation of inactive alcohol-enzyme complex, was observed in many studies of lipase-catalyzed esterification [126, 134, 157, 166, 168]. However, for example, a slight increase in conversion with increasing of ethylene glycol concentration from 0.08 M to 2.7 M in the reaction of esterification with stearic acid in hexane by immobilized *R. miehei* was also reported [169]. It was even claimed that polyhydric alcohols, such as glycerol and ethylene glycol, may be used as effective protective agents against protein deactivation in the case of *R. miehei* lipase [160].

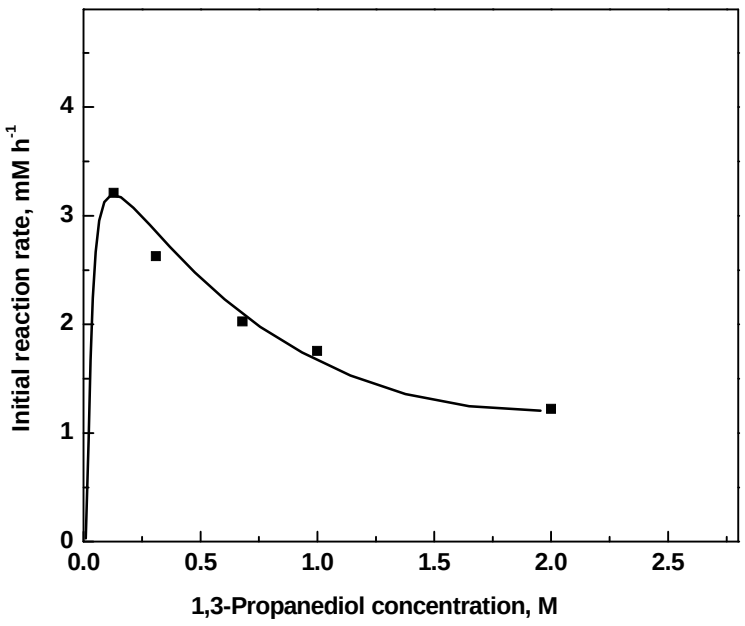


Figure 3.32. Dependence of the initial reaction rate on 1,3-propanediol concentration in the tetradecane/fermentation broth system with *R. miehei*.

The reaction of esterification between acid and diol is believed to follow Bi-Bi Ping-Pong mechanism (**Figure 3.33**) [131, 134, 170, 171]. According to this mechanism an acid first binds to an enzyme forming E-A complex, which eliminates water as the first product P and transforming to the new active enzymatic complex F, which then binds to the alcohol B, resulting in the formation of an F-B complex which in the end gives the ester product Q. This mechanism is also known as substituted-enzyme mechanism [172].

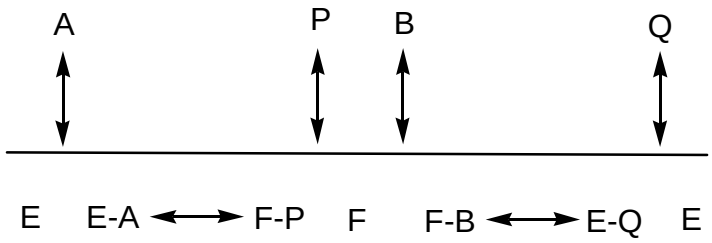


Figure 3.33. Bi-Bi Ping-Pong enzymatic mechanism.

Oliviera *et al* suggested that the reaction is described by the ternary-complex mechanism given in **Figure 3.34** [173]. In this case an acid and an alcohol first bind to an enzyme forming the E-A-B complex, which after transformation changes to the E-P-Q complex which consequently ejects both products. Depending on whether the acid or the alcohol first or both randomly bind to an enzyme, two different types of the mechanism are distinguished: compulsory-order and random-order.

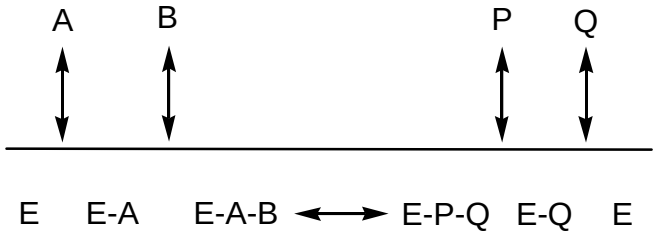


Figure 3.34. Ternary-complex enzymatic mechanism.

Two different equations correspond to the Bi-Bi Ping-Pong and the ternary-complex mechanisms:

$$V = \frac{V_{\max}[A][B]}{[A][B] + K_M^B[A] + K_M^A[B]} \qquad \text{Bi-Bi Ping-Pong} \qquad (1)$$

$$V = \frac{V_{\max}[A][B]}{[A][B] + K_M^B[A] + K_M^A[B] + K_i^A K_M^B} \qquad \text{ternary-complex} \qquad (2)$$

where V is the reaction rate, $V_{\max}$ is the maximum reaction rate ($V_{\max} = k_{enz} \cdot C_{enz}$), $[A]$, $[B]$ are the concentrations of acid and alcohol, K_M^A, K_M^B are the Michaelis constants for A, B when all others substrates are at saturation, K_i^A is the equilibrium dissociation constant of E-A complex.

Considering the Bi-Bi Ping-Pong mechanism (inhibition may also occur for the ternary-complex mechanism but is not considered here), if one or both substrates bind to the enzyme forming inactive, so called dead-end complex, then equation (1) should be rewritten as:

$$V = \frac{V_{\max}[A][B]}{[A][B] + K_M^B[A]\left(1 + \frac{[A]}{K_i^A}\right) + K_M^A[B]\left(1 + \frac{[B]}{K_i^B}\right)}, \qquad (3)$$

where K_i^A, K_i^B are the inhibition constants for acid and alcohol.

In our case only inhibition by alcohol was detected; hence the resulting equation is:

$$V = \frac{V_{\max}[A][B]}{[A][B] + K_M^B[A] + K_M^A[B]\left(1 + \frac{[B]}{K_i^B}\right)}. \quad (4)$$

Rearranging equation (4) leads to equation (5):

$$V = \frac{V_{\max}}{\left(1 + \frac{K_M^A}{[A]}\right) + \frac{K_M^A}{[A]K_i^B}[B] + \frac{K_M^B}{[B]}}. \quad (5)$$

Experimental data can be easily used to determine K_M^A at fixed [A] when equation (5) simplifies to Michaelis-Menten equation. When inhibition is involved, as with substrate B, equation (5) becomes parabolic, and can be resolved only with a broad range of experimental data. For that at least 25 experiments need to be carried out. In our case there is not enough experimental data to determine the kinetic parameters with high accuracy.

Effect of water content in the two-phase reaction

If we compare conversion profiles obtained for the different initial percentage (v/v) of the aqueous phase shown in **Figure 3.35**, an increase in conversion and a decrease in reaction rate can be observed with the decrease in water content in the reaction medium. Thus, at 20 % of the aqueous phase a 26 % conversion can be achieved but in 150 h. When 50 % of water is present, only 12 % conversion is attained in 75 h. No changes in the conversion or rate were observed if water content was increased to 70 %.

The observed increase in the reaction rate can be explained by the increase in the interface area between the two phases due to better emulsion formation at a higher water content and

therefore better interface activation of *R. miehei*, which is known to have a special "lid" structure that can be activated at oil/water interface [174].

At the same time, lowering the water content favours a shift in the equilibrium towards product formation, thus higher conversions can be obtained (**Figure 3.35**). Water concentrations of *ca* 20-30 % allow conversions of 20-25 % with low 0.1 M initial concentration of 1,3-propanediol to be achieved. However, other approaches such as using emulsifiers or vigorous stirring should be applied to increase the contact area between the two phases to increase reaction rates *via* this mechanism. These conclusions are supported by the literature data: a low conversion but higher initial reaction rates were observed at high water concentration in *n*-hexane/water system in the reaction of oleic acid and ethanol esterification by *M. miehei* or *R. niveus* [129].

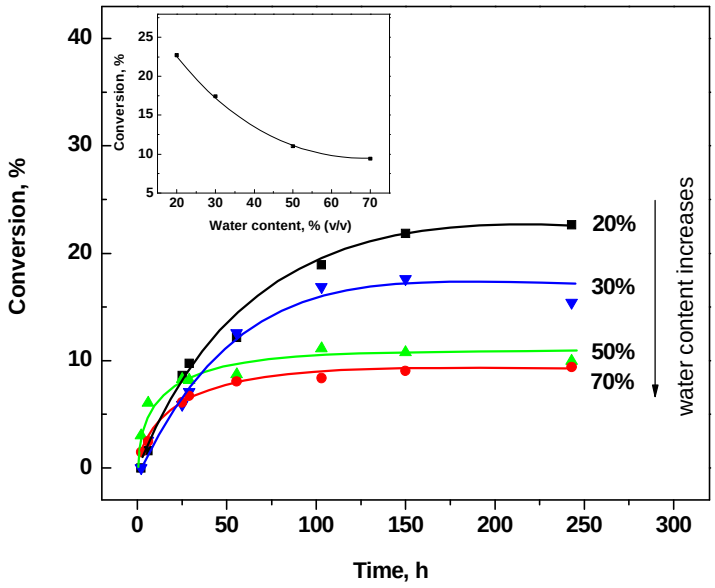


Figure 3.35. Esterification profiles in the tetradecane/fermentation broth system at different concentrations of water (V_{org} = V_{aq} = 3 ml, C_{PD} = 0.12 M_{aq}, 20 µL *R. miehei* free, 37 °C).

It is useful to examine literature on enzymatic esterification reactions in biphasic systems in general and on the activity of *R. miehei* in particular. A maximum 77 % conversion in 4 h was obtained in the reaction of esterification of a fatty acids mixture with an equimolar amount of ethanol by aqueous solution of Novozyme 435 lipase (water content 46 % v/v) at 50 °C whereas free *C. rugosa* showed a lower activity, with 30-40 % conversion [161]. Foresti *et al* reported the highest among some lipases tested conversion of 70-80 % in the esterification between the stoichiometric amounts of oleic acid and ethanol by aqueous solution of native *C. antarctica B* (water content 14 % v/v) at 45 °C in 7 h time [175]. In this work the authors suggested that water favors reaction rate and that equilibrium shifts towards the ester formation due to extraction of the formed water into the aqueous phase. Kraai *et al* obtained

100 % conversion in the esterification between 0.6 M_{org} oleic acid and 0.94 M_{org} butanol in n-heptane in 20 min by 0.2 g L_{aq} of *Rhizimucor miehei* lipase [132]. Maximum equilibrium conversion of 57 % was achieved by Oliviera *et al* in the reaction between oleic acid (1.55 M) and buffer solution of ethanol (0.64 M) (water content 50 % v/v) at 30 °C catalyzed by *Rhizimucor miehei* lipase immobilized onto Accurel EP700 in different types of reactors [176]. Znidarsic-Plazl demonstrated a 35 % conversion obtained in 36.5 s in a laminar flow microreactor in the biphasic n-hexane/aqueous esterification of the stoichiometric amounts of isoamyl alcohol and acetic acid (water content 50 % v/v) at 45 °C by *C. antarctica* lipase B [177]. About 60 % conversion in the reaction of esterification of oleic acid with n-butanol catalyzed by *C. rugosa* was reported by Shin when the reaction was carried out in a buffer aqueous solution at 37 °C for 8 h between 0.1 M of each substrate (water content is 97 % v/v) [130]. If an emulsifier was used the conversion reached 80 % due to, as the author suggested, "enhancement of the accessibility of oleic acid with lipase and shifts equilibrium to high level" because of better emulsion formation. The apparent K_m increased if an emulsifier was used, suggesting a decreasing binding affinity between the lipase and oleic acid, whereas V_{max} also increased, caused by the increase in the interfacial surface and therefore leading to higher conversion.

As one can see, there is a significant discrepancy within the reported results for biphasic systems with water content as high as 50 % v/v even for the same esterification reactions and usually no clear explanation is provided to explain the effect of water on equilibrium.

The influence of water in biphasic systems in many cases is more complicated compared to the micro-aqueous systems and determined not only by equilibrium limitations, but also by the properties of the organic solvent used (or the fatty acid used in the solvent-free systems), partitioning substrates between the two phases and their inhibitory effect on the enzyme used.

Even more importantly, as was pointed out by Wehtje *et al*, the apparent V_{max} and K_m should be determined and compared for different systems instead of substrate- and enzyme-concentration-dependant initial activities [131]. Wehtje found out for esterification reactions by different enzymes, that with an increase in water content ($0 < a_w < 1$) K_m for the acid substrate showed only a slight increase but K_m for the alcohol increased 10-20-fold due to the increasing competition of water as a nucleophile to bind to the acyl-enzyme complex.

To understand the influence of water on conversion in biphasic aqueous/organic system we shall consider thermodynamic equilibrium in more details in the next chapter.

Thermodynamic equilibrium in biphasic aqueous-organic enzymatic synthesis

When an enzymatic reaction is carried out in biphasic aqueous-organic system it is believed that the reaction takes place in the aqueous phase, whereas all reactants and products are partitioned between the two phases (**Figure 3.36**).

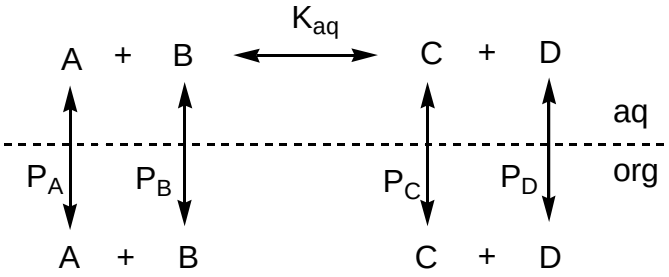


Figure 3.36. Bimolecular reaction in biphasic aqueous-organic system.

Apparent equilibrium constant for the biphasic system can be written as [178]:

$$K_{biphasic}^{ap} = \frac{C_C^{total} C_D^{total}}{C_A^{total} C_B^{total}} = \frac{\frac{(C_C^{aq} V_{aq} + C_C^{org} V_{org})}{V_{aq} + V_{org}} \cdot \frac{(C_D^{aq} V_{aq} + C_D^{org} V_{org})}{V_{aq} + V_{org}}}{\frac{(C_A^{aq} V_{aq} + C_A^{org} V_{org})}{V_{aq} + V_{org}} \cdot \frac{(C_B^{aq} V_{aq} + C_B^{org} V_{org})}{V_{aq} + V_{org}}} =$$

$$= \frac{C_C^{aq} C_D^{aq}}{C_A^{aq} C_B^{aq}} \cdot \frac{(1 + \frac{C_C^{org}}{C_C^{aq}} \cdot \frac{V_{org}}{V_{aq}}) \cdot (1 + \frac{C_D^{org}}{C_D^{aq}} \cdot \frac{V_{org}}{V_{aq}})}{(1 + \frac{C_A^{org}}{C_A^{aq}} \cdot \frac{V_{org}}{V_{aq}}) \cdot (1 + \frac{C_B^{org}}{C_B^{aq}} \cdot \frac{V_{org}}{V_{aq}})} = K_{aq} \cdot \frac{(1 + \alpha P_C) \cdot (1 + \alpha P_D)}{(1 + \alpha P_A) \cdot (1 + \alpha P_B)} = K_{aq} m$$

,

where $m = \frac{(1 + \alpha P_C) \cdot (1 + \alpha P_D)}{(1 + \alpha P_A) \cdot (1 + \alpha P_B)}$, $P_i = \frac{C_i^{org}}{C_i^{aq}}$ is partition coefficient, $\alpha = \frac{V_{org}}{V_{aq}}$ is phase volume ratio. For the reactions of esterification one of the product is water, therefore:

$m = \frac{(1 + \alpha P_{ester}) \cdot (1 + \alpha)}{(1 + \alpha P_{acid}) \cdot (1 + \alpha P_{alc})}$, if suggest that $C_{water}^{total} = C_{water}^{aq}$ in a highly hydrophobic solvent.

On the other hand the equilibrium constant can be re-written *via* fractional conversion:

$$K_{biphasic}^{ap} = \frac{\xi^2}{(n_A^0 - \xi) \cdot (n_B^0 - \xi)} = K_{aq} m \ , \qquad (6)$$

where n_A^0, n_B^0 are the initial numbers of moles of reactants A and B, ξ is fractional conversion.

Resolving the equation $(\frac{1}{K_{aq} m} - 1)\xi^2 + (n_A^0 + n_B^0)\xi - n_A^0 n_B^0 = 0$ (7)

we can obtain $\xi = n_A^0 \frac{(1+S) - \sqrt{(1-S)^2 + \frac{4S}{K_{aq}m}}}{2(1-\frac{1}{K_{aq}m})}$, where $S = \frac{n_B^0}{n_A^0}$. (8)

Then conversion of the reaction can be determined as:

$$x = \frac{(1+S) - \sqrt{(1-S)^2 + \frac{4S}{K_{aq}m}}}{2(1-\frac{1}{K_{aq}m})} \quad . \tag{9}$$

The equation (9) was developed in [179, 180]. Thus, theoretical conversion can be estimated, if the equilibrium constant and partition coefficients for reactants and products are known.

If we take S = 1, *i.e.* stoichiometric ratio of initial reactants, equation (9) can be simplified:

$$x = \frac{1}{1+\sqrt{\frac{1}{K_{aq}m}}}. \tag{10}$$

Using the developed equations we can investigate how conversion depends on partition coefficients of substrates and products and on K_{aq}. We will make a broad assumption that the ester product has the same partition coefficient as one of the substrates, for example, when a highly hydrophobic long-chain fatty acid is used, we might suggest that the ester product will be also highly hydrophobic. In this case *m* will depend only on the partition coefficient of the remained substrate:

$$m = \frac{1+\alpha}{1+\alpha P_{subs}}. \tag{11}$$

It appeares that if we assume $P_{ester} = P_{acid}$ the high solubility of an alcohol (low $P_{alcohol}$ values) in the aqueous phase favours formation of the product as it can be seen in **Figure 3.37**, where

conversion decreases drastically with $P_{alcohol}$ increasing. Because it was assumed, that reaction takes place in aqueous phase, high concentrations of both reactants favours esterification. Partitioning of substrates into organic phase will decrease the conversion.

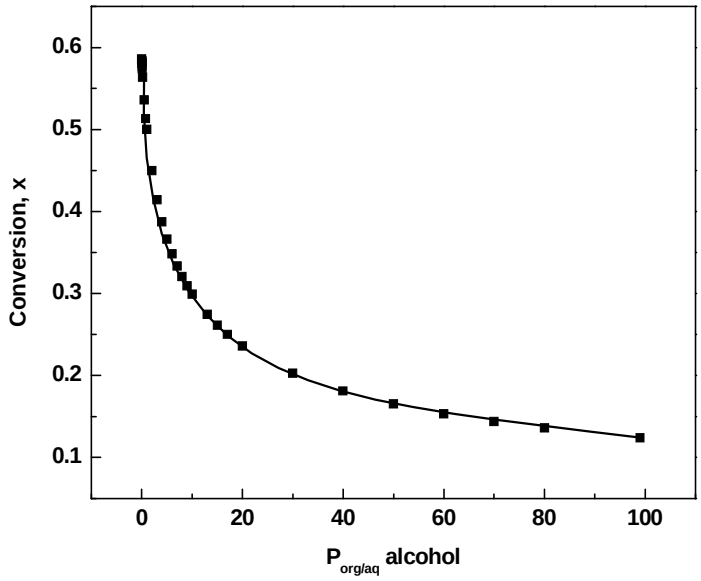


Figure 3.37. Dependence of conversion on partition coefficient of an alcohol, $P_{org/aq}$ (acid) = $P_{org/aq}$ (product), K_{aq} = 1, α = 1, S = 1.

A much stronger dependence of conversion on K_{aq} can be observed in **Figure 3.38, 3.39**. At K_{aq} > 20 conversion reaches high 86 - 93 % compared to < 58 % at K_{aq} < 1.

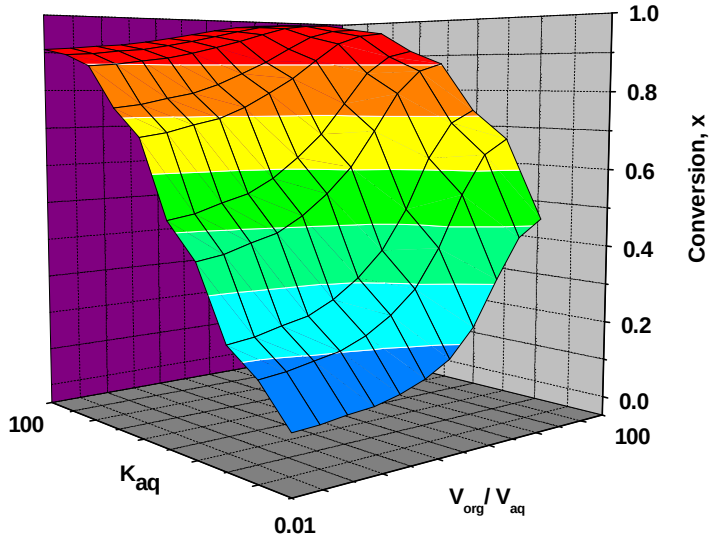


Figure 3.38. Dependence of conversion on K_{aq} and V_{org}/V_{aq} ($P_{org/aq}$ (acid) = $P_{org/aq}$ (product) = 99, $P_{org/aq}$ (alcohol) = 0.001, S = 1).

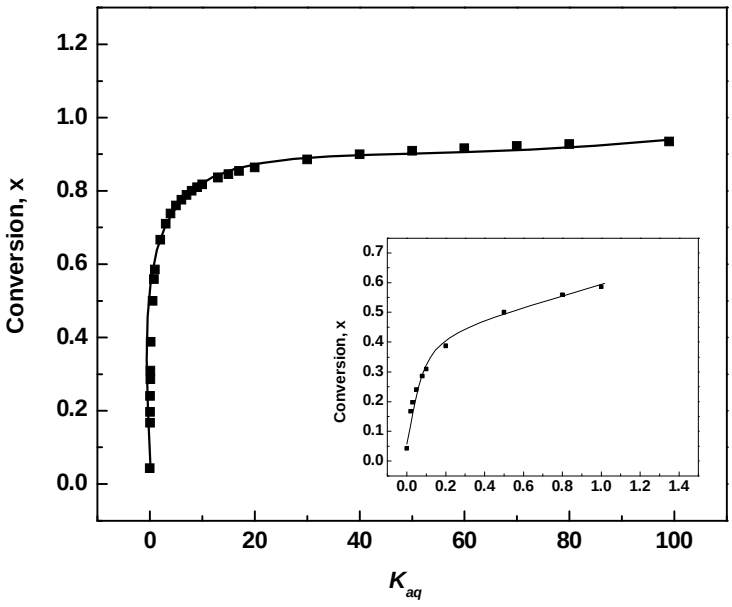


Figure 3.39. Dependence of conversion on K_{aq}, $P_{org/aq}$ (acid) = $P_{org/aq}$ (product) = 99, $P_{org/aq}$ (alcohol) = 0.001, α = 1, S = 1.

It can be seen in **Figure 3.38** that conversion depends on K_{aq} much stronger compared to phase volume ratio V_{org}/V_{aq}.

Figure 3.40 shows that an increase in the organic phase percentage (v/v) from 10 % to 80 % (corresponds to $\alpha = V_{org}/V_{aq} = 0.11$ and 4 on the x axis of the graph) leads to less than 20 % increase in conversion (the change will be even less at $K_{aq} < 1$), whereas changing of K_{aq} from 0.001 to 1 allows to increase conversion up to 60 %.

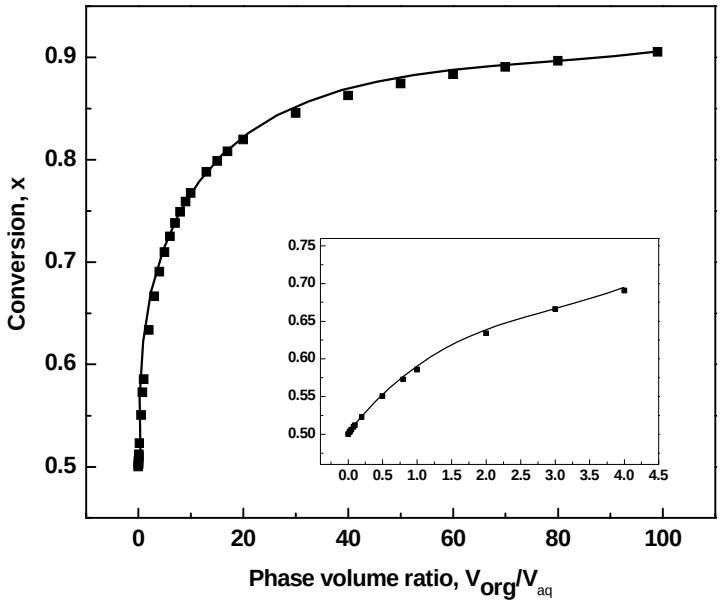


Figure 3.40. Dependence of conversion on V_{org}/V_{aq}, $P_{org/aq}$ acid = $P_{org/aq}$ (product) = 99, $P_{org/aq}$ alcohol = 0.001, K_{aq} = 1, S = 1.

For the reaction of 1,3-propanediol esterification two equivalents of a fatty acid need to be taken (**Figure 3.41**), therefore equation (9) must be changed to (12).

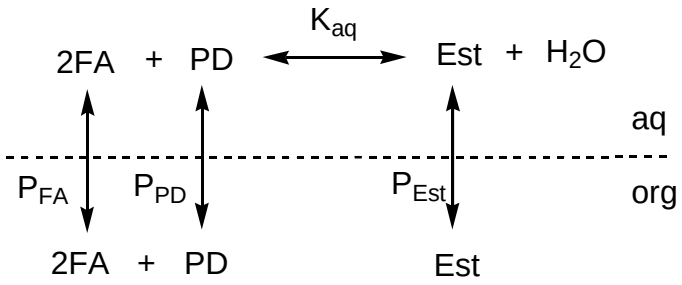


Figure 3.41. Enzymatic reaction of esterification between 1,3-propanediol and a fatty acid in biphasic aqueous-organic system.

$$x = \frac{(2+S) - \sqrt{(2-S)^2 + \frac{4S}{Km}}}{2(2 - \frac{1}{Km})} \qquad (12)$$

Fitting the experimental data obtained in our study to equation (12) at S = 2.5, P_{diol} = 0.01, $P_{ester} = P_{acid}$, $\alpha = V_{org}/V_{aq} = 1$, results in K_{aq} = 0.003. Based on the obtained dependence of conversion on V_{org}/V_{aq}, K_{aq} can be calculated from (12) and compared with the experimental data (**Figure 3.42**). Below 50 % content of an organic solvent in the biphasic medium, the experimental curve coincides with the modelled one. At concentrations above 50 % of the organic phase, the experimental conversion is higher compared to the calculated one. The discrepancy can be attributed to the incorrect assumption that $P_{ester} = P_{acid}$.

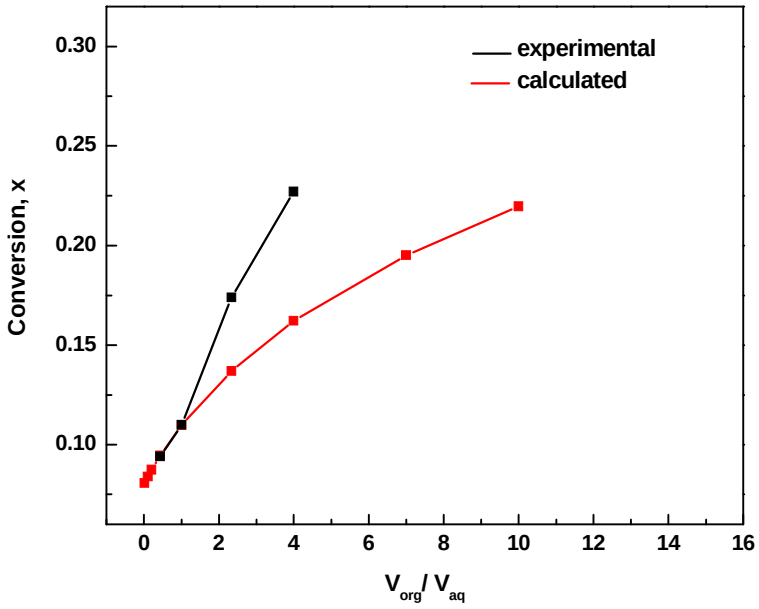


Figure 3.42. Estimated and experimental dependence of conversion on V_{org}/ V_{aq} in esterification of 1,3-propanediol with linoleic acid.

To conclude we can say that the aqueous equilibrium constant K_{aq}, which depends on the nature of substrates, has the strongest influence on conversion in the biphasic aqueous/organic medium. To increase conversion, substrates should be mostly dissolved in the aqueous solution whereas product needs to partition into the organic phase. Also, low water content favours high yield, but high conversion is achievable even at high water content when aqueous equilibrium constant K_{aq} is large, and carrying out the reaction in almost monophasic organic solvent with low water content does not provide high conversion when aqueous equilibrium constant is small. 1,3-Propanediol esterification with linoleic acid is characterised by low equilibrium constant K_{aq} = 0.003. In order to increase conversion, partitioning of acidic substrate into the aqueous phase and of the ester product into the organic phase must be

increased; simultaneously the water content has to be lowered. Using a more hydrophilic acid and an organic solvent with a higher P_{ester} will enhance conversion. However, hydrophilic and therefore short-chain, acids are known to be inhibitory for enzymes as they dissolve in the aqueous protective layer, decrease pH and therefore cause denaturation of the protein. Indeed, the reaction of 1,3-propanediol esterification with butyric acid by free *R. miehei* in the biphasic tetradecane/aqueous medium gave low equilibrium conversion of 6 %.

Another way to increase conversion is to find an organic solvent with the highest solubility for the product so that formed product will partition into organic phase during the reaction thus shifting the equilibrium.

Effect of different organic solvents

Using a hydrophobic solvent as a second phase in the biphasic system allows to reduce the inhibitory effect of substrates and/or products. For example, some hydrophobic ethers and ketones (diethyl ether, diisoproryl ether, diethyl ketone, di-*n*-propyl ketone) were favourable solvents in the reaction of lactic acid esterification with ethanol due to their miscibility with lactic acid and therefore preventing the enzyme inactivation from acidity of lactic acid [181]. At the same time toxicity of the solvent itself for enzyme also should be taken into account. Moreover, hydrophilic solvents are known to be able to strip aqueous layer from enzyme surfaces, thus reducing their activity [182].

In our study we mainly used tetradecane as a highly hydrophobic solvent in which linoleic acid can be dissolved. Apart from tetradecane, diphenyl ether was also tested as a second

hydrophobic organic solvent in the biphasic reaction medium. As shown in **Figure 3.43** more than twice higher conversion of 1,3-propanediol can be obtained if the aromatic solvent was used, compared to the C_{14} hydrocarbon. Comparing ethers and hydrocarbons, diphenyl ether was found to be the best solvent in the reaction of bis(2,2,2-trifluoroethyl) sebacate and 1,4-butanol polycondensation at 37 °C *Mucor miehei* lipase, although the author does not offer any explanation [141]. A correlation between an increase in conversion and the increase in hydrophobicity of the solvent used in the esterification of isoamyl alcohol with isovaleric acid by immobilized *R. miehei*, was reported, but excluding hydrophobic solvents with close log P (hexane, heptane, isooctane) [158]. In our case, a 20 % conversion was obtained in 10 h in a less hydrophobic diphenyl ether (log P = 4.1) compared to 8 % obtained in tetradecane (log P = 8.5), suggesting perhaps an influence of other parameters, such as, for example, water concentration in the organic solvent, 1,3-propanediol partitioning between the two phases or an ability of solvent to form an emulsion.

Figure 3.43 shows that in the case of pure linoleic acid in the absence of an extractive organic solvent, a 75 % conversion can be achieved in 20 h time. Hence, solvent-free fatty acid lipase-catalyzed esterification with 1,3-propanediol dissolved in aqueous solution is a promising approach for active extraction of the diol from fermentation broth.

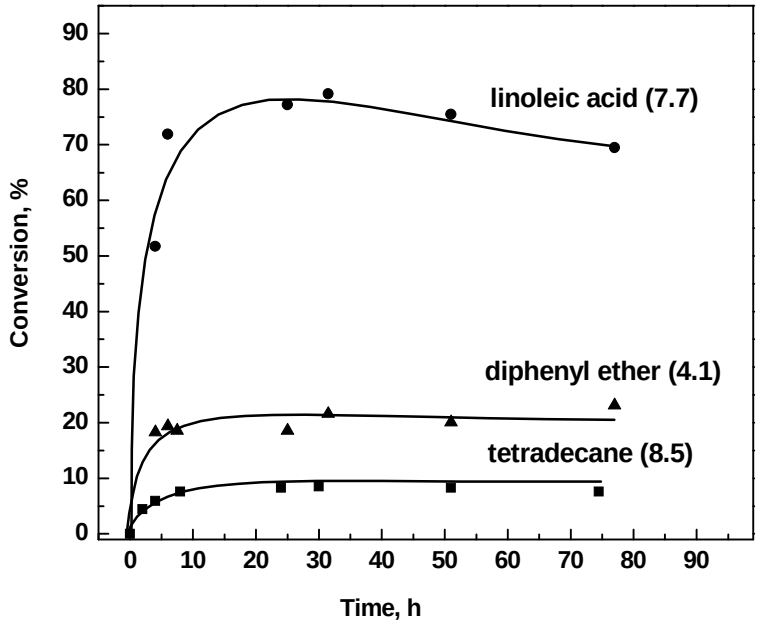
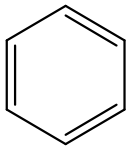
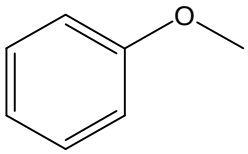
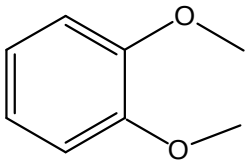
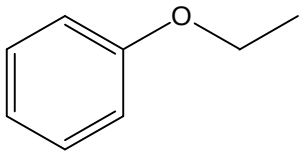
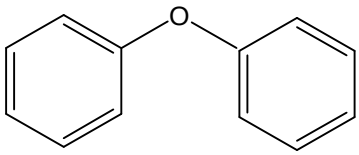
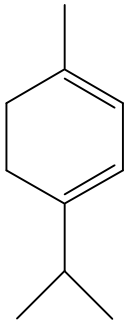
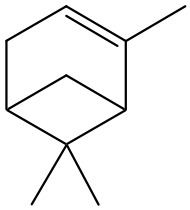
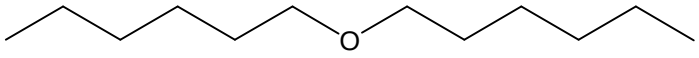
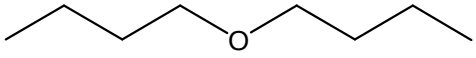
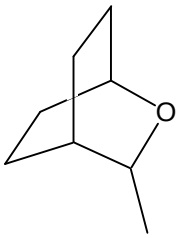
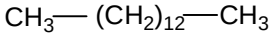
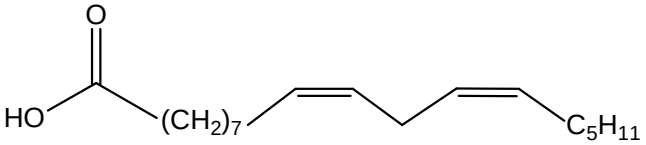


Figure 3.43. Esterification profiles obtained for different solvents in biphasic system with fermentation broth (V_{org} = V_{aq} = 3 ml, C_{PD} = 0.12 M_{aq}, C_{LA} = 0.3 M_{org}, 20 µL *R. miehei* free, 37 °C). Numbers in brackets correspond to solvent's log P.

To investigate the influence of organic solvents on enzyme activity, several solvents were tested in the reaction of esterification. All solvents used in this project can be divided into three groups: aromatic (benzene, anisole, ethoxybenzene, dimethoxybenzene, diphenyl ether), ethers (dibutyl ether, dihexyl ether and eucalyptol) and solvents containing double bonds (α – pinene, α – terpinene, linoleic acid). Linoleic acid and tetradecane should be considered with more attention as the first one is a reactant itself and therefore taken in high excess, and the second one is highly hydrophobic non-toxic hydrocarbon which shows poor solubility properties. The solvents investigated are summarised in **Table 3.9**.

Table 3.9. Organic solvents tested in the reaction of linoleic acid esterification with 1,3-propanediol in biphasic system.

	Name	Structure
Bz	Benzene	
An	Methoxybenzene (Anisole)	
dMB	Dimethoxybenzene	
EB	Ethoxybenzene (Phenetol)	
dPhE	Diphenyl ether	
T	α - Terpinene	

P	α – Pinene	
dHE	Dihexyl ether	
dBE	Dibutyl ether	
Euc	Eucalyptol	
Tetr	Tetradecane	
LA	Linoleic acid	

Conversion profiles obtained with different solvents are shown in **Figure 3.44**. The lowest conversions of about 3-4 % were obtained for ether solvents: dibutyl ether, hexyl ether and eucalyptol. Experiments with aromatic solvents gave the highest conversions of 15-22 %, whereas 10 % was achieved in the reaction with tetradecane as an organic solvent. An interesting behaviour was observed for α–pinene and α–terpinene: a twice lower conversion of about 7.5 % was obtained for the second one compared to 14 % for the first, due to probably low purity of the α–terpinene used. The highest conversion of 75 % was obtained if pure linoleic acid was used as the second phase, as was shown in **Figure 3.43**.

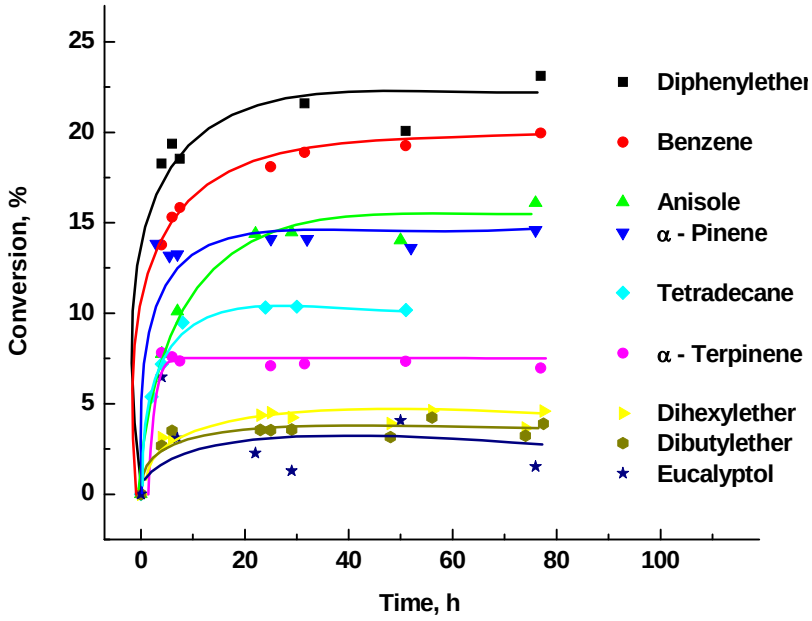


Figure 3.44. Conversion profiles obtained in biphasic system with fermentation broth and different organic solvents (V_{org} = V_{aq} = 5 ml, C_{PD} = 0.12 M_{aq}, C_{LA} = 0.3 M_{org}, 33 µL *R. miehei* free, 37 °C).

To understand the influence of different solvents on enzyme activity, water content and 1,3-propanediol partition coefficients were measured and also calculated using COSMO-Therm.

Firstly, if we look at the dependence of conversion on log P estimated by COSMO-Therm, shown in **Figure 3.45**, we can see that all data lay within the range of 3-22 % with no distinctive correlation. Linoleic acid can be distinguished as the only highly hydrophobic solvent (log P = 7.7) in which the highest conversion of 75 % was obtained due to the shift of equilibrium towards product formation at high concentration of the initial reactants.

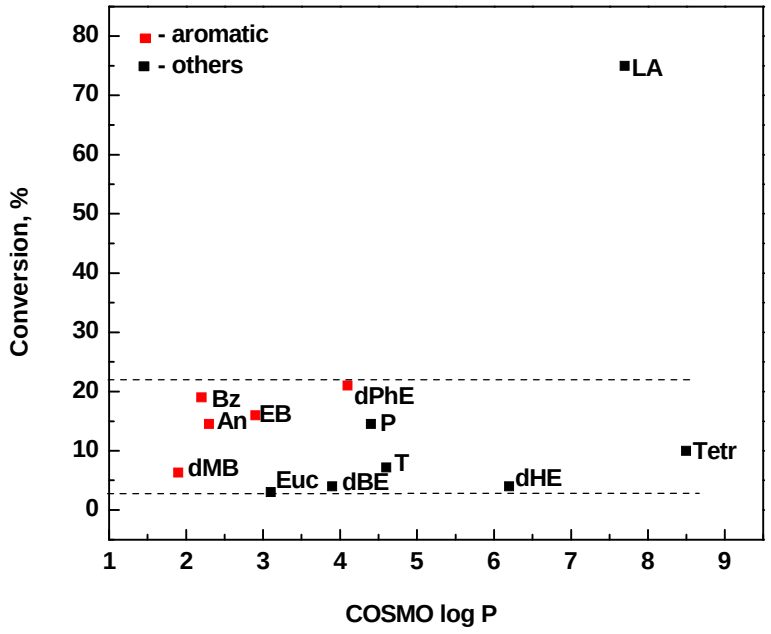


Figure 3.45. Dependence of conversion obtained in the biphasic systems with fermentation broth and different organic solvents on log P calculated with COSMO at 25 °C.

There is an inverse correlation between water content in the organic phase measured at the end of the reaction and estimated log P of the organic solvent used: the higher log P corresponds to the lower water concentration (eucalyptol and linolelic acid are exceptions) (**Figure 3.46)**. However, predominantly, all solvents tested showed low water content regardless of the variation in log P within the range of 2-9. Therefore, with all the solvents being highly hydrophobic it is incorrect to take log P parameter to estimate the influence of hydrophobicity of an organic solvent on conversion or reaction rate. The same conclusion that log P is not a convenient parameter for comparison of different organic solvents, though in regard to

enzyme stability, was made by Filho *et al*, when organic solvents with different functionality - hydrocarbons, ethers, alcohols and aromatic - were tested [183].

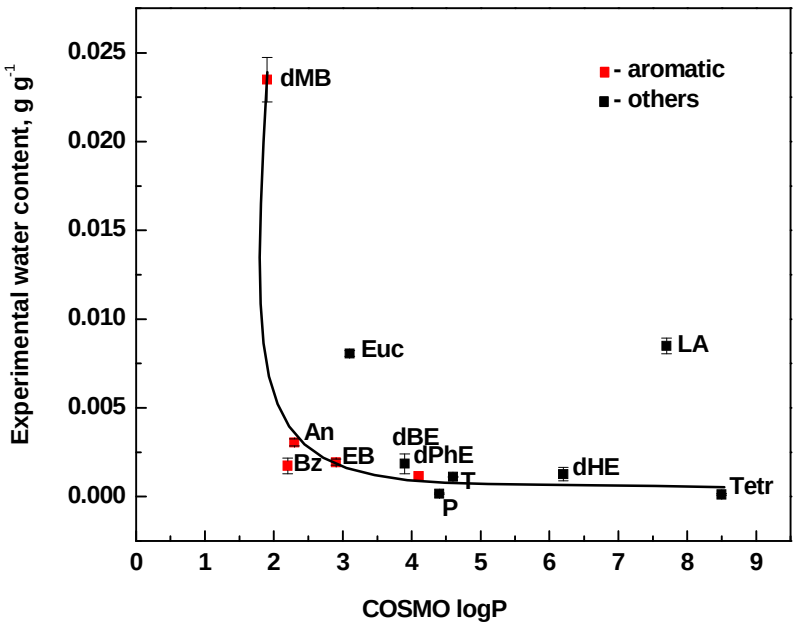


Figure 3.46. Dependence of water concentration measured after the esterification of linoleic acid with 1,3-propanediol was finished in the biphasic fermentation broth/organic solvent system with *R. miehei* on log P calculated with COSMO at 25 °C.

It is more accurate to compare conversion with the amount of water dissolved in the hydrophobic solvents instead of log P. Such dependence is shown in **Figure 3.47**.

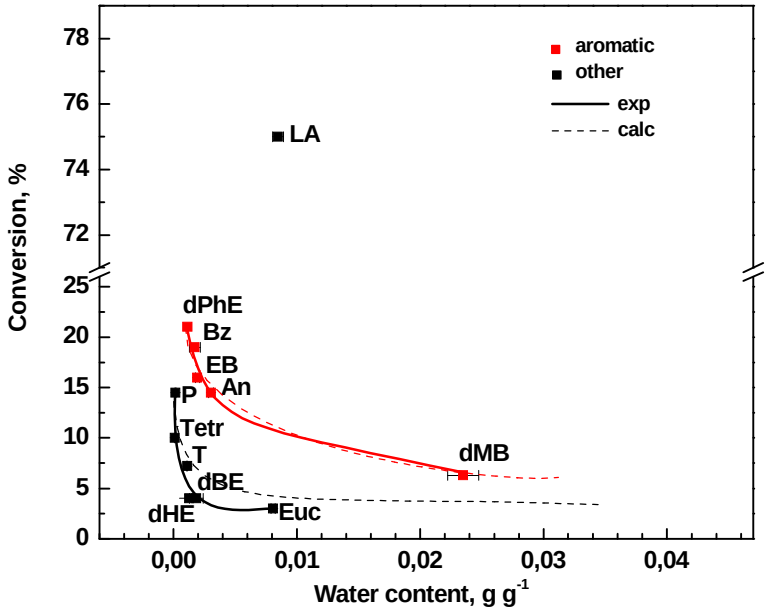


Figure 3.47. Dependence of conversion obtained in the biphasic systems with fermentation broth and different organic solvents on water content measured and calculated with COSMO at 37 **°C.**

A direct inverse correlation between conversion and water content in the hydrophobic solvents tested was obtained in the reaction of linoleic acid esterification with 1,3-propanediol in the biphasic aqueous/organic system. Higher conversions correlate with a lower water content and both, the measured and estimated water concentrations, follow the same trend. This correlation reflects equilibrium limitation: being a product, water dissolved in an organic solvent shifts the equilibrium towards lowering the product formation. However, according to equation (12) conversion depends only on V_{org}/V_{aq} ratio, and concentration of water in the organic phase does not influence the overall yield. Our study showed results contrary to this statement.

Therefore, we suggest that the theoretical equation needs to be improved to take into account the effect of water dissolved in the organic solvent.

More accurately, the reaction can be considered as taking place at the boundary between the two phases and not solely in an aqueous solution. Reis *et al.* pointed out that concentration of substates and products at the interface differs from that in the bulk solution; therefore the molar concentrations cannot be used to describe the two-phase systems [125]. Taking into account pH, ionic strength, temperature is insufficient in this case. Other parameters, such as surface tension for example need to be investigated. Indeed, enzyme inactivation was found to correlate with aqueous-organic interfacial tension in the biphasic systems with the organic solvents with close log P and different functional groups [184]. Moreover, enzymes themselves and fatty acids as substrates are surface-active molecules that could change properties of the interface. In general, new methods and models need to be developed to study interface interactions.

In **Figure 3.47** two different types of solvents can be easily distinguished in properties: aromatic solvents (benzene, anisole, phenetol, dimethoxybenzene and diphenylether) seem to favour ester formation as higher conversions were obtained for this group of solvents (shown red in **Figure 3.47**). A better partitioning of the product in aromatic solvents might be responsible for this enhancement.

Dependence of conversion on the 1,3-propanediol partition coefficient both experimental and calculated is shown in **Figure 3.48**. There is a common trend among all solvents tested: with

higher 1,3-propanediol partitioning into organic phase, lower conversions were obtained. This is in agreement with theoretical dependence of conversion on partition coefficient of alcohol (**Figure 3.37**). As the reaction takes place in the water phase, extraction of 1,3-propanediol into organic phase reduces its aqueous concentration and causes lowering of conversion.

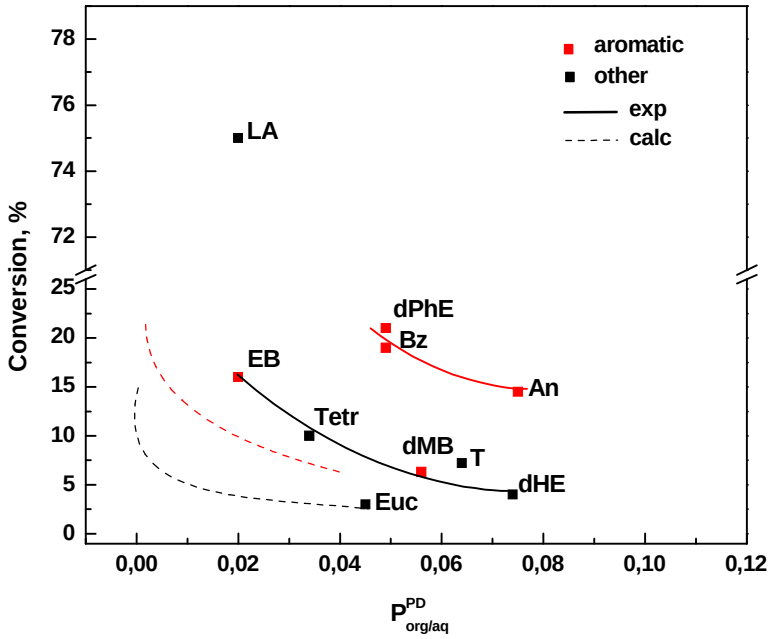


Figure 3.48. Dependence of conversion obtained in the biphasic systems with fermentation broth and different organic solvents on 1,3-propanediol partition coefficient measured and calculated with COSMO at 25 °C.

As one can see, aromatic solvents like diphenyl ether, benzene and anisole stand aside from the rest of the solvents, having some beneficial effect on 1,3-propanediol esterification, following at the same time the common trend. Dimethoxybenzene and phenetol appeared to lay on one curve with ether, double-bonded and hydrocarbon solvents on the graph. The longer hydrocarbon chain of ethoxy group in phenetol significantly worsens its solubility

properties for 1,3-propanediol compared to anisole that has methoxy group. Dimethoxybenzene has two methoxy groups to form hydrogen bonds with 1,3-propanediol but shows lower solubility for the diol than anisole. It is not clear why lower conversion was obtained in cases of dimethoxy-benzene and phenetol in comparison to other aromatic solvents.

The benificial effect of aromatic solvents on 1,3-propanediol esterification, when higher conversions were achieved at the same solubilities for water and 1,3-propanediol as for ethers and tetradecane, is an example of the influence of solvent functionality rather than of a single physiochemical parameter, like log P for example. “Chemical functionality is a pragmatic label for a set of molecular properties, such as the presence of certain heteroatoms and residues, the absence of others, molecular geometry, electronic density distribution and frontier orbitals, and consequent macroscopic properties of solvents, such as surface tension, solubility, solvatation energy” [183]. Obviously, the presence of aromatic rings (electron-rich π - systems) in diphenyl ether, benzene and anisole played a role either in solely better partitioning of the product into the organic phase or in the enhancement of the enzyme activity and stability. These effects need to be further studied.

To summarize the results discussed above we can say that *R. miehei* is a suitable catalyst for biphasic organic/aqueous esterification of 1,3-propanediol with linoleic acid at 37 °C. The lipase appeared to be tolerant to complex fermentation medium. It was observed that the optimum enzyme concentration is 132,000 U L_{aq}^{-1}; higher loading does not enhance the reaction rate. 1,3-Propanediol was found to be inhibitory for the enzyme at the concentration

as low as 0.3 M. Thermodynamic study showed that the conversion of *ca* 10 % was obtained in the biphasic tetradecane/aqueous medium due to low aqueous equilibrium constant K_{aq} which depends on the nature of the substrates. To increase the conversion an organic solvent with better partitioning of the product is required. Indeed, using an aromatic solvent as diphenylether allows to increase the conversion of 1,3-propanediol esterification to 22 %. Joint effect of better partitioning of the product and high concentration of non-inhibitory reactant as linoleic acid provided the highest conversion of 75 %.

3.4 1,3-Propanediol transesterification by lipases

Different types of lipases, Novozyme 435, CLEA and free *R. miehei* were tested in the reaction of some esters transesterification with 1,3-propanediol at different reaction conditions in mono and biphasic systems.

A typical reaction is shown in **Figure 3.49** with dibutyl-phthalate (BPh) as initial diester for transesterification. Apart from dibutyl-phthalate (BPh), bis-2-ethyl-hexyl-sebacate (BES) and di-octyl-sebacate (OS) were used (shown in **Figure 3.50**). The obtained results are summarized in **Table 3.10**.

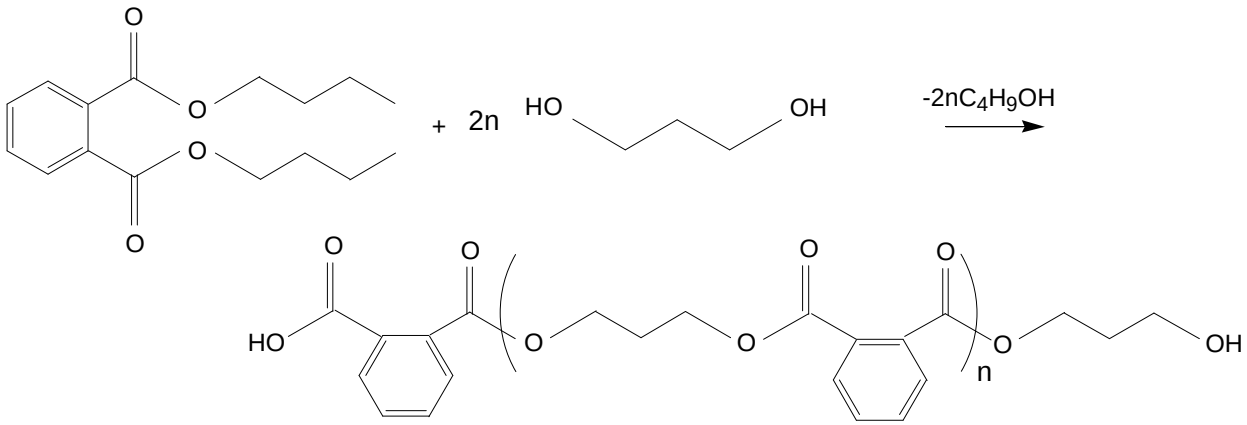
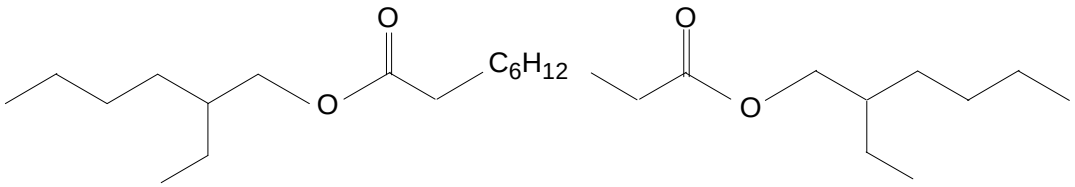
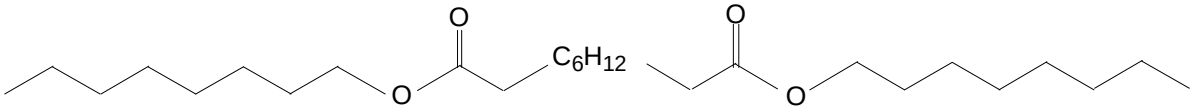


Figure 3.49. Dibutyl-phthalate (BPh) transesterification with 1,3-propanediol leading to the formation of polymeric ester.



bis-2-ethyl-hexyl-sebacate (BES)



di-octyl-sebacate (OS)

Figure 3.50. Structures of the two esters of sebacic acid used in the reaction of transesterification with 1,3-propanediol.

As one can see from **Table 3.10**, only when pure reactants were taken initially was di-esterification of the diester observed in the case of catalysis by Novozyme 435 (row 2). Although Novozyme 435 is a powder, some amount of water, enough to hydrolyze an ester, could be present in the reaction mixture. When either reactants were dissolved in tetradecane

or a biphasic system was used no product was detected regardless of the nature of the diester or the catalyst used.

An example of a successful transesterification of dimethylsebacate with 2-ethyl hexanol by Novozyme 435 and *R. miehei* lipases was shown by Gryglewicz [185]. The conversion of 84-89 % was obtained in 20 h time in the reaction between pure diester and alcohol at 50 °C with evaporation of methanol formed during the reaction. When aryl substrates *m*-dimethylphtalate and *o*-dimethylphtalate were used, much lower conversions of 30 and 4 % were obtained in 350 h.

In our system no transesterification occurred. The reason for this might be steric hindrance caused by bulky octyl, ethyl-hexyl, butyl groups in the esters tested. Also in the biphasic system hydrolysis prevails over transesterification in the presence of water.

Table 3.10. A summary of results obtained in transesterification of different esters (BPh, BES, OS) with 1,3-propanediol by different lipases at 37 ºC.

N	System	Catalyst	Time, h
1[a]	Tetradecane: BES +PD		54
2[b]	BES +model mixture	10 % (w) Novozyme 435	146
3[c]	Tetradecane: BES +broth		54
4[a]	BES + PD	11 % CLEA *Rhiz. m*	260
5[a]	Tetradecane: BPh + PD	2.5 % CLEA *Rhiz. m*	10 days
6[a]	Tetradecane: BPh + PD	1.8 % CLEA *Therm. l*	10 days
7[a]	Tetradecane: BPh + PD	10.6 % CLEA *lipase B*	9 days
8[c]	Tetradecane: BPh + broth	10 μl free *Rhiz. m*	72
9	Tetradecane: BES+broth	10 μl free *Rhiz. m*	72
10[d]	Tetradecane: BES+broth		106
11[d]	Tetradecane: BPh+broth	1 ml free *Rhiz. m*	106
12[c, d]	Tetradecane: OS+broth		106

[a] No product according to NMR

[b] 7 % of 2-ethyl-1-hexanol was formed

[c] No product according to HPLC

[d] Strong emulsion was formed

Procedure: an appropriate amount of diester (ester:diol = 1:1) was dissolved in 3 mL of tetradecane, either pure 1,3-propanediol or 3 mL of broth solution was added and mixed with a catalyst.

3.5 Different reactors for enzymatic biphasic esterification

Eesterification of linoleic acid with 1,3-propanediol was studied in two different types of reactors: segmented flow and membrane hollow fibre. In both cases either linoleic acid itself or tetradecane were used as the organic phase. Fermentation broth or a buffer solution of 1,3-propanediol (KPi buffer, pH = 5.6, 50 mM) were used as the aqueous phase.

3.5.1 Segmented flow reactor

Two types of enzymes were tested in the segmented flow reactor: free and CLEA *R. miehei* lipase. Reaction conditions and results obtained are shown in **Table 3.11**.

Table 3.11. Reaction conditions and results obtained for the linoleic acid esterification with 1,3-propanediol at 37 °C in biphasic system in the segmented flow reactor (C_{PD} = 0.12 M_{aq}, LA/PD = 2, residence time t = 20 h, Vorg/Vaq = 1).

Enzyme	Enz. conc., U L^{-1}_{aq}	Conversion, %
2.4 % (w/w_{PD}) CLEA *R. miehei*	17,560	0
1 ml free *R. miehei*	6,667	6

The use of the free enzyme aqueous solution as a catalyst allowed to achieve 6 % conversion in 20 h which is lower compared to a batch reaction carried out in a vessel with vigorous mixing of two phases at 700 rpm. If we take into account that a very high concentration of the enzyme (1 mL compared to 20 μL for batch reactor) was taken, no beneficial effect can be observed in using a segmented flow reactor to carry out biphasic esterification of linoleic acid

and 1,3-propanediol. If a powdery CLEA enzyme was used, no product was observed, due to probably low dispersion (accessibility) of the catalyst in the reaction medium. Other difficulties, such as unstable segmented flow, problems in handling and cleaning of long narrow tubing, degradation of the peristaltic pump tubing after a prolonged contact with tetradecane are worth mentioning.

The same approach of carrying out an enzymatic transformation within a micro-rector (ID = 0.6 mm), where a biphasic segmented flow can be generated, was used for kinetic studies of an immobilized CALB in the reaction of 4-nitrophenyldecanoate hydrolysis in a decane/water medium [186]. The authors claimed 96 % conversion in the micro-reactor compared to 23 % obtained in a batch reactor with the free lipase, but in the latter system the hydrolysis occurred in the diffusion-limited regime when the biphasic solution was not mixed properly but agitated at 100 rpm. Therefore, no emulsion was formed and, as a result of that, low conversion was obtained. The same effect of lower conversion at a lower stirring speed was observed in our study for 1,3-propanediol esterification with decanoic acid in the biphasic system with Novozyme 435 (see **Table 3.7**, chapter 3.3.2).

In general, micro-reactors could provide high surface/volume ratio for reactions with immobilized enzymes. However a further optimization and simplification of the operating procedure need to be carried out.

3.5.2 Hollow fiber membrane reactor

A hollow fiber reactor was run in two different modes: with free and immobilized lipases. In the first case either powdery CLEA or free enzyme solution was added to tetradecane or a broth solution, and both phases were recirculated along the shell and lumen sides of the membrane. In the second case the enzyme was immobilized onto a membrane. In both cases conversion was estimated *via* changes of initial reactants in the supplying/receiving flask.

Figure 3.51 shows comparative graphs for the batch emulsified reactor and PES hollow fiber reactor with CLEA enzyme as a catalyst (it was shown previously that CLEA and free *R. miehei* have the same activity). A higher 11.4 % conversion was reached in the PES membrane reactor compared to the batch one, but a 4.4 times lower reaction rate was observed. A lower reaction rate cannot be solely attributed to the 2.5 times lower enzyme concentration, but can be explained probably by the lower contact area between the two phases ($S_{membrane}$ = 15 cm^2) and diffusion limitations.

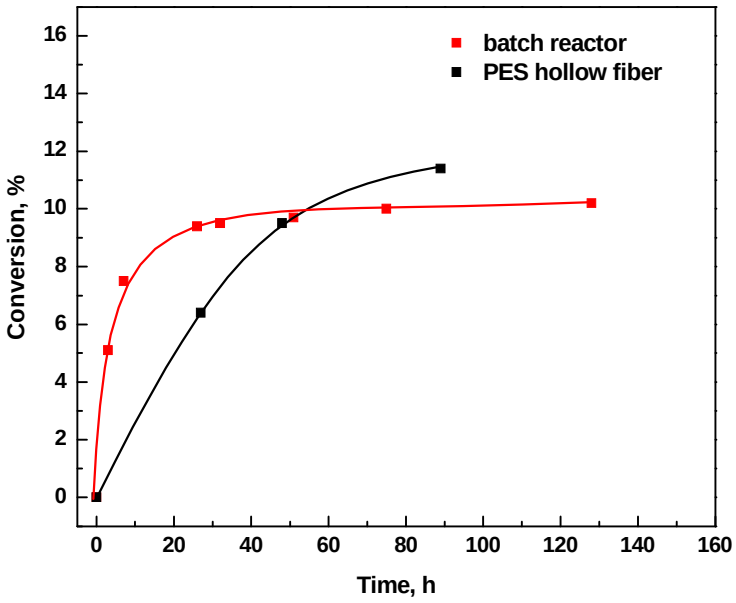


Figure 3.51. Esterification profiles obtained for batch reactor (V_{org} = V_{aq} = 3 ml, C_{PD} = 0.12 M_{aq}, C_{LA} = 0.3 M_{org}, 10 % (w/w_{PD}) CLEA *R.* miehei lipase (73,100 U L_{aq})) and PES hollow fiber membrane reactor (V_{org} = 3 ml, V_{aq} = 6 ml, C_{PD} = 0.12 M_{aq}, C_{LA} = 0.24 M_{org}, 4 % (w/w_{PD}) CLEA *R.* miehei lipase (57,450 U L_{aq}), 0.1 mL min^{-1}).

Figure 3.52 shows the conversion profile obtained for 1,3-propanediol esterification carried out in the carbon membrane (Novacarb) hollow fiber reactor in biphasic linoleic acid/aqueous broth system in comparison with the emulsified batch reactor. A much lower reaction rate is observed for the enzyme in the hollow fiber reactor pointing at diffusion limitations and low contact area between the phases.

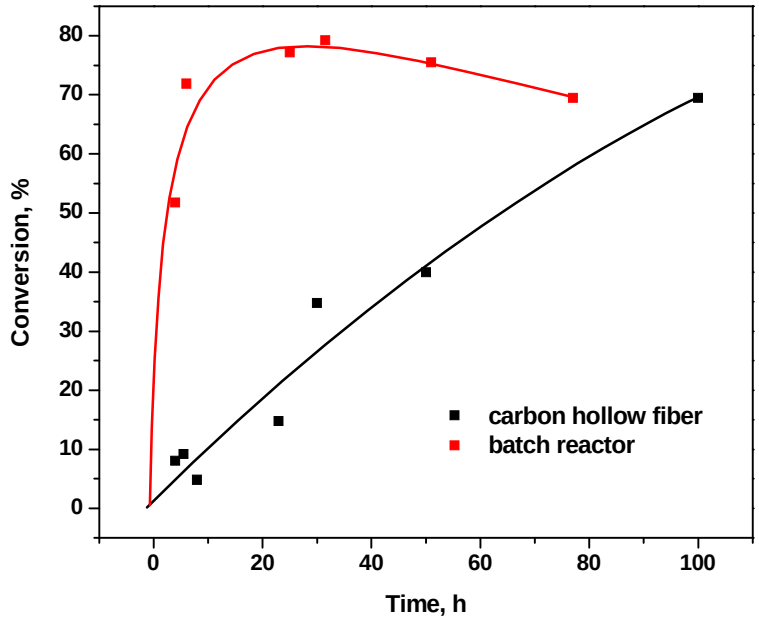


Figure 3.52. Esterification profiles obtained for batch reactor and Novacarb hollow fiber membrane reactor (V_{LA} = V_{aq} = 3 ml, C_{PD} = 0.12 M_{aq}, 20 µL *R. miehei* free lipase (133,000 U L_{aq}), 0.1 mL min^{-1}).

3.5.3 Hollow fiber membrane reactor with immobilized enzyme

It is known that the contact area between two phases in a biphasic system increases because of the formation of emulsion. However, enzymes cannot be reused in such systems and de-emulsification is problematic. Using a hollow fiber reactor with an immobilized enzyme makes it possible to reuse the enzyme and avoid the problem of product recovery in the case of emulsion formation. Immobilization also increases stability of enzymes, though lowering their activity in some cases [187].

Figure 3.53 shows comparative curves obtained for esterification of 1,3-propanediol and linoleic acid in biphasic aqueous/tetradecane hollow fiber reactors with different membranes. Carbon and PES membranes appeared to be poor choices for the catalytic system, whereas the PP membrane was the only suitable support material for the enzyme. An enzyme activity test carried out before and after immobilization showed that no lipase was supported onto the carbon and PES membranes, therefore no activity was detected during the experiments. In general, a lower initial reaction rate can be observed for the hollow fiber reactors compared to the batch reactor.

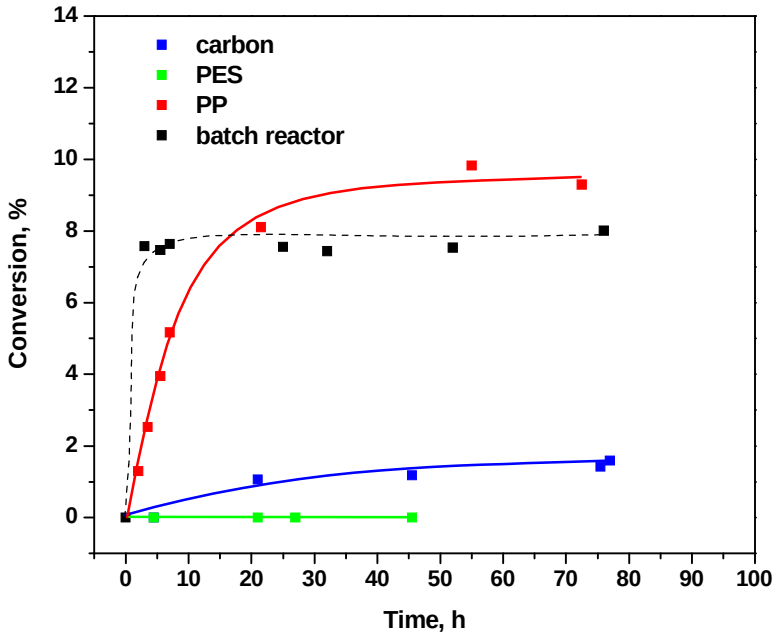


Figure 3.53. Esterification profiles for batch and different hollow fiber membrane reactors. Batch reactor: 37 °C, PD/LA = 1/2, 3 mL tetradecane, 3 mL KPi buffer solution (50 mM, pH = 5.6), C_{PD} = 0.12 M_{aq}, 20 µL *R. miehei* free (133,000 U L_{aq}^{-1}), 700 rpm. Hollow fiber membrane reactors: 37 °C, PD/LA = 1/2.5, 5 mL tetradecane, 5 mL KPi buffer solution (50 mM, pH = 5.6), C_{PD} = 0.12 M_{aq}, 14,800 Units *R. miehei* immobilized (183 U cm^{-2}) (PP membrane), F_{org} = 0.1 mL min^{-1}, F_{aq} = 0.8 mL min^{-1}.

Parameters and conditions of immobilization of the enzyme onto the PP membrane are given in **Table 3.12**. Immobilization was carried out by circulation of free *R. miehei* enzyme solution (20,000 U g^{-1}) through the lumen side of the membrane at 0.1 mL min^{-1} for several hours followed by washing with KPi buffer (50 mM, pH = 5.6). The amount of retained enzyme was estimated based on the enzyme activity assay before and after immobilization. For the membranes tested the surface area did not vary significantly and was within 80-90 cm^2. As one can see in **Table 3.12** the amount of the enzyme immobilized onto the membranes reaches a maximum value of 15,000 Units (corresponds to 170 U cm^{-2}) regardless whether the time of immobilization was 5 or 19 h. Apparently, there is an optimum amount of enzyme that can be immobilized onto this type of membrane, this optimum can be achieved already in 5 h and cannot be further increased with increasing the time of immobilization. The amount of *R. miehei* enzyme immobilized on the PP membrane reaches 9.2 mg cm^{-2}, which is higher compared to 2 mg cm^{-2} of CALB enzyme reported for the same PP membrane [188].

Table 3.12. Parameters of the PP membranes with the immobilized enzyme.

Name	N	L, cm	S, cm^2	$Time_{imm}$, h	Retained. enz., %	C_{enz}, U	C_{enz}, U cm^{-2}	C_{enz}, mg cm^{-2}
M1	40	12	90	5	25.5	15,300	170	8.5
M2	50	10	94	19	36	14,400	153	7.6
M3	36	12	81	19	37	14,800	183	9.2

Further investigation of stability and activity of the catalytic PP membranes in the biphasic aqueous/organic esterification of 1,3-propanediol with linoleic acid showed that the membrane

remained stable up to 4 cycles for 200 h. In these experiments linoleic acid was used as an organic phase and KPi buffer with dissolved in it; 9 g L^{-1} (0.12 M) of 1,3-propanediol was used as the aqueous phase that mimics the fermentation broth. The organic phase was circulated along the shell side of the membrane and the aqueous phase was pumped along the lumen side of the membrane such that enzyme was submerged into the aqueous phase and was in contact with the organic phase through the network of pores, filled with linoleic acid due to the hydrophobic nature of the membrane. To prevent breakthrough of the membrane and penetration of organic phase into the aqueous phase, flow rate of the aqueous phase was maintained at 0.8 mL min^{-1}, that is 8 time higher than the flow rate of the aqueous phase F_{org} = 0.1 mL min^{-1}. This results in sufficient transmembrane pressure to prevent breakthrough of the organic phase.

The amount of the enzyme supported onto the PP membrane was 14,400 Units, 153 U cm^{-2} or 2,880,000 U L_{aq}^{-1}, which is 22 times higher compared to the amount of the enzyme used in batch experiments - 132,000 U L_{aq}^{-1}. Thus, immobilization allowed us to increase the enzyme load significantly. We showed that the initial reaction rate almost does not change at enzyme loadings higher than 132,000 U L_{aq}^{-1} (**Figure 3.27**), therefore the batch reactor can be compared with the hollow fiber reactor as the enzyme concentration in both cases is so high that the initial reaction rate does not depend on the enzyme loading. High load of enzyme on the membrane can cause diffusion resistance for the substrates and the product, but at the same time provides constant supply of the enzyme in the case if enzyme desorption occurs.

In **Figure 3.54** one can see that the first two cycles give 75 % conversion of 1,3-propanediol in 20 h. After the first run the enzyme retains its initial activity, as the initial reaction rate does not change significantly in the first two cycles (shown 1 and 2 in **Figure 3.54**). However, a lower initial reaction rate and conversion were obtained when the membrane was used for the third cycle. Nevertheless the membrane remained active even after 120 h and if the fermentation broth was used as aqueous solution, 50 % conversion of 1,3-propanediol was achieved.

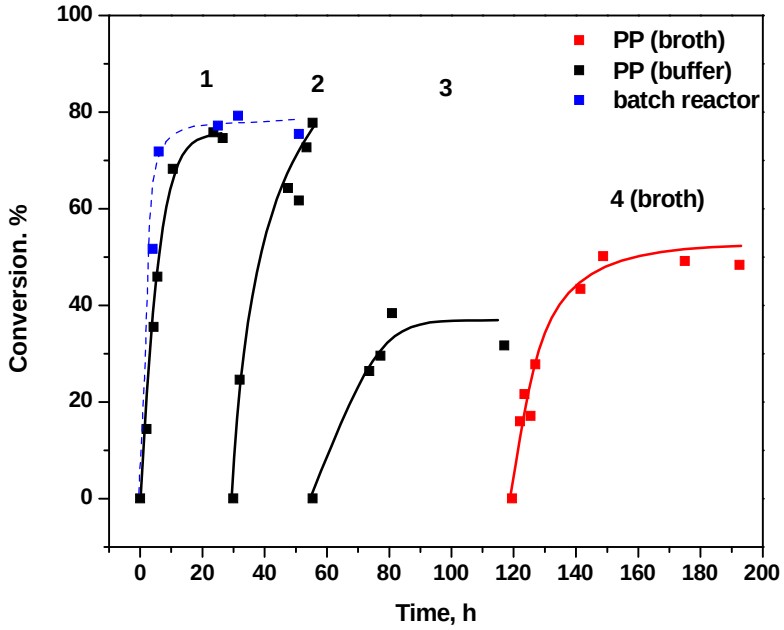


Figure 3.54. Conversion profiles for 4 cycles of biphasic esterification of 1,3-propanediol carried out in hollow fiber PP membrane reactor and its comparison with a batch reactor. Bath reactor: 3 mL linoleic acid, 3 mL broth solution, C_{PD} = 0.12 M_{aq}, C_{LA} = 3.2 M_{org}, 20 µL *R. miehei* free (400 U), 37 °C. Hollow fiber membrane: 5 mL linoleic acid, 5 mL KPi buffer solution (50 mM, pH = 5.6), C_{PD} = 0.12 M_{aq}, C_{LA} = 3.2 M_{org}, 14,400 U *R. miehei* immobilized (153 U cm^{-2}), F_{org} = 0.1 mL min^{-1}, F_{aq} = 0.8 mL min^{-1}, 37 °C.

If performance of the hollow fiber reactor with the immobilized enzyme is compared with that of the batch reactor one can say that the same conversion of *ca* 75 % was achieved in both cases, though 12-15 % of that is monoester for the hollow fiber reactor. In general there should not be differences in the equilibrium conversion, as solubility parameters do not change with the mode of the reactor. To compare the apparent reaction rates, the interfacial surface area between organic and aqueous phases in the biphasic batch reactor was estimated based on the size of LA droplets formed in the aqueous solution (**Figure 3.55**).

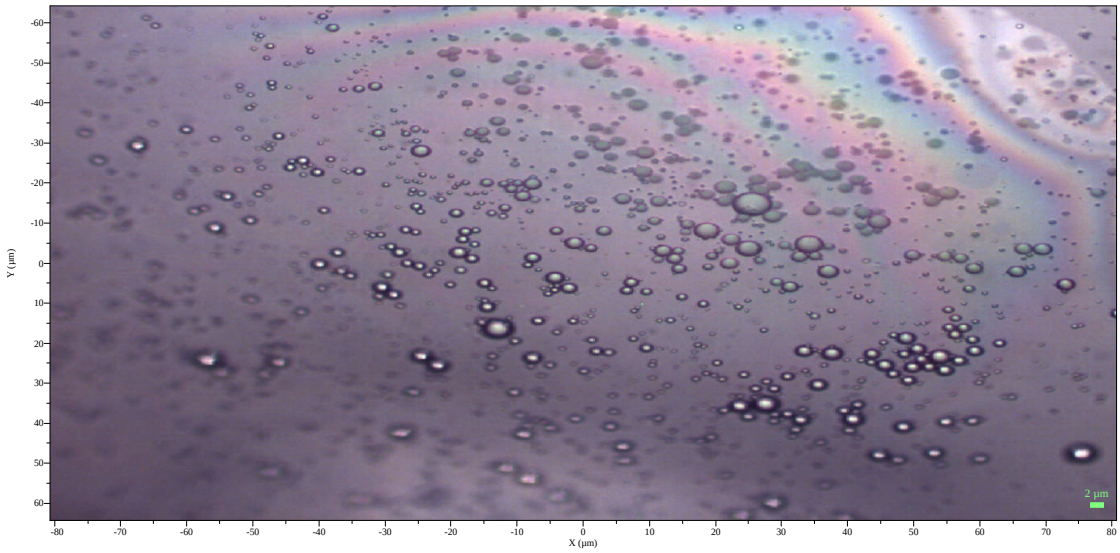


Figure 3.55. A light microscope picture of the emulsion formed in the LA/aqueous solution biphasic medium.

The interfacial area was estimated as 23 cm^2 in total 6 mL of the batch reactor, which corresponds to S/V ratio of 3.8 cm^{-1}. The interfacial area in the hollow fiber reactor is 94 cm^2, or S/V = 67 cm^{-1}. As we can see, the hollow fiber reactor has *ca* 18 times higher area to volume ratio. If we take into account that *R. miehei* enzymes are interfacially active, it follows that the higher S/V ratio is beneficial for esterification. Reaction rates evaluated from **Figure**

3.54 are 8.4 and 12.9 mmol L^{-1}_{org} h^{-1} for the hollow fiber and the batch reactors accordingly. Because samples in the case of the hollow fiber reactor were taken from the supplying tank, we need to take into account the dilution factor:

$r_r = r_b \cdot \frac{V_b + V_r}{V_r} = 8.4 \cdot \frac{3.6 + 1.4}{1.4} = 30 \left[mmol\ L_{org}^{-1}\ h^{-1} \right]$, where r_r is the initial reaction rate in the hollow fiber reactor, r_b is the initial reaction rate in the supplying vessel, V_r is the volume of the hollow fiber reactor, V_b is the volume of the batch. Now we can refer reaction rate to the interfacial area in both cases and obtain reaction rates in mol cm^{-2} h^{-1} units. Reaction rates expressed in different units for both batch and hollow fiber reactors are summarized in **Table 3.13**.

Table 3.13. Comparative table of the initial reaction rate expressed in different units for batch and hollow fiber reactors.

Reactor type	S, cm^2	Enzyme concentration, U L_{aq}^{-1}	Production rate, μmol h^{-1}	Reaction rate, mmol L^{-1}_{org} h^{-1}	Reaction rate, μmol cm^{-2} h^{-1}
Batch	23	132,000	38.7	12.9	1.68
HF	94	2,880,000	42	30	0.45

As we can see from **Table 3.13** the same initial reaction rate of *ca* 40 μmol h^{-1} was observed for both reactors, but the volume reaction rate is higher for the hollow fiber reactor, suggesting that a higher concentration of the product can be obtained in the hollow fiber reactor compared to the batch reactor during the same period of time. Normalising reaction rate to the interfacial

area showed that probably not all contact area in the membrane hollow fiber reactor is active in the reaction of esterification.

As a result we can say that among all the reactor systems tested only a hollow fiber reactor with *R. miehei* lipase immobilized onto PP membrane appeared to be beneficial compared to the batch emulsified reactor. Firstly, high enzyme capacity can be provided in the hollow fiber membrane reactor due to its high surface area. Secondly, the membrane appeared to be stable up to 200 h. Thirdly, the volumetric reaction rate is twice higher for the hollow fiber reactor compared to batch reactor allowing to obtain higher concentration of the product in a shorter period of time.

4 Conclusion

With increasing interest in using biocatalysts for feedstocks transformation, it became clear that the bottleneck of biotechnological processes is the down-stream product recovery. The time- and energy-consuming and waste-generating stage of product extraction and purification hampers the development of feedstock processing to value-added products. A new approach was proposed to overcome this problem by combining a bio-process with the subsequent transformation of intermediate chemicals to useful products within the same reactor.

In the framework of this methodology the main objective of the project was to develop effective bio-chemical transformation of glycerol.

We chose glycerol microbial fermentation by *C. Butyricum* as a case study of a biotechnological process. This was based on the abundance of the feedstock and the difficulty of separation of the product 1,3-propanediol from the aqueous broth. We showed that to obtain high yield of 1,3-propanediol, the fermentation should be carried out in a pH – controlled fermentor to avoid inhibition of the bacteria by acidic by-products. It is possible to use raw glycerol straight after biodiesel synthesis after a simple procedure of purification from fatty acids residues. The highest final concentration of 31 g L^{-1} (0.4 M_{aq}) of 1,3-propanediol was achieved with a total productivity of 1.4 g L^{-1} h^{-1}. The productivity was further increased to 2.8 g L^{-1} h^{-1} in the optimized continuous fermentation.

We further screened an effective catalyst/solvent pair for esterificatin of 1,3-propanediol. Among the catalysts tested – (Zr catalyst (1), Amberlyst-15, Nafion, Novozyme 435) – all,

except the Zr catalyst (1), were active in **monophasic** esterification of 1,3-propanediol with decanoic acid. But only Novozyme 435 remained active in a **biphasic** tetradecane/ aqueous broth system yielding 10 % of the diester at 37 °C. ***Thus our study showed that enzymes are the more suitable catalysts for the tandem reactions of glycerol to 1,3-propanediol derivatives compared to chemical catalysts.*** Low temperature and the presence of water are the main reasons for inactivity of the chemical catalysts in the reaction studied. But this rule cannot be extended to all tandem systems as it is still possible to develop monophasic aqueous bio-chemical transformation with a chemical catalyst active in water. At this point the paradigm of bio-chemical tandem systems shifts from biphasic organic/aqueous to monophasic aqueous reaction medium.

Further study of the enzymatic biphasic tetradecane/aqueous esterification of 1,3-propanediol with linoleic acid catalysed by *R. miehei* lipase showed that the enzyme remains active if complex fermentation broth is used as an aqueous phase. The optimal temperature and enzyme concentration were determined. 1,3-Propanediol was found to be inhibitory for the enzyme at concentrations as low as 0.3 M. A thermodynamic study showed that the low conversion of *ca* 10 % obtained in the biphasic medium is due to low aqueous equilibrium constant which depends on the nature of substrates. Organic solvents with better partitioning of the product, like aromatic solvents, with diphenylether being the best, can be used to increase conversion to 20-25 %. The joint effect of a better partitioning of the product and high concentration of non-inhibitory reactants, such as linoleic acid, provided the highest conversion of 75 % in the biphasic linoleic acid/fermentation broth enzymatic esterification of 1,3-propanediol.

We also attempted to transesterify 1,3-propanediol with esters of sebacic diacid in order to obtain polymers, but hydrolysis prevails over transestrification in the biphasic organic/aqueous media.

Finally, a hollow fiber reactor with *R. miehei* lipase immobilized onto polypropylene membrane was designed and tested in the biphasic linoleic acid/aqueous esterification of 1,3-propanediol. Other membranes as Novacarb and PES appeared to be poor choices for enzyme immobilization. The hollow fiber membrane reactor was characterized by a high enzyme capacity, stability for at least 200 h (4 cycles) and higher compared to batch emulsified reactor reaction rate per volume.

As a result we demonstrated feasibility of a subsiquent transformation of feedstock to value-added products *via* bicatalytic process, by the example of microbial fermentation of glycerol combined with enzymatic esterification of 1,3-propanediol with linoleic acid to obtain esters. This method makes it possible to avoid 1,3-propanediol extraction and purification steps, thus reducing time, energy and waste. Moreover this system is flexible as changing substrates will allow to obtain different products. In general, the demonstrated approach and the developed system can be easily utilized in process intensification of biorefineries.

Outlook

Although, we have developed a catalytic system for 1,3-propanediol esterification compatible with microbial fermentation, the catalytic transformation was carried out in a sequential mode. With the hollow fiber membrane developed it is possible to perform active extraction of 1,3-propanediol *in situ* in a batch (or better in fed-batch) fermentor during the fermentation process. The experimental set up is simple as the membrane module can be immersed into a fermentor.

Although in the reaction of 1,3-propanediol esterification with linoleic acid a high conversion of 75-80 % was obtained, the product was dissolved in the unreacted linoleic acid. Because conversion does not depend on lower than 50 % percentage of the organic phase a lower initial concentration (and therefore lower volume of organic phase) of linoleic acid can be used. Combined with continuous esterification optimized for the highest conversion in one run through the membrane, the product of higher purity can be obtained.

Different esters can be synthesized using the system developed in this work. To obtain a polymer, a suitable diacid needs to be found for the reaction of esterification with 1,3-propanediol; transesterification with esters of diacids is suppressed by hydrolysis and is not suitable for polymers synthesis, as we showed in our study.

Appendix

Preliminary study of C. butyricum growth

To optimize the fermentation process of glycerol transformation to 1,3-propanediol, preliminary study of *C. butyricum* growth was carried out under the direction of Dr. Martin Rebros in Manchester Interdisciplinary Biocentre.

Firstly, the inoculation procedure was optimised. The bacteria were grown from a single colony in the reinforce clostridium medium (RCM). This is commercial, rich in nutrients, fermentation medium. Dependence of optical density at 660 nm on time of inoculation process is shown in Figure 1.

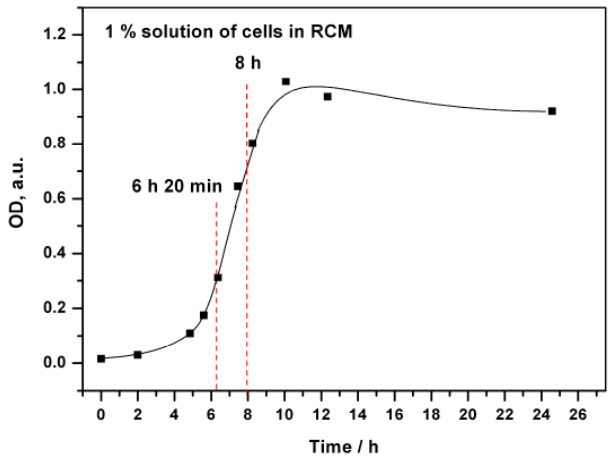


Figure 1. Growth of bacteria from a single colony in reinforce clostridium medium (RCM) during a day. The best area for inoculum development in rich glycerol medium is shown by dash lines on the plot.

In Figure 1 we can see that log phase lasts about 3 h, then the amount of cells doubles in ~ 1 h during exponential phase which takes 4 h, followed by death phase from 12 h after the beginning of fermentation. Therefore the best time for transportation of the inoculum into glycerol-rich medium is 6.20 – 8 h since the start of inoculation. Further study showed that instead of reinforce clostridium medium, glycerol medium prepared for fermentation could be used for inoculum growth. Due to prolonged lag phase such inolculum could be transferred after 12-14 h from the beginning of inoculation from a single colony.

Figure 2 shows that contaminant cells appeared in the inoculum medium and grew during experiment reaching 40 % content compared to the main *Clostridium* bacteria. This probably could affect the growth rate of the former. Therefore, special precautions must be taken to avoid contamination.

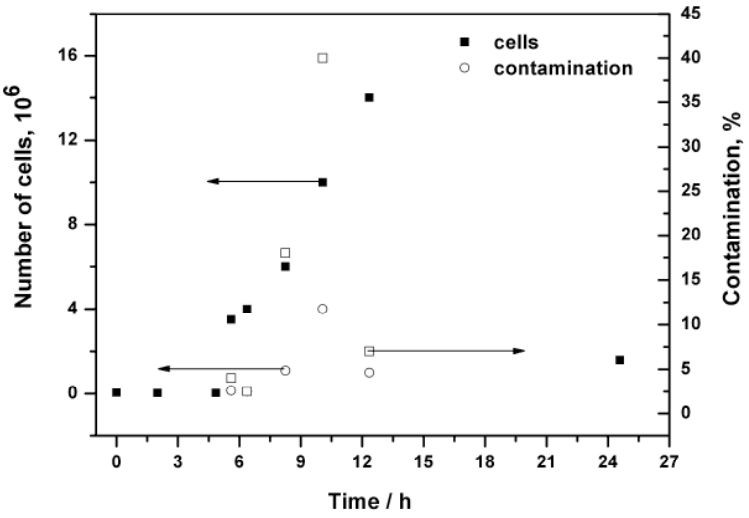
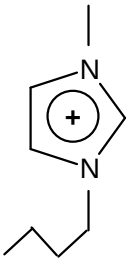
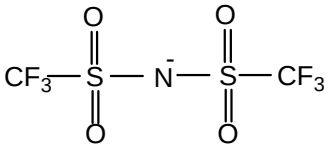
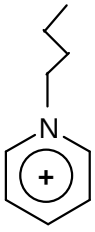
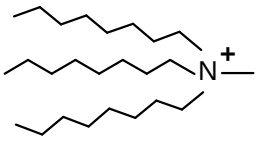


Figure 2. Number of cells, grown during inoculation, contaminating cells and their percentage versus time of experiment.

Synthesis and characterization of ionic liquids

In our work we chose bis(trifluoromethylsulfonyl)imide anion ionic liquids for investigation and testing as they are immiscible with water and therefore able to form a two-phase system for bio- and chemical tandem. We paid our attention mostly to three bis(trifluoromethyl-sulfonyl)imide anion ILs with different cations shown in Table 1.

Table 1. Abbreviations, names and structures of used ILs.

Abbreviation	*Name*	*Cation*	*Anion*
$BmimNTf_2$	1-buthyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide		
$BuPyNTf_2$	1-buthylpyridinium bis(trifluoromethylsulfonyl) imide		
$AmNTf_2$	methyltrioctylammonium bis(trifluoromethylsulfonyl) imide		

All ionic liquids synthesised in this work were obtained by metathesis of lithium bis(trifluoromethylsulfonyl)amine with the appropriate salt, mainly chloride of the cation needed:

$$XCl + LiNTf_2 \longrightarrow XNTf_2 + LiCl\downarrow \text{ where } X - Am, BuPy, Bmim.$$

This reaction is exothermic (leading to precipitation of LiCl) and carried out in water as initial salts are soluble in water but the product is not. In the case of $AmNTf_2$, acetone was used as reaction solvent and evaporated afterwards. After decantation the ionic liquid should be washed with water carefully to purify from chloride ions. As reported by Seddon *et al*, impurities of chlorides and water have a significant influence on the physical and chemical properties of ionic liquid, and a wide variety of different incomparable data can result from that [189, 190]. In our work, a 10 fold decrease in Cl^- ion concentration in, for example, 4 mL of $BuPyNTf_2$ was obtained by washing with 80 mL of fresh water (1:20). If we evaluate the concentration of Cl^- ions titrated in grams per gram of IL we can obtain 4 ppm (w/w), which is quite a low number. At the same time ~ 30 % of IL was lost during washing.

The next step of IL purification is drying at 70-80 °C under reduced pressure for 1-2 days. Moreover, IL should be kept under an Ar atmosphere, closed tightly, in dried atmosphere because exposure to air leads to absorption of water as was detected by Karl Fischer titration. It can be seen from Figure 3 that after 35-50 min of exposure to the air water content in hydrophobic ILs increases 2-3.5 times. To get a real value of water concentration in freshly dried ILs, samples from flasks under Ar were taken for Karl Fischer titration. The results are 32, 28, 48 ppm w/w water in $BmimNTf_2$, $BuPyNTf_2$ and $AmNTf_2$ ionic liquids accordingly.

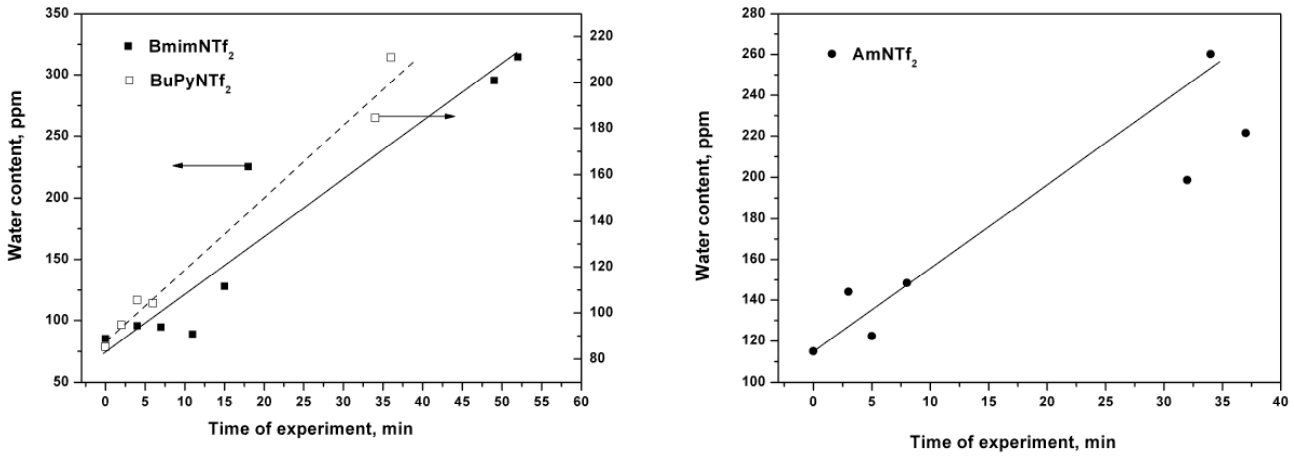


Figure 3. Water content in $BuPyNTf_2$, $BmimNTf_2$ and $AmNTf_2$ ionic liquids determined by Karl-Fisher titration depending on the duration of exposure to air.

Tests of miscibility of $BmimNTf_2$ and $AmNTf_2$ with some organic solvents (CH_2Cl_2, hexane, toluene, diethyl ether, ethanol, THF, ethyl acetate, 2-propanol, pentane, CH_3CN, methanol, acetone) showed that $BmimNTf_2$ was immiscible with hexane and diethyl ether only, and $AmNTf_2$ was miscible with all tested solvents except formic acid (see Table 2). Therefore question of miscibility of IL with any solvent cannot be considered in terms of polarity coefficient only, but other aspects should be taken into account, such as capability of solvent to form ionic pairs, presence of O, N atoms, protons to form hydrogen bonding, heterocyclic or aromatic rings, alkyl hydrophobic chains and so on. For example, insolubility of oxychlorides at room temperature in NTf_2 anion ILs can be explained by the presence of 8 molecules of water in their coordination sphere, preventing from dilution in hydrophobic IL.

Table 2. Miscibility of ILs with organic solvents.

Solvent	**$BmimNTf_2$**	**$AmNTf_2$**
Non-polar		
Dichloromethane	+	+
Hexane	-	+
Toluene	+	+
Diethyl ether	-	+
Pentane	+	+
Ethyl acetate	+	+
Polar Aprotic		
Tetrahydrofuran	+	+
Acetonitrile	+	+
Acetone	+	+
Polar Protic		
Methanol	+	+
Ethanol	+	+
2-Propanol	+	+
Formic acid		-

As a result of this part of work we can say that ILs can be prepared by simple reaction between appropriate salt with lithium bis(trifluoromethylsulfonyl)amine, but purification from chloride ions should be carried out thoroughly that leads inevitably to loss of product during repeated washing procedure with copious amount of water. More attention should be paid also

to drying and storage of ILs because even hydrophobic ILs are able to adsorb water from air at room temperature as was confirmed by Karl Fischer titration. The fact, that prediction of miscibility of ILs with organic solvents related to protic, aprotic polar or non-polar groups cannot be done without experimental test, points at diversity of interactions between cations, anions in ionic liquid with molecules of organic solvent. Among interactions that could be hydrogen bonding, electrostatic, van der Waals interactions and others one or all of them can act as a main reason for miscibility with different organic solvents.

PLLA synthesis in ILs by Zr catalyst (1)

$BmimNTf_2$ and $AmNTf_2$ ionic liquids were tested in the reaction of ring-opening polymerization of L-lactide by a Zr catalyst (1). This catalyst is characterized by hexagonal coordination of Zr atom by C_3-symmetric aminotriphenolate ligands (see Figure 4) [147]. The Zr catalyst (1) allows to obtain highly heterotactic PLLA in rac-lactide polymerization under solventless conditions at 130 °C, or at ambient temperature using toluene as a solvent [147]. However this catalyst is sensitive to air and moisture.

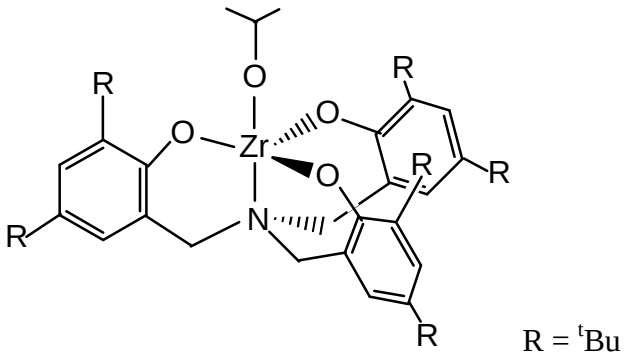


Figure 4. The structure of Zr catalyst (1) used for L-lactide polymerization.

To check activity and possibility of stabilization of the catalyst in hydrophobic ILs, L-lactide polymerization was carried out using $BmimNTf_2$ and $AmNT_2$ as a solvent medium instead of toluene. Reaction conditions and results are shown in Table 3.

Table 3. Catalyst loading, temperature, time and yield for PLLA synthesis by Zr catalyst (1) in hydrophobic ILs.

Solvent	Catalyst, %	Temperature, °C	Time, h	Yield, %
$BmimNTf_2$	0.5	25	24	No product
$BmimNTf_2$ [a]	0.55	100	24	70
$AmNTf_2$	0.6	100	24	No product

[a] in this case after filtration of polymer and evaporation of MeOH reaction was repeated with L-lactide, but no product was obtained.

As one can see from Table 3 only in the case of $BmimNTf_2$ ionic liquid and at 100 °C 70 % of PLLA was obtained using 0.5 % (mol) of the Zr catalyst (1). The yield obtained is comparable with the same in toluene as a solvent [147]. ^{1}H NMR analysis gave sufficient pattern of two main peaks at 1.6 ppm $(-CH_3)_n$ and 5.0 ppm $(-CH)_n$ (see Figure 6) if compare to NMR spectrum of L-lactide (Figure 5). HPLC analysis confirmed that PLLA had M_n = 10,760, M_w = 12,265 and PD = 1.14 comparing to for example M_w = 57,650 and PD = 1.13 of PLLA obtained by the same catalyst in melt polymerization at 130 °C. Mass spectrometry showed good distribution of polymer fragments with M(m/z) equal around 4550.

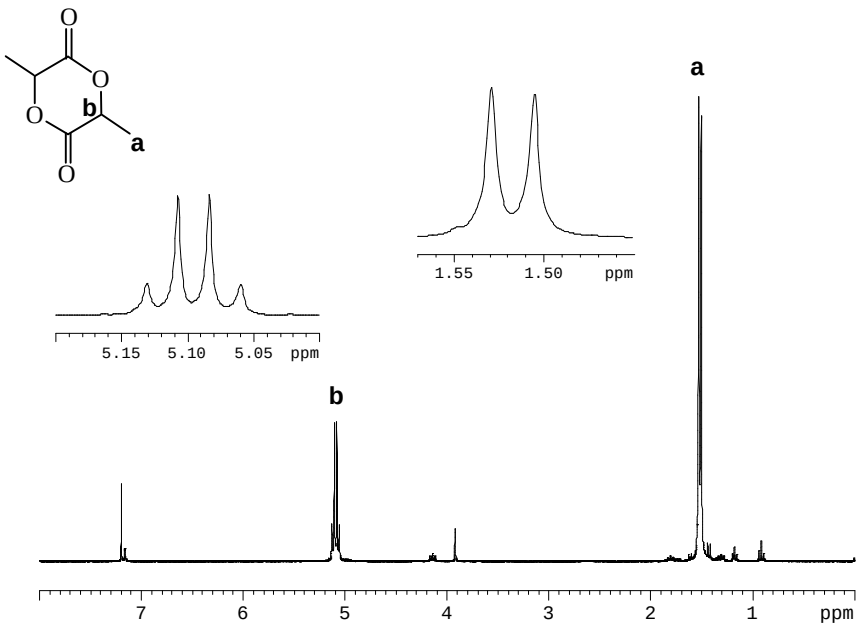


Figure 5. ^{1}H NMR spectrum of L-lactide in $CDCl_{3.}$

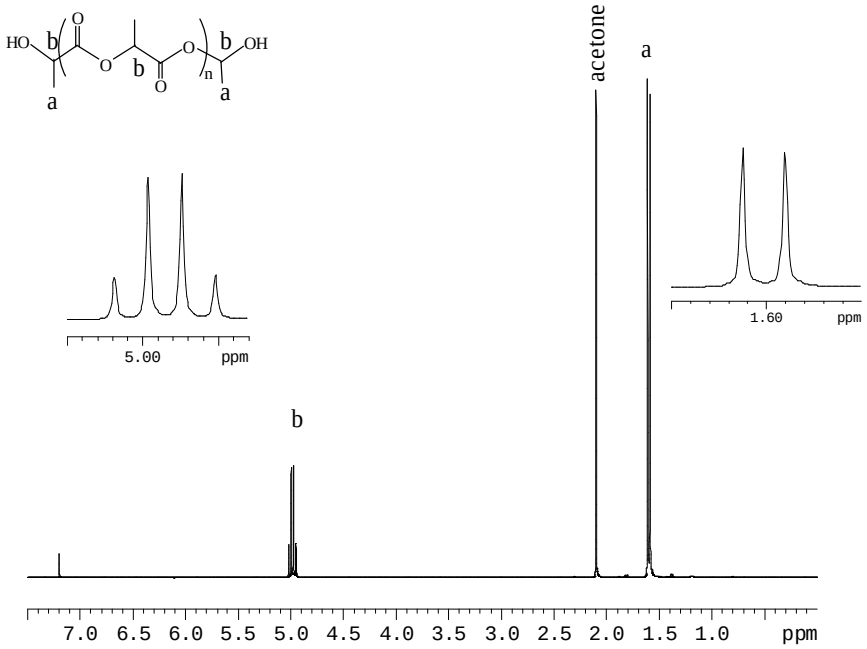


Figure 6. ^{1}H NMR analysis of PLLA obtained by ROP of L-lactide at 100 °C in $BmimNTf_2$ using Zr catalyst (1).

ROP carried out at room temperature does not lead to PLLA formation probably due to low solubility of L-lactide and catalyst in highly viscous ionic liquid. Inactivity of the Zr catalyst (1) in $AmNTf_2$ even at 100 °C could be due to insufficient purity of this ionic liquid, because the higher concentration of water compared to $BmimNTf_2$ could be crucial for deactivation of the catalyst. At the same time it is interesting to mention that in the presence of alcohol in reaction medium carbene radical can be generated from imidazolium ring. This carbene catalyzes reaction of ring-opening polymerization as shown by Dove *et al* [191]. To check activity of Bmim IL in ROP of lactide itself, further investigation should be carried out.

As a result we can conclude that ionic liquid can be used for L-lactide ring-opening polymerization by the Zr catalyst (1) (0.5 % mol), but only at 100 °C. At the same time the catalyst is sensitive to impurities in the solvent, and ionic liquid does not enhance stability of the catalyst.

1,3-Propanediol esterification in ionic liquids

The use of Zr oxychlorides to esterify alcohols was shown to be a simple procedure with the possibility to reuse catalyst [192]. In our work reaction of 1,3-propanediol esterification with phenylbutyric acid was carried out using $ZrOCl_2$ catalyst and different solvents as a reaction medium (Figure 7).

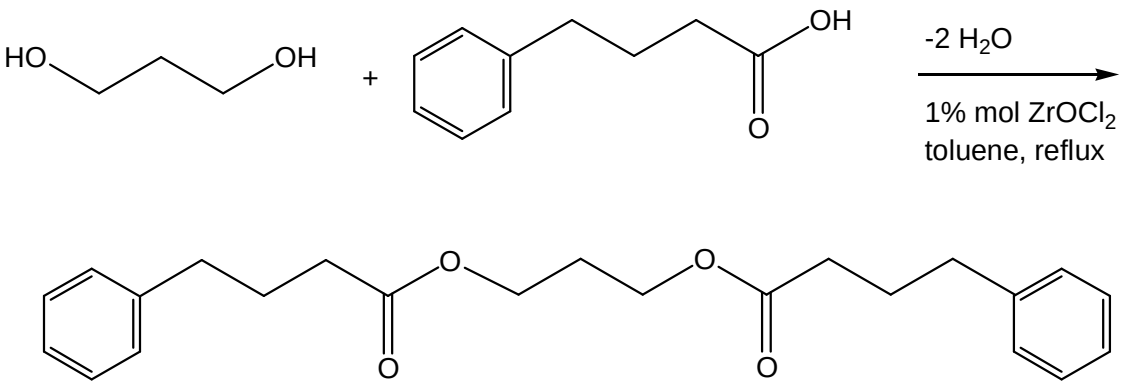


Figure 7. Esterification of phenylbutyric acid with 1,3-propanediol.

In Table 4 conditions and results of 4-phenylbutyric and 1,3-propanediol esterification are presented.

Table 4. Results and reaction conditions for PD esterification in different solvents by $ZrOCl_2$ catalyst. In toluene 1 % mol of catalyst was used, in the rest experiments – 2 % mol.

Solvent	Temperature, °C	Extractive solvent	Yield of unpurified[a] product, %	Yield after reusing cat, %
Toluene	170	No[b]	90	83.5
Tetradecane	110	diethyl ether	102	-
$BmimNTf_2$	150	diethyl ether	110	113
$AmNTf_2$	110	formic acid	129	no product
$BmimNTf_2$	150	diethyl ether	150[c]	-

[a] high yields of unpurified product (more than 100 %) are due to residual solvent.

[b] product was obtained after toluene evaporation under reduced pressure, extraction of catalyst was carried out by 1M HCl.

[c] acetic acid was used instead of phenylbutyric acid.

The use of IL instead of toluene gives one moderate benefit such as using extractive immiscible with IL solvent - diethyl ether or formic acid – instead of catalyst extraction by HCl solution. Evaporation of extractive solvent gives the product which, however, has high impurities of IL. Dissolving ILs in extractive solvents is confirmed by high (more than 100 %) yields of unpurified product and presence of peaks related to IL in ^{1}H NMR spectra (Figure 8) compared to the ester obtained in toluene (Figure 9).

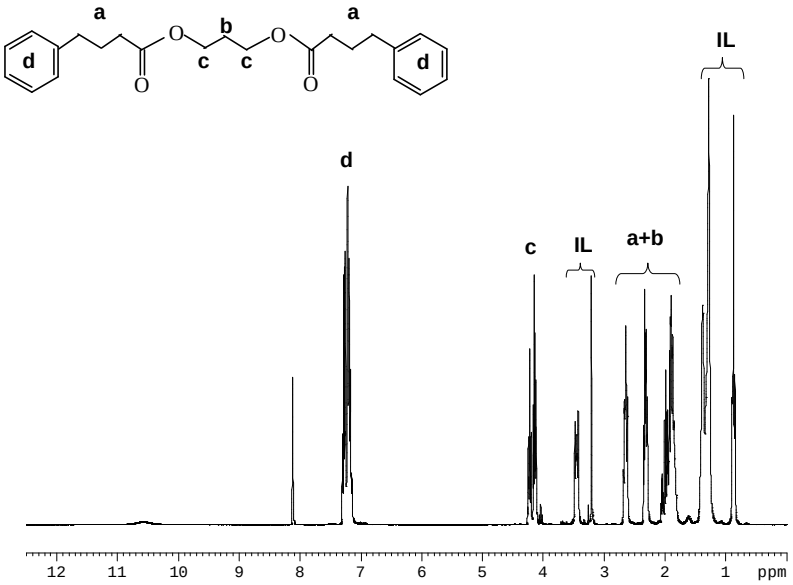


Figure 8. ^{1}H NMR analysis of diester obtained by eserification of 1,3-propanediol and phenylbutyric acid in $AmNTf_2$ at 100 °C using Zr oxychloride catalyst.

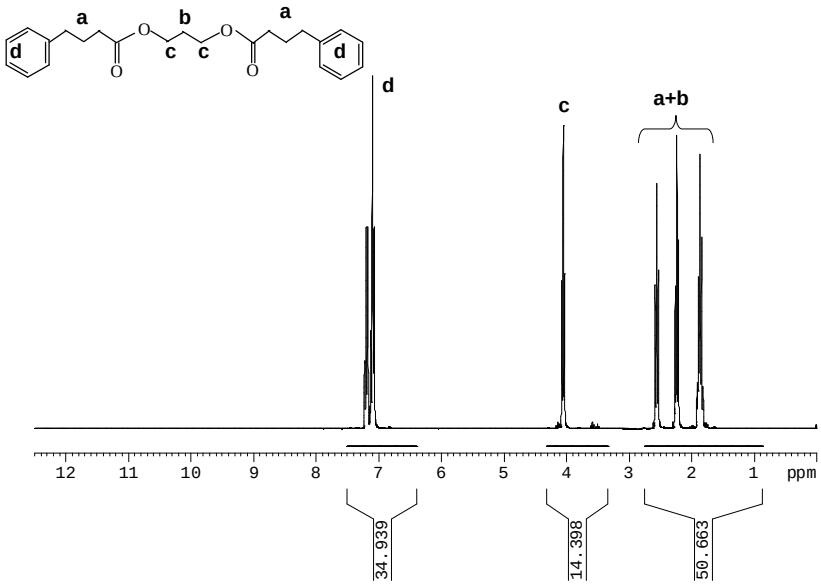


Figure 9. ^{1}H NMR spectrum of ester obtained in toluene (2 % mol $ZrOCl_2$ catalyst, 24 h, 150 °C).

In the case of $AmNTf_2$ ionic liquid it is difficult to extract product without extraction of the catalyst. As only one solvent immiscible with $AmNTf_2$ within variety of organic solvents formic acid was used to extract ester, but at the same time $ZrOCl_2$ catalyst dissolves in acidic solutions. This was confirmed by attempt to reuse reaction medium after first run giving no product (see Table 4) comparing to experiment with $BmimNTf_2$ as a solvent.

Carrying out the reaction of esterification between 1,3-propanediol and acetic acid in $BmimNTf_2$ ionic liquid at 150 °C leads to ester formation which also contains considerable amount of IL (see Table 4).

Finally, we can say that IL as a solvent for reaction of 1,3-propanediol esterification shows some benefits comparing to toluene such as: lower reaction temperature, simplicity of experiment and extraction of the product. At the same time purification of the ester is demanded.

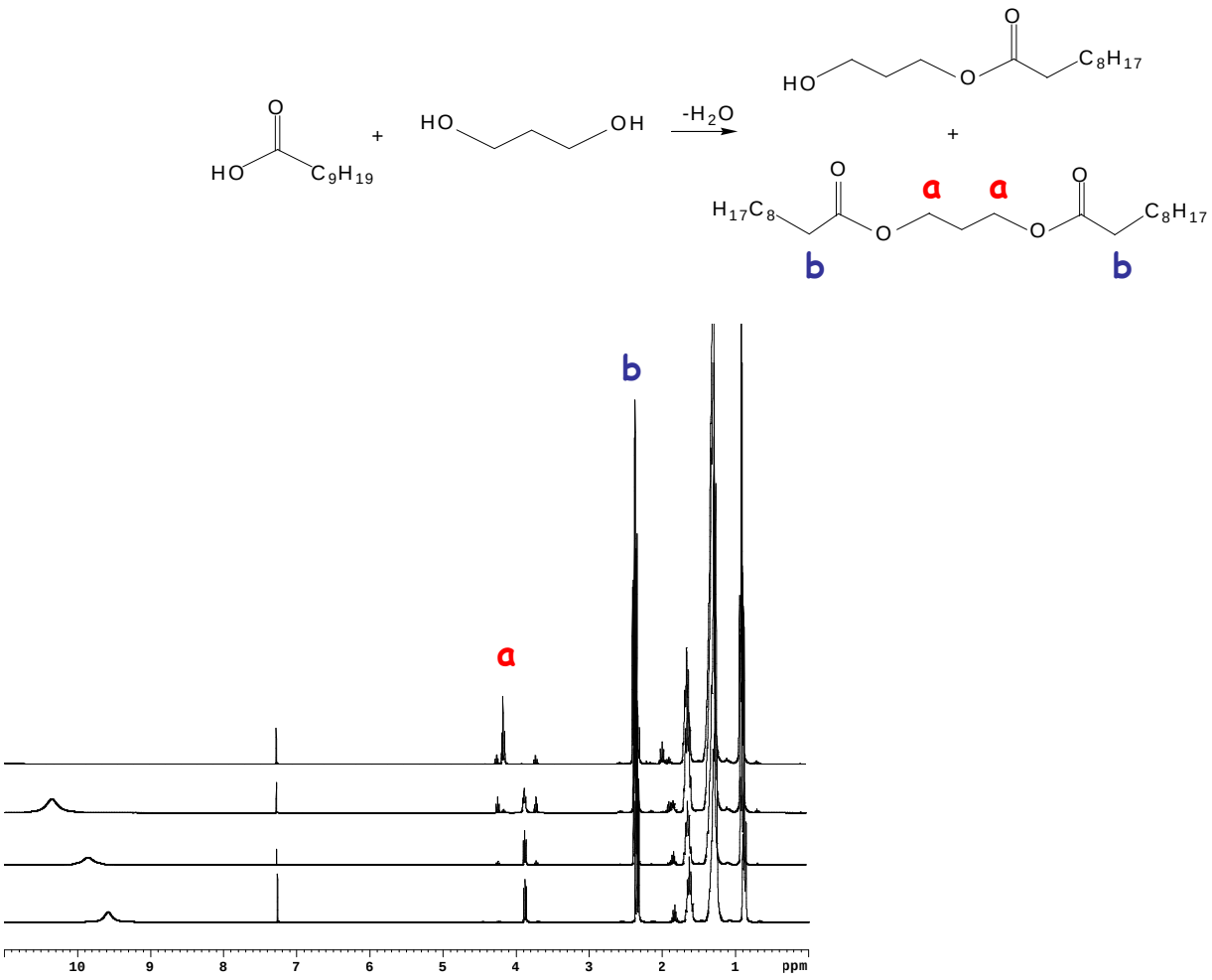


Figure 10. Changes in NMR spectrum during the reaction of 1,3-propanediol esterification with decanoic acid.

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