STUDIES ON ENZYMATIC SYNTHESIS OF NATURAL ETHYL ACETATE IN NON-CONVENTIONAL MEDIA

by

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Értekezés doktori (PhD) fokozat elnyerése érdekében
*a Veszprémi Egyetem
Doktori Iskolájához tartozóan*.

Írta:
Amir Kabiri Badr

**Készült a Veszprémi Egyetem …….Vegyészmérnöki Tudományok Doktori Iskolája keretében

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Elfogadásra javaslom (igen / nem)

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Megjegyzés: a * közötti részt az egyéni felkészülők, a ** közötti részt a szervezett képzésben résztvevők használják, *** esetleges

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Introduction

According to recent trends, more and more natural flavours are being used in the food, cosmetic, pharmaceutical and beverage industries, since consumers prefer foodstuffs that can be labelled natural. The term ”natural” often has positive connotations, while terms employing the word ”artificial” have a negative impact. The range of flavours that can be obtained directly from fruits is limited and their compositions depend highly on climate, weather and soil conditions. There is, therefore, an increasing demand for reliable synthesis routes to large amounts of high-quality natural flavour compounds. Biotechnological methods are suitable for the synthesis of these compounds.

Esters are a very important group of flavours. Synthesis of esters can be carried out by using lipase enzyme in non-aqueous systems, e.g. in organic solvent. A further challenge is, however, to avoid the application of any organic solvent during the process. Thus, the flavour ester compound can be expected to be free of any solvent traces, and the products may be regarded as even more “natural”.

In this PhD work the esterification reaction of acetic acid and ethanol was studied. The process was realised by using a biocatalyst: an immobilized lipase enzyme. The esterification resulted in a highly volatile flavour ester compound: ethyl acetate and water as a by-product. During the research work kinetics of the reaction both in organic solvent and solvent-free system, controlling of the water content and recovery of the ester formed were studied as well as realisation of the process in integrated system was investigated to enhance the effectiveness.

1. Literature review

1.1. Enzyme catalysis

The name enzyme originated from Greek word of ζγμειν which means to ferment, in 1687 W. Kühne used the name enzyme to indicate ferments or "active agents of fermentations". Later on in 1893 F. W. Ostwald indicated that enzymes are biological catalysts. A year later E. Fischer proposed very famous concept of "Lock and key" [Lehninger A. L., 1975]. This concept gives us very simple explanation for the biological asymmetry of enzyme mechanisms and led to a very popular misconception of "one enzyme – one substrate". In 1913 L. Michaelis and M. Menten described the enzymatic kinetics [Cabral, J. M. S., Best, D., Boross L., Tramper, J., 1994], studying the rate of reaction catalysed by invertase at constant temperature and pH.

Enzyme nomenclature derives from what the enzyme does rather than the enzyme is [Dixon M., Webb E. C., 1964]. The suffix "ase" is added either to the reaction, which is catalysed or to the substrate name. There are six major classes of reactions which enzymes catalyse: *Oxidoreductases, Transferases, Hydrolases, Lyases, Isomerases* and *Ligases*.

Enzymes are catalysts that increase the rate of a chemical reaction without undergoing a permanent chemical change [Suelter, C.H., 1985; Fersh, A., 1984; Godfrey, T., Reichelt, J., 1983]. While a catalyst influences the rate of a chemical reaction, it does not affect reaction equilibrium. A characteristic of enzymes is their frequent need for cofactor [Gray C. J., 1971]. Cofactor is defined as non-protein compound, which combines with an otherwise inactive protein to give a catalytically active complex e.g. metal ions.

Some enzymes are very specific, that means it can catalyse only one reaction involving only certain substrate [Blanch, H., Clark, W., 1997]. Specificity of enzyme is conformation which allows formation of the "active side", a hydrophobic cavity responsible for the catalytic ability of the enzyme [Bailey, J. E., Ollis, D. F., 1986].

Immobilized enzymes are enzymes that are attached to, or entrapped within, a macroscopic support matrix so that the resulting catalyst can be reused [Howell, J. A., Knapp, J. S., Velicangil, O., 1978]. Immobilized enzymes offer several potential advantages over soluble enzymes. Immobilized enzymes are typically macroscopic catalysts that are retained in the reactor; therefore, continuous replacement of the enzyme is not necessary, and separation of the enzyme from other components in the reaction mixture is simplified [Atkinson, B., Mavituna, F., 1984]. Immobilized enzymes can be employed in a wide range of different reactor configurations and because high concentration of catalyst can be obtained, correspondingly high volumetric productivities are possible [Crueger W., Crueger A., 1984].

1.2. Enzyme kinetics

Reaction rate as a function of substrate concentration

Consider S as a molar concentration of substrate and P as a molar concentration of product [Keleti T., 1985; Plowman, K.M., 1972; Cornish-Bowden, A., 1976; Wong, J.T.F., 1975]. The reaction is $\mathbf{S} \rightarrow \mathbf{P}$, the reaction rate V, in the quasi-state approximation is defined by

$$V = -\frac{dS}{dt} = \frac{dP}{dt} \qquad (1)$$

The dimension of the V is moles per unite volume per unite time. At the beginning of the reaction [P] is small**.** As [P] grows, the back reaction rate increases until equilibrium is reached.

Keeping enzyme concentration constant and measuring the product formation as a function of time (Figure 1.1), before the reaction is near the equilibrium, at different initial substrate concentrations is a way to measure the kinetic properties of a given enzyme [Sevella, B., 1993].

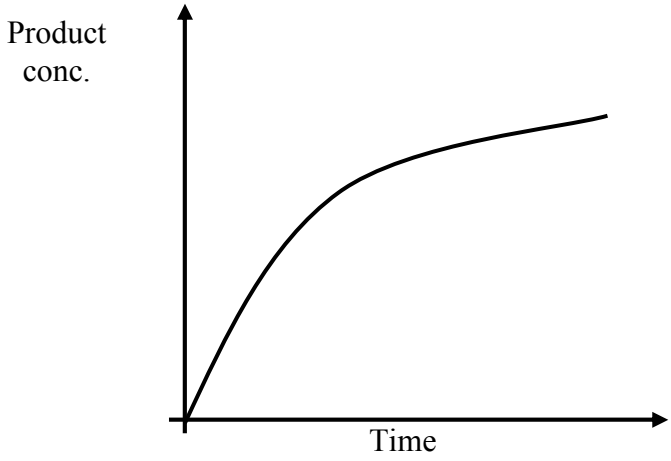


Figure 1.1.: Measure of reaction rate in an enzymatic process

The initial rate V_0 is plotted vs. substrate concentration and the behaviour of it can be seen in Figure 1.2. The velocity is substrate [S] dependent, as [S] is increased, the enzyme molecule are saturated with substrate and the reaction rate depends on the amount of the enzyme. Since the enzyme concentration is constant, the reaction rate approaches to V_{max} in high substrate concentrations [Segel, I. H., 1975].

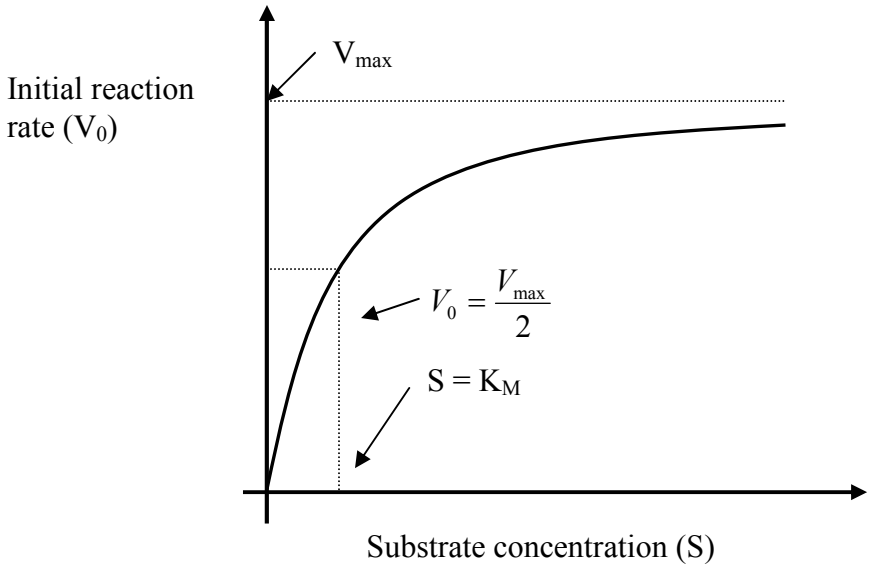
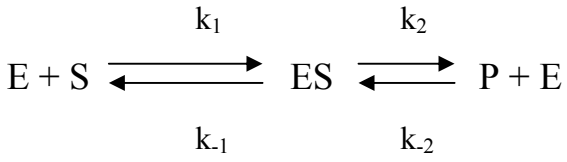


Figure 1.2.: Measure of kinetic parameters

The kinetic behaviour of the enzyme can be modelled mathematically known as Michaelis-Menten equation.



Considering the initial velocity of the reaction, there will be negligibly small amount of product [P] present ([P] < 5% of [S]), so back reactions are negligible, that is k_{-2} [P] = 0. The initial velocity is:

$$V_0 = k_2[ES] \qquad (2)$$

Where

[S] Initial concentration of substrate

[P] Product produced

[E] Total amount of enzyme added to the reaction

Transforming the equation and introducing the two parameters (V_{max} - maximal reaction rate, K_M - Michaelis constant), Michaelis-Menten equation as it is normally known is obtained:

$$V_0 = \frac{V_{\max}[S]}{K_M + [S]} \qquad (3)$$

It has to be recalled that this equation is derived for V_0, when very little product is formed and the back reaction can be ignored.

When [S] is small ([S]<<K_M), the activity is in linear range. In this case [S] + K_M is approximately equal to K_M and Eq. (3) becomes:

$$V_0 = \frac{V_{\max}[S]}{K_M} \qquad (4)$$

Which means that at low[S] values V_0 is linearly proportional to [S].

In the case where [S] = K_M, Eq. (3) becomes:

$$V_0 = \frac{V_{max}}{2} \qquad (5)$$

By this, K_M is defined as the substrate concentration that gives half the maximum rate.

V_{max} and K_M are the two parameters, which define the kinetic behaviour of an enzyme as function of [S]. V_{max} is a measure of how fast the enzyme can go at full speed. It is a rate of reaction and will have unites of concentration per unit of time.

K_M is a measure of roughly how much substrate is required to get to full speed. If $[S] >> K_M$ then V_0 will be close to V_{max}. K_M is a concentration and will have units of mol/L.

The quantities K_M and V_{max} are experimentally determined and different for each enzyme. K_M and V_{max} can be estimated from the graph of initial velocity versus [S]. The procedure to conduct is as follows:

1. Run a series of reactions with constant $[E]_{tot}$, varying [S], and measure V_0.
2. Graph V_0 *vs* [S].
3. Estimate V_{max} from the asymptote.
4. Calculate $V_{max}/2$.
5. Read K_M from graph.

It is hard to extrapolate to infinite [S] and guess V_{max}. For this reason, the **Lineweaver-Burk** plot is used. This equation is obtained by rearranging the Michaelis-Menten equation in linear form.

$$\frac{1}{V_0} = \frac{K_M}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (6)$$

By plotting $1/V_0$ *vs.* 1/[S] data, a straight line is obtained, where the intercept to y-axis is $1/V_{max}$ and the slope is K_M/V_{max}. From this graph it is easy to estimate K_M and V_{max} (Figure 1.3).

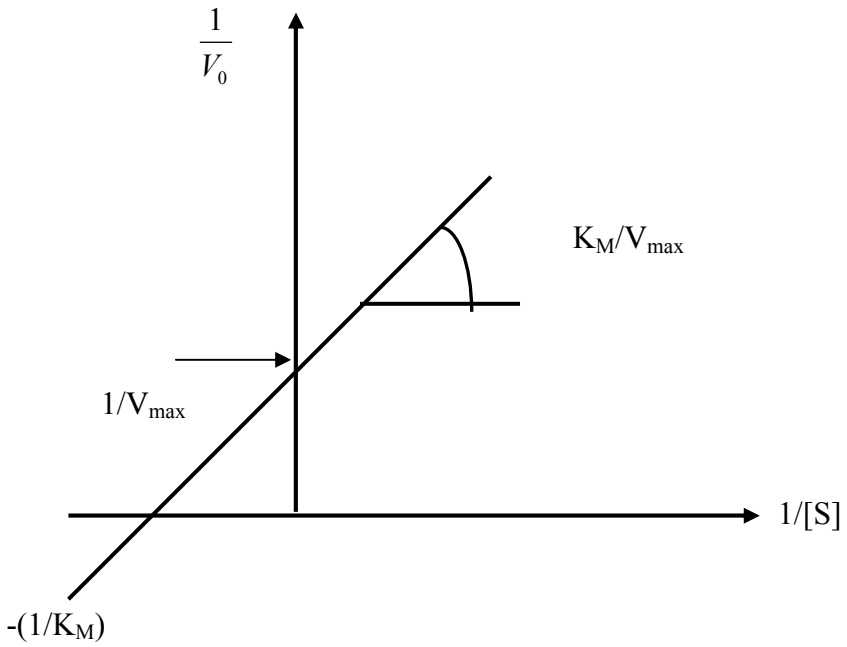


Figure 1.3.: Lineweaver-Burk plot.

Inhibitions

Any substance that decreases the rate of an enzyme-catalysed reaction is considered as an inhibitor [Segel, I. H., 1975]. Inhibition of enzyme helps us to understand specificity, chemical and physical structure of the active site and kinetic mechanism of the reaction. The main roles of enzyme inhibitors in our daily life are preservatives, antibiotics, toxins, poisons and pharmaceutical products. There are two main groups of inhibition, reversible and irreversible. Reversible inhibitors can be eliminated by detaching while irreversible types of inhibition are those which action is not changed by detaching of the inhibitor. Reversible group can be divided into three subgroups:

1.Competitive inhibition: compound that joins with free enzyme in a way that stops substrate binding.
2.Noncompetitive inhibitor does not effect on substrate binding so inhibitor and substrate can bind differently.
3.Uncompetitive inhibition: Substance that reversibly combine with the enzyme substrate complex not the free enzyme.

Substrate inhibition

In several enzymatic processes it was found that the reaction rate did not increase beyond a certain, optimal substrate concentration and it did not remain approximately constant above the saturation concentration, but the rate decreased. It means that a type of inhibition occurred. The phenomenon was called substrate inhibition, though its name - more accurately – is an inhibition caused by substrate excess [Keleti T., 1985].

For the description of substrate inhibition the classical Michaelis-Menten equation was modified, as follows:

$$v_o = \frac{v_{max} S}{K_M + S + S^2 / K_I} \qquad (7)$$

where K_I is the inhibition constant, which characterizes the process from the inhibition point of view.

Effect of temperature on enzyme activity

A chemical transformation $A \rightarrow B$, involves the random activation of molecules in the A population to specific, high-energy conformation that is designated the transition state. Those molecules that are in the transition-state conformation will, at a relatively fixed frequency, undergo the transformation to product, B. Thus the rate of the reaction will be proportional to the concentration of the transition-state species. The concentration of the transition state species, in turn, depends on the amount of thermal energy required to produce the transition-state species of the reacting molecules. In enzyme-catalysed reactions, the activation energy is less than in the corresponding uncatalysed reaction [Segel, I. H., 1975]. The transition state level is more readily with a result that more molecules enter the transition state and form product B. The enzyme, of course, does not alter the ΔG for the reaction but only reduces the activation energy that molecule A must attain before it can undergo change.

The familiar Arrhenius-equation relates the specific reaction rate constant, k, to temperature (T):

$$k = k_o \, e^{-E_A/RT} \qquad (8)$$

where k_o- pre-exponential factor

E_A- activation energy

R- gas-low constant (8.31 J/mol K)

Another form of the equation is

$$\ln k = \ln k_o - E_A/R.1/T \qquad (9)$$

The equation predicts that the rate of the reaction, being it enzymatically catalysed or not, will be increased with increasing temperature. However, since enzymes are proteins and any protein will be denatured if the temperature is raised sufficiently, enzyme catalysed reactions show an increase in rate with increasing temperature only within relatively small and low temperature range. The combined effects of temperature on the enzyme-catalysed reaction and the enzyme protein result in a typical curve (reaction rate versus temperature, Figure 1.4) having a maximum. The activation energy characterising the enzymatic reaction can be determined by the linear transformation of the Arrhenius-equation.

The optimum of temperature of the enzyme-catalysed reaction will depend on several factors, including how long the enzyme is incubated at the test temperature true before the substrate is added and the type of organism from which the enzyme was derived. Longer incubations before addition of substrate will allow more time for inactivation of the enzyme protein; enzymes from thermophilic organisms generally will be much more stable at elevated temperature than enzymes from other organisms.

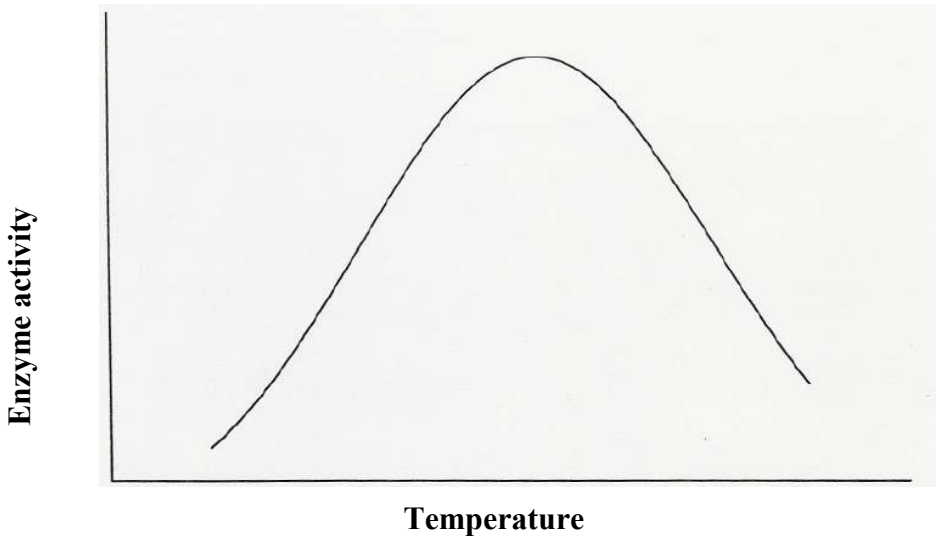


Figure 1.4.: Reaction rate versus temperature in enzymatic process

Multi-reactant enzymes

The "simple" Michaelis-Menten model was developed originally for one-substrate type enzymatic reactions. However, in several reactions two or more substrates are involved, and two or more products are formed [Segel, I. H., 1975]. In these cases the kinetics can be studied by either (i) simplifying the system, and only one (the most important) substrate (and/or product) is considered or (ii) all the compounds are taken into account and using a more complicated kinetical description (e.g. bi-bi mechanism....). These kinds of kinetic mechanism can be described with Michaelis-Menten model, where more parameters are available than in original one. There are several graphical methods existing to identify model, as well as to calculate the model parameters. They are usually based on linearization of the original Michaelis-Menten model, like double reciprocal plot (Lineweaver-Burk method). Inhibition effects can be investigated in the same bases [Laszlo, E., 2004].

In many cases simplification of the kinetical approach is accepted, since it makes the application of industrial reaction design easier from the engineering and procedure points of view.

1.3. Lipases

Lipases as triacylglycerol ester hydrolases, (EC. 3.1.1.3) are ubiquitous enzymes that catalyse the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols, and glycerol [Malcata, F.X., 1996; Gandhi, N.N., 1997; Derewenda, Z.S., 1994; Linko, Y.Y., Lamsa, M., Huhtala, A., Rantanen, O., 1995;]. These enzymes are distributed among higher animals, microorganisms and plants in which they fulfil a key role in the biological turnover of lipids. They are required as digestive enzymes to facilitate not only the transfer of lipid from one organism to another, but also the deposition and the mobilization of fat that is used as an energy reservoir within the organism. They are also involved in the metabolism of intracellular lipids, and, therefore, in the functioning of biological membranes.

Lipases have been extensively investigated with respect to their bio chemical and physiological properties, and lately for their industrial applications [Liese, A., Filho, M.V., 1999; Schulze, B., Wubbolts, M.G., 1999]. The increasing interest in lipase research over the past decades has likely occurred for three reasons [Kazlauskas, R.J., Bornscheuer, U.T., 1998; Bornscheuer, U.T., 2002]. The first is related to the molecular basis of the enzyme catalytic function. The second reason is linked to the enzyme's medical relevance and its importance in regulation and metabolism. Lastly, it was discovered that lipases are powerful tools for catalysing not only hydrolysis, but also various reverse reactions, such as esterification, transesterification and aminolysis in organic solvents [Brink, L. E. S., Tramper, J., 1985; Boutur, O., Dubreucq, E., Galzy, P., 1995; MacNaughtan, M. D., Daugulis, A. J., 1993]. The possible reaction ways are presented in figure 1.5 [Paiva A.L., Balcao, V. M., Malcata, F. X., 2000]. Such biocatalysts present some important advantages over classical catalysts. Indeed, their specificity, regioselectivity and enantioselectivity allow them to catalyse reactions with reduced side products, lowered waste treatment costs and under conditions of mild temperature and pressure. Accordingly, considerable attention has been given lately to the commercial use of lipases.

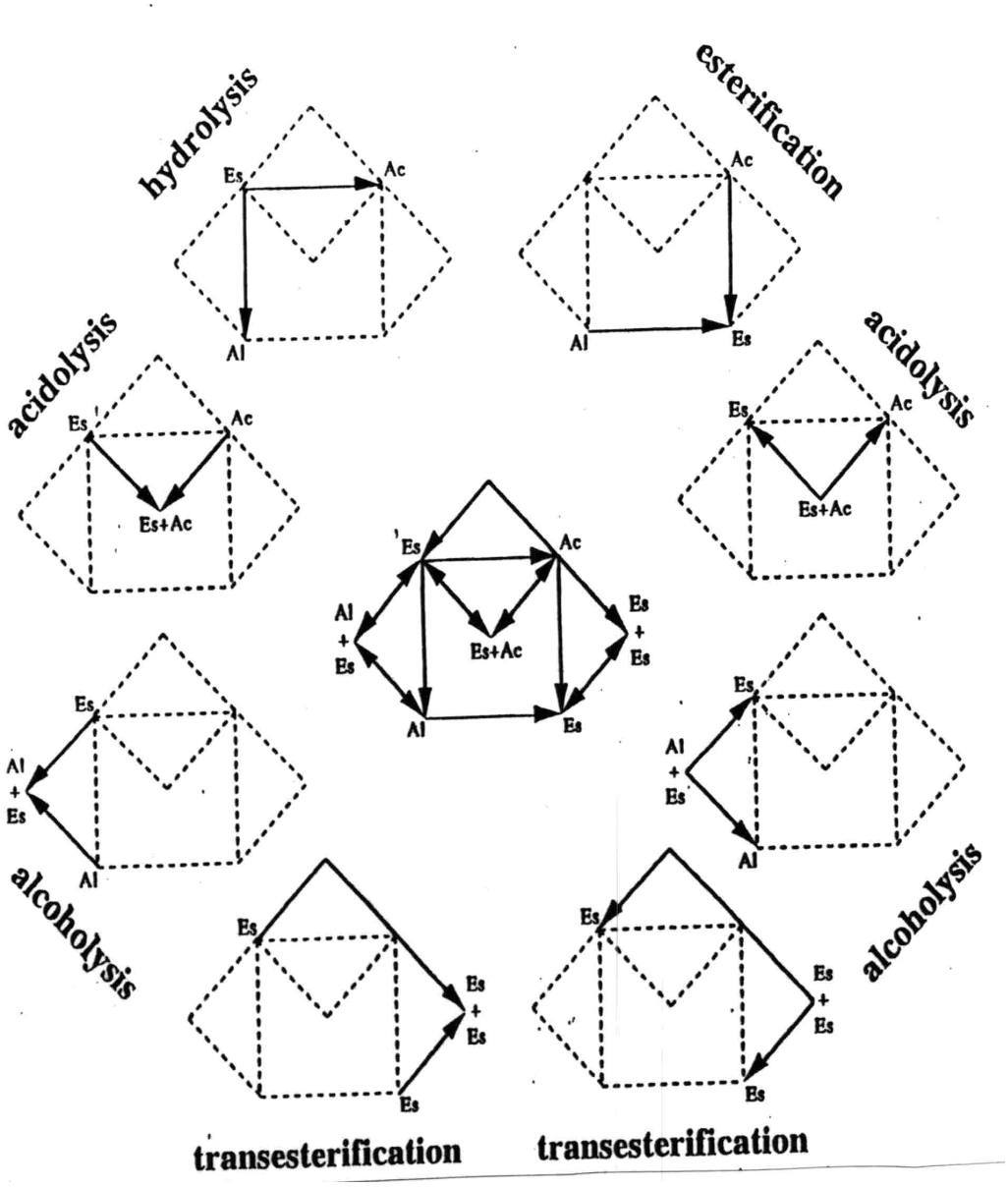


Figure 1.5.: Possible reaction catalysed by lipase enzyme

[Paiva A.L., Balcao, V.M., Malcata, F.X., 2000]

Because of their capability to preserve their catalytic activity in organic solvents, the activities of lipases as catalysts have been investigated to determine their potential for the conversion of surplus fats and oils into higher value products for food and industrial uses [Sharma, R.; Chisti, Y., 2001]. Further examples of their applications are numerous and are found in the resolution of racemic mixtures, the synthesis of pharmaceuticals and new surfactants, the bioconversion of oils, fats, etc [Marlot, C., Langrand, G., 1985]. However, the low stability, low activity or selectivity encountered occasionally with a number of these enzymes, and the relatively prohibitive cost of native enzyme have been the chief obstacle hindering more rapid expansion of industrial lipase technology on a large scale. Therefore, customisation of lipases by chemical and physical modifications has more recently been attempted to improve their catalytic properties in hydrolysis and synthesis involving aqueous and non-aqueous solvents.

1.4. Lipases applied in esterification

Like all other catalysts, lipases increase the rate of the reaction they catalyse without affecting the position of chemical equilibrium. Processing variables such as pH, time, concentration and temperature of compounds present in the reaction medium can have a strong effect on the enzyme activity. Lipases are capable of catalysing the reverse reaction [Lecointe, C., Dubreucq, E., Galzy, P., 1994; Csányi, E., Bélafi-Bakó, K., Venyige, T., Sisak, Cs., 1997] such as ester synthesis:

$$R_1\text{-COOH} + R_2\text{-OH} \leftrightarrow R_1COOR_2 + H_2O$$

acid alcohol ester water

In all reactions carried out by lipases, equilibrium conversion depends on the water content of the reaction mixture; and it can be controlled easily by changing the water content. Water is needed both for maintenance of the enzyme structural integrity and for generation of the catalytic intermediate [Lortie, R., 1997]. Although ester synthesis can be done chemically with acid or base catalysis [Bernal, M. P., Coronas, J., 2000 and 2002], the use of enzyme technology offers the advantages of mild conditions, reduced side reactions, and specificity.

Effect of water

In the esterification reactions water is a by-product, having an important role in the chemical equilibrium. Moreover water level is very important parameter affecting lipase enzyme activity in reaction medium apart from the medium itself. The hydration level of the enzyme greatly influences the flexibility of the protein and thereby the catalytic activity [Wehtje, E., Costes, D., Adlercreutz, P., 1997]. Therefore, it is necessary to measure and control the water level in enzyme-catalyzed reactions. The yield or amount of the product formed can vary greatly depending on reaction medium and water content of the medium.

Water present contributes to the structural integrity, active site polarity, and protein stability. It provides hydrophobic interactions with polar residues on the enzyme molecule, which would otherwise be interacting with each other, creating an incorrect conformational structure. Water can also limit the solubility of hydrophobic substrates around the enzyme. The actual amount needed varies significantly depending on the origin of the lipase. In esterification reactions, the water content affects the equilibrium position of the reactions as well as the distribution of the products in the media. In addition to activity, water affects the thermo-inactivation of enzymes. The water content of the catalyst is more important in dictating catalytic activity than the total water content in the system. Large amounts of water around the enzyme in excess of the amount needed for a complete hydration layer may provide some protection from denaturation by substrates; however, this may cause mass transfer problems for substrates especially for hydrophobic compounds. In contrast, water acts as lubricant to ease unfolding and refolding which often results in denaturation in unfavorable environments, thus, the proper amount of water present in the solvent is critical to ensure high catalytic activity [Yahya, A. R. M., Anderson, W. A., Moo-Young, M., 1998]. However small amount of the water in the reaction medium is essential for the enzyme activity, an increase or decrease in water concentration will directly affect the productivity. Therefore optimum level of water has to be maintained throughout the reaction period.

1.5. Flavour ester production (ethyl acetate)

According to Webster's Dictionary flavour is defined as "the blend of taste and smell sensations evolved by a substance in the mouth". Some important flavour key compounds are listed in figure 1.6, where aromatic, heterocyclic and other components having completely different structure can be seen.

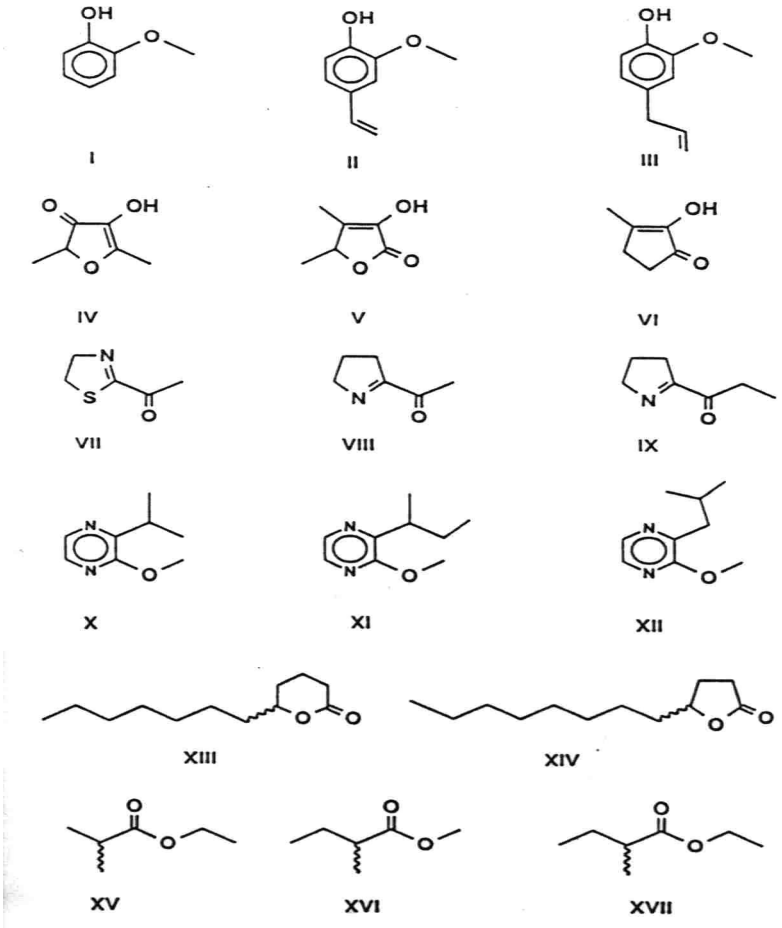


Figure 1.6.: Chemical structure of important flavour compounds

I = Guaiacol, II = 4-Vinyl guaiacol, III = Eugenol, IV = Furaneol, V = Sotolone, VI = Cyclotene, VII = 2-Acetyl-2-thiazoline, VIII = 2-Acetylpyrroline, IX = 2-Propanoylpyrroline, X = 2-Isopropyl-3-methoxypyrazine, XI = 2-sec-Butyl-3-methoxypyrazine, XII = 2-Isobutyl-3-methoxypyrazine, XIII = 5-Dodecanolides, XIV = 4-Dodecanolides, XV = Ethyl 2-methylpropanoates, XVI = Methyl 2-methylbutanoates, XVII = Ethyl 2-methylbutanoates

Esters of short chain carboxylic acids and alcohols (e.g. ethyl 2-metylpropanoates, methyl 2-methylbutanoates and 2-isobutyl-3-methoxypyrazine) also belong to the highly important aroma compounds that are used in different industries [Berger R.G., 1995, [Manjon, A., Iborra J. L. and Arocas, A., 1991]. Those flavour esters are either extract from plant or produced chemically. The third way that currently interested is enzymatic aroma production. These flavour components can be synthesized by microbial lipases as it was presented earlier [Leblanc, D., Morin, A., Gu, D., Zhang, X.M., Biasaillon, J.G., Paquet, M., Dubeau, H., 1998]. The flavours are extremely important due to their nature and have many applications especially in food, pharmaceutical and cosmetic industries.

Ester synthesis by an enzyme (lipase) requires appropriate reaction condition, suitable lipase and substrate concentration. For single aliphatic acid and alcohol substrate the number of carbons are very decisive. Those with less than C_4 have been shown to produce lower ester production and yield then larger molecular weight substrates. This can be described as the fitting effect of substrate on active site of enzyme. This phenomenon is related with the findings that – among the short chain esters - the yields of *ethyl* and *methyl acetate* were found as the lowest [Langrand, G., Rondot, N., Triantaphylides, N., Baratti, J., 1990] regardless of the lipase preparation used.

In this work the purpose was to study the ethyl acetate production by lipase. Natural ethyl acetate has been manufactured enzymatically by using a Lipozyme IM ® lipase preparation in organic solvent (hexane), so far [Armstrong, D.W., Yamazaki, H., 1997; Gubıcza, L., Szakács-Schmidt, A., 1995]. Nowadays, however, the trend goes towards „more natural" compounds, *i.e.* the natural products should be free of solvent traces, as well. Our purpose was to carry out the esterification by another lipase preparation in both organic solvent and solvent-free system [Frank, W., Welsh, F., Williams, G., 1989].

Kinetics of enzymatic esterifications has been studied by several scientists. Many of them found that esterification reaction of *long chain acids* and *short chain alcohols* was inhibited by the *alcohol* compound of the reaction, regardless whether the reaction was carried out in organic solvent (even in supercritical solvent) or in solvent-free system.

Some examples from the relevant papers are listed in Table 1. On the other hand, *acid* inhibition was observed during esterification of *short chain acids* and *longer chain alcohols*, as it was presented in some papers, summarized also in Table 1.1.

Table 1.1: Type of substrate inhibitions in esterification reaction by lipase

Substrate acid	**Substrate alcohol**	**Solvent**	**Enzyme**	**Inhibition observed**	**References**
Palmitic acid	Isopropyl alcohol	No data	Lipozyme IM	Alcohol	[Kee, C.Y., Hassan, M., Ramachandran, K.B., 1999]
Palmitic acid	Isopropyl alcohol	Sollent-free	Novozyme 435	Alcohol	[Garcia, T., Sanchez, N., Martinez, M., Arcil, J., 1999]
Fatty acid	Sulcatol (6-ethyl -5-heptene-2-ol)	Hexane	Immobilized *Candida rugosa*	Alcohol	[Janssen, A. E. M., Sjursnes, B.L., Vakurov, A.V., Halling, P.J., 1999]
Oleic acid	Ethanol	Supercirit. CO_2	Lipozyme IM	Alcohol	[Goddard, R., Bosley, J., Al-Duri, B., 2000]
Acetic acid	Geraniol	No data	Surfactant coated lipase	Acid	[Huang , S.Y., Chang, H. L., 1999]
Acetic acid	Isopentyl alcohol	Heptane	Lipozyme IM	Acid	[Krishna, S. H., Divakar, S., Prapulla S.G., Karanth, N.G., 2001]

Taking into account the findings summarized above, the most important open question regarding the kinetics of the reaction was whether the acid or the alcohol substrate causes stronger substrate inhibition in this system. Using organic solvent or solvent-free media, the aim was to compare the experimental results.

1.6. Application possibilities of integrated systems

Application of integrated systems in enzymatic reactions may enhance the productivity of the whole process. One of the most important purposes of integrated systems is the product recovery. It is especially advantageous if product inhibition occurs during the reaction [Bélafi-Bakó, K., Gubicza, L., 2000; Prenosil, J.E., 1995]. An example is shown in Figure 1.7. [Bailey, J.E., Ollis, D.F., 1986].

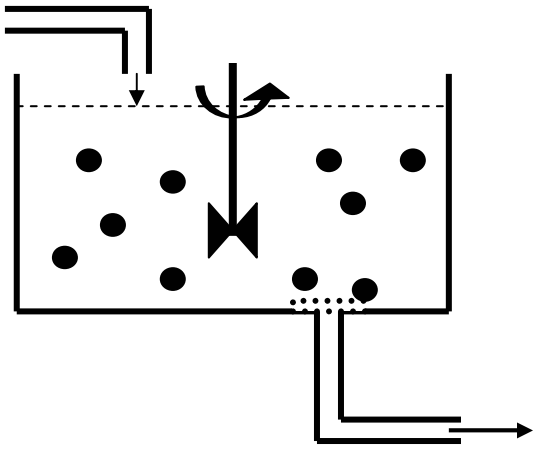


Figure 1.7.: Retention of enzymes in solution using porous membrane

If integrated separation steps are planned to connect to the enzymatic reaction system, several requirements should be taken into account, since these kind of reactions are carried out under mild conditions and the biocatalysts are usually rather sensitive [Bélafi-

Bakó, K., Harasek, M., Friedl, A., 1995; Bluemke, W.; Schrader, J., 2001]. Thus combining a traditional separation unit operation with an enzymatic reaction should be designed very carefully. Difficulties of the integration involve:

- biocatalysts reuse (proper stability, activity of the enzyme)
- coordinating the operation conditions simultaneously for the reaction and the separation step (pH, temperature, agitation, flowing rate…etc.)
- dosage of substrate(s) consumed as a function of the product(s) recovered and removed from the system (continuous mode of operation)

Among the separation processes extraction, adsorption, distillation are the often applied techniques in integrated systems [Bélafi-Bakó, K., Nagy, E., 1996]. Recently membrane separation processes have been becoming more and more popular due to their beneficial features (operating under mild conditions, no additive requirements, no hazardous wastes are formed, energy-saving and environmental-safe methods etc.).

In case of esterification of short chain acids and alcohols by lipase, ester and water are produced. In general ester has not shown strong inhibition effect towards the reaction (although its continuous recovery contributes to the high productivity of the system), water, however, plays an extremely important role in the reaction. To realize the natural ethyl acetate production, the aim was to connect product separation steps for both products: ester and water. In this way recovery of the valuable product, ester can be carried out, while water content can be kept at a constant level, optimal for the enzymatic reaction.

Possibilities for water removal

Several techniques have been suggested to remove excess water and keep its level constant. One of the most elegant methods is the application of salt hydrate [Kvittingen, L., Sjursnes, B., Anthonsen, T., Halling, P., 1992; Wehtje, E., Costes, D., Adlercreutz, P., 1997]. Water produced can be removed by circulating the reaction mixture through a packed column filled with zeolite, cellite or other water-adsorbing material [Fonteyn, F., Lognay, G., Marlier, M., 1998; Mensah, P., Gainer, J.L., Carta, G., 1998]. However, in the

latter case a chemical reaction between the acid and the adsorbent can not be excluded. Several further solutions have been suggested in the literature for water removal, such as pervaporation [van der Padt, A., Sewalt, J.J.W. and van't Riet, K., 1993] headspace evacuation, sparging of dry inert gas through the reaction medium [Jeong J.C., Lee, S.B., 1997]. However, the large-scale application of these methods has not been reported so far.

Possibilities for ester recovery

Much less techniques for ester recovery as an integrated step have been found in literature than for water removal, probably due to the special features of ethyl acetate (volatile compound, solubility etc.). Extraction is not quite suitable for the purpose, since ethyl acetate can be dissolved almost in every solvent, but its recovery and concentration is difficult then since ethyl acetate forms azeotropic mixture with lots of solvents. Distillation or air stripping might be used, however the effectiveness seemed far not enough for the recovery of the product formed in a laboratory scale reactor, moreover distillation is limited by the azeotropic formation mentioned.

Among the mild membrane separation processes [Mulder, M. H. V., 1996; Scott, K., 1995] pervaporation seemed a promising technique exploiting the volatile character of the target compound. Pervaporation [Huang, R.Y.M., 1991] is the only membrane separation method, where the feed is in liquid phase, while permeate is obtained as a vapour and condensed in cooled traps. It means, that evaporation occurs in the membrane material. The selectivity of the process therefore is based on the solution-diffusion mechanism.

Pervaporation has been mainly used for the separation of aqueous solutions, as well as azeotropic mixtures. Although pervaporation has already applied in esterifications and in natural aroma manufacture [Lipnizki, F.; Olsson, J.; Trägardh, G., 2002a and 2002b; Lim, S. Y.; Park, B., 2002; Liu, Q.; Zhang, Z., 2001; Xuehui, L.; Lefu, W., 2001], no membrane has been available commercially for ester recovery either from organic solvent or from solvent-free system; it has not been developed so far. Therefore organophilic

membranes, elaborated for other separation purposes were planed to select and use in this project.

Pervaporation membranes are characterized by two basic parameters: flux and selectivity. Flux is defined as amount of permeate passing through the membrane per unit area and time, while definition of the selectivity α is as follows:

$$\alpha_{ij} = \frac{y_i / y_j}{z_i / z_j} \qquad (10),$$

Where y_i and y_j are the concentrations of the components i and j in the permeate, z_i and z_j are the concentrations in the feed.

2. Aims of the work

In this PhD work the main aim was to develop a complete system for manufacturing natural ethyl acetate, one of the most important flavour esters. The synthesis of natural ethyl acetate can be carried out by using natural initial compounds (ethanol and acetic acid from biological origin) and enzymatic catalysis. During realization of the process itself, however, several difficulties arose. Having studied the relevant literature, it is clear that inhibition phenomena should be taken very carefully, including the role of water (by-product of the reaction), not only from the reaction equilibrium point of view, but due to its effect on the enzyme conformation. To solve these problems it seemed reasonable to set up an integrated system. For this purpose kinetics of the reaction itself (especially the possible inhibitions), product removal techniques (separation processes) have to be investigated in details as a background for the experimental work. Considering the possibilities for the integrated system, the following tasks were set:

- description of the kinetics of the esterification reaction in organic solvent and solvent-free system by lipase (Michaelis-Menten model, inhibitions, temperature)
- studying techniques to keep water content in the reaction mixture at a constant level
- investigation of pervaporation as a tool for ester recovery
- building of an integrated system for ethyl acetate manufacturing in solvent-free media, where separation units for both water removal and ester recovery are coupled to the system.

As a result of elaborating these tasks, natural ethyl acetate synthesis can be realised in an integrated system, which will provide useful background for other natural flavour ester production, as well.

3. Materials and Methods

3.1. Materials

The following materials were used during the experimental work:

- Acetic acid ($C_2H_4O_2$) analytical grade, Daniel GmbH, Germany,

  M=60,05 g/mole; ρ=1,05 kg/l; boiling point = 117-118°C
- Ethanol (C_2H_6O) 96° analytical grade, Győri Szeszgyár és Finomító RT, Hungary

  M=46,07 g/mole; ρ=0,79 kg/l; boiling point = 78°C
- Ethyl acetate ($CH_3COOC_2H_5$) analytical grade, Reanal, Hungary

  M=88,11 g/mole; ρ=0,9 kg/l; maximum water content=0,2%,
  boiling point = 78°C
- Pentane (CH_3-CH_2- CH_2- CH_2- CH_3) analytical grade, organic solvent,

  Reanal, Hungary
- n-Heptane (CH_3-CH_2- CH_2- CH_2- CH_2- CH_2- CH_3) analytical grade, organic

  solvent, Reanal, Hungary
- Pervaporation membrane GFT PV 1060 (organophilic) Sulzer (originally Carbone

  Lorraine), Germany
- Novozyme 435 (lipase enzyme) Novo Nordisk A/S, Denmark

Novozyme 435 lipase preparation from Novo Nordisk (Denmark) was used, manufactured by recombinant DNA technology. The gene coding for the lipase has been transferred from selected strain of *Candida antarctica* to the host organism, *Aspergillus oryzae*. The enzyme produced is immobilised onto a macroporous acrylic resin, diameter 0.3-0.9 mm, its reported activity is 7000 PLA.g^{-1} (PLA means Propyl Laurate Activity and its unit is defined as 1 μmol propyl laurate formed per minute per gram of catalyst under

standard conditions). The structure of the lipase preparation from *Candida antarctica* is shown in Figure 3.1. [www.expasy.org/cgi-bin/niceprot].

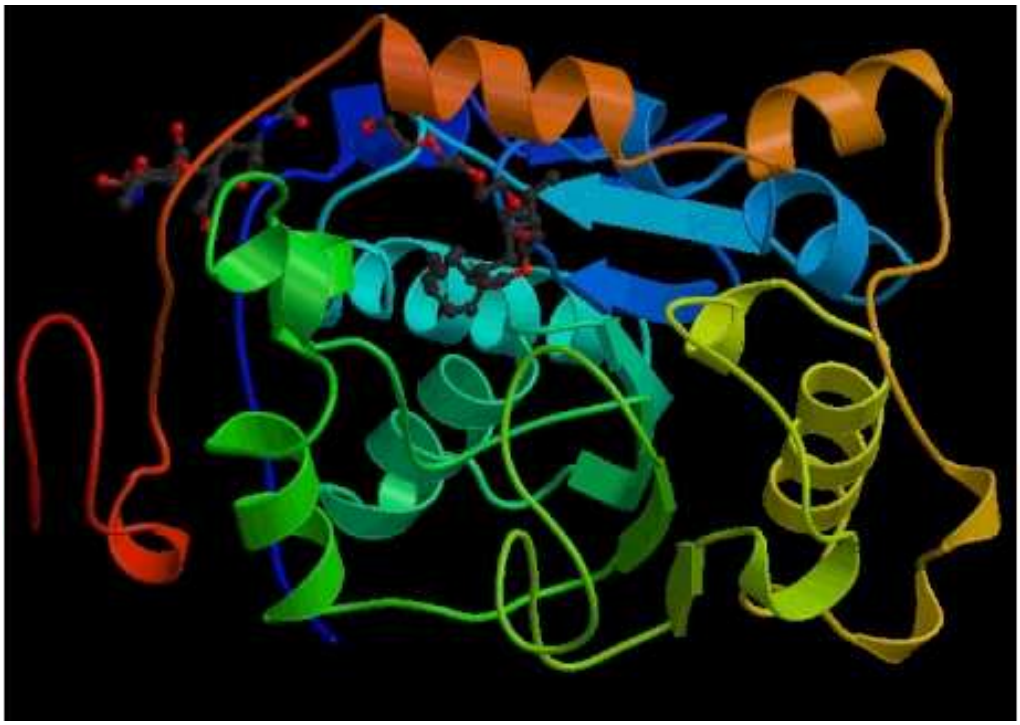


Figure 3.1.: Structure of *Candida antarctica* lipase

- Zeolite 3A Ajkai Timföldgyár és Finomító RT, Hungary
- Karl Fischer A component (pyridine sulphur dioxide solution), Reanal, Hungary
- Karl Fischer B component (iodine in methanol solution), Reanal, Hungary
- KOH (0.1 M) solution (prepared at research institute lab.)
- Phenolphthalein indicator (prepared at research institute lab.)

3.2. List of equipments used

- Shaking flask (New Brunswick Scientific Co. INC. EDISON, N.J. USA)
- Gas chromatograph (Hewlett Packard Series II, 5890)
- Peristaltic pump (IKA, Schlauch pumpe, Janke & Lunkel, IKA Labortechnik)
- Vacuum pump (Vacuum Brand GMBH + Co., Germany)
- Karl Fischer titration (Mettler DL 35)

3.3. Analytical Methods

Determination of ethanol and ethyl acetate by gas chromatography

For the determination of ethanol and ethyl acetate Hewlett Packard 5890A gas chromatography equipped with an integrator type HP 3396A was used. The detector used was a flame ionisation (FID) detector. The type of capillary column applied in this work was a HP-FFAP (crosslinked FFAP) [30m*0.53mm*1.0μm]. The carrier gases used in GC analysis were as follows:

Nitrogen	5.9 ml/min	3 bar
Air	287 ml/min	2.5 bar
Hydrogen	59 ml/min	1.8 bar

The temperatures applied during the GC measurements are summarized in Table 3.1.

Table 3.1.: The temperatures used in GC analysis

Oven temperature	50 °C
Initial temperature	50 °C
Final value	50 °C
Detector temperature	250 °C
Injection temperature	250 °C
Range	4

To determine ethanol and ethyl acetate by GC, first some solutions of ethyl acetate and ethanol were prepared (5 w/w %, 10 w/w % and 20 w/w %), and a calibration curve was prepared. The amount of sample introduced to GC, was 5 μl and the retention times of the compounds were between 5 and 10 minutes.

Water determination by Karl-Fischer method

The determination of the water content is based on the reaction described by R. W. Bunsen in 1853.

$$I_2 + SO_2 + 2\ H_20 \rightarrow 2\ HI + H_2SO_4$$

Karl Fischer discovered [Fischer, K., 1935] that this reaction could be used for water determination in a non-aqueous system containing an excess of sulphur dioxide. Methanol proved to be suitable as a solvent. In order to achieve an equilibrium shift to the right, it is necessary to neutralise the acids that are formed during the process. Karl Fischer used pyridine for this purpose. Smith and his co-workers formulated a two-step reaction [Smith, D.M., Bryant, W.M., Mitchell, J., 1939):

$$I_2 + SO_2 + 3\ Py + H_2O \rightarrow 2\ Py\text{-}H^+I^- + Py\cdot SO_3$$

$$Py\ SO_3 + CH_3OH \rightarrow Py\text{-}H^+CH_3SO_4^-$$

According to these equations, methanol not only acts as a solvent but also participates directly in the reaction itself. In an alcoholic solution, the reaction between iodine and water takes place in the stoichiometric ratio of 1:1.

In an alcohol-free solution, the reaction between iodine and water takes place in the stoichiometric ratio of 1:2:

$$I_2 + SO_2 + 3\ Py + H_20 \rightarrow 2\ Py\text{-}H^+I^- + PySO_3$$

$$PySO_3 + H_2O \rightarrow Py\text{-}H^+HSO_4^-$$

Determination of acetic acid by KOH titration

Approximately 0.3 grams of samples were taken and 3 ml of distilled water with 3-4 drops of phenolphthalein were added to the sample. Than it was titrated with KOH (0.1 mol/l) solution till it became slightly pink colour. The moles of acetic acid were calculated on the basis of following equation

$$n_{\text{acetic acid}} = (f \times N \times 10^{-3} \times V_{KOH}) / m_{\text{measured}} \qquad (11)$$

where,

$n_{aceticacid}$: moles of acetic acid in sample

f : KOH factor

N : Molarity of KOH

V_{KOH} : volume of KOH consumed

$m_{measured}$: grams of sample

Before starting the titration, the factor of KOH solution was determined by 0.1 M HCl with a known factor.

3.4. Experimental methods

Shaking flask experiments

Enzymatic esterifications in both organic solvent and solvent-free system were carried out in shaking flasks containing 25 cm^3 reaction mixture and 0.2 g enzyme preparation. The initial water content of all the reaction mixtures was adjusted to 0.50 mass% and checked carefully. The flasks were shaken at 150 min^{-1} and 40 ^oC temperature in a New Brunswick G24 incubator shaker.

The experiments aimed to determine the kinetics of the reactions (to measure progress curves), to demonstrate the possible inhibitions, and to describe the effect of the temperature on the enzymatic processes (range 25-80 ^oC).

Water removal

Removal of water produced during the esterification reaction inorganic solvent was carried out by hetero-azeotropic distillation, which is a well-known method to remove water content from reaction mixtures. During the process a solvent – which forms hetero-azeotropic mixture with the components - is present in the system and makes the boiling point of the hetero-azeotropic mixture lower than those of the individual compounds. In our case pentane was used for this purpose, since water-pentane hetero-azeotropic mixture was formed, having a boiling point of 34.6 ^{o}C.

The other possibility to remove water was the adsorption, which is suitable for water removal from solvent – free system as well. A column packed with zeolite 3A was placed after reactor and the reaction mixture was circulated regularly through the column. Water was adsorbed by the pore of zeolite and the water content of the reaction mixture was kept in a constant level during the reaction.

Pervaporation

Pervaporation was studied for removal of ethyl acetate, product of the enzymatic esterification of ethanol and acetic acid. A GFT pervaporation test cell was improved and an organophilic pervaporation membrane was used for the experiments.

Membrane was placed into the pervaporation unit and supported by a perforated plate. The membrane unit was built into an apparatus including traps, vacuum pump, manometer and thermostate. The permeate side of pervaporation unit was kept under vacuum (8 kPa). Permeate was collected in three parallel traps, immersed in acetone dry ice mixture (-60°C) to condense ethyl acetate. Experiments were carried out to test the membranes firstly, where selectivity and flux were determined.

Integrated system

Enzymatic esterification was realised in integrated system where pervaporation unit for ester removal and packed bed column for water removal were connected to the reactor. The outline of the system is shown in Figure 3.2.

During the experiments 1:20 molar ratio of acetic acid and ethanol mixture was fed directly to the reactor containing the biocatalyst. The catalyst (lipase) for the esterification was placed in a round bottom, glass reactor with a working volume of 200 ml. A column packed with zeolite 3A was placed between the reactor and pervaporation unit and whole system were jacketed and thermostated. The reaction mixture was allowed to pass through the packed column with zeolite in order to adsorb water, while was circulated continuously through the primary side of the pervaporation cell.

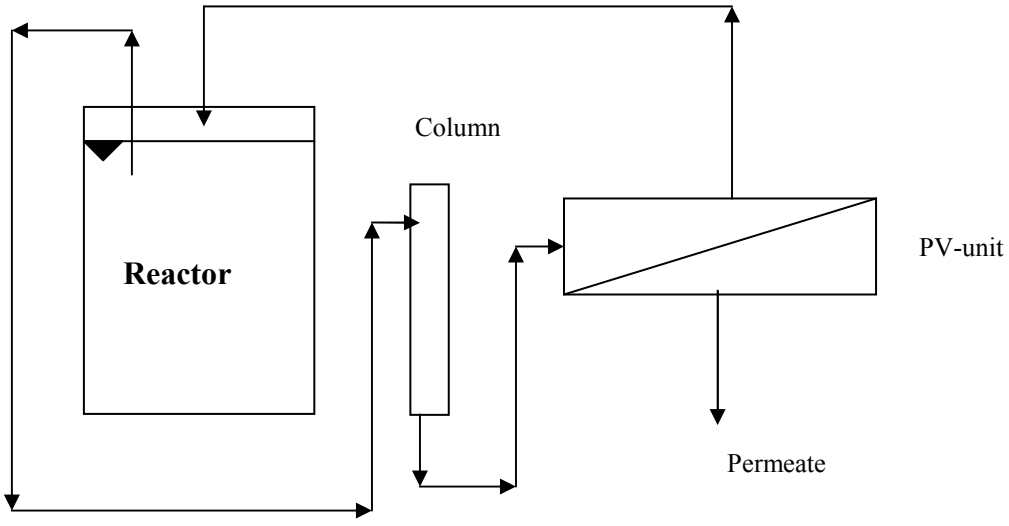


Figure 3.2.: Outline of the integrated system

4. Results

4.1. The esterification reaction

Statement:

Kinetics and the effect of temperature were studied in the esterification of ethanol and acetic acid by lipase both in organic solvent and in solvent–free system. It was found that strong acid inhibition occurs during the reaction, and Michaelis-Menten parameters (v_{max}, K_M, K_I) were determined for the solvent-free system. The activation energy of the system was calculated, as well [Own publications: 5, 9].

4.1.1. Introduction

The aim of the work in this section was to study the reaction conditions of natural ethyl acetate. Therefore enzymatic esterification of acetic acid and ethanol was investigated:

$$CH_3\text{-}COOH + CH_3\text{-}CH_2\text{-}OH \rightarrow CH_3\text{-}COOCH_2\text{-}CH_3 + H_2O$$

As it can be seen beyond ester compound, water is obtained as by-product. The reaction is catalysed by lipase enzyme. To describe and analyse the particular reaction in details, numerous experiments were conducted.

4.1.2. Experiments in n-heptane solvent

Experiments in n-heptane solvent were carried out with various initial acid (substrate, S) concentrations in the range of 0.05 and 4.2 mol/l for four different initial ethanol concentrations, each, using Novozyme 435 immobilised lipase preparation, applying 0,50 w/w% initial water content since it was found optimal earlier (therefore the effect of water was not studied here in details). The amounts of ester produced as a function of reaction time (progress curves - some examples are shown in Figure 4.1) were determined by gas chromatography.

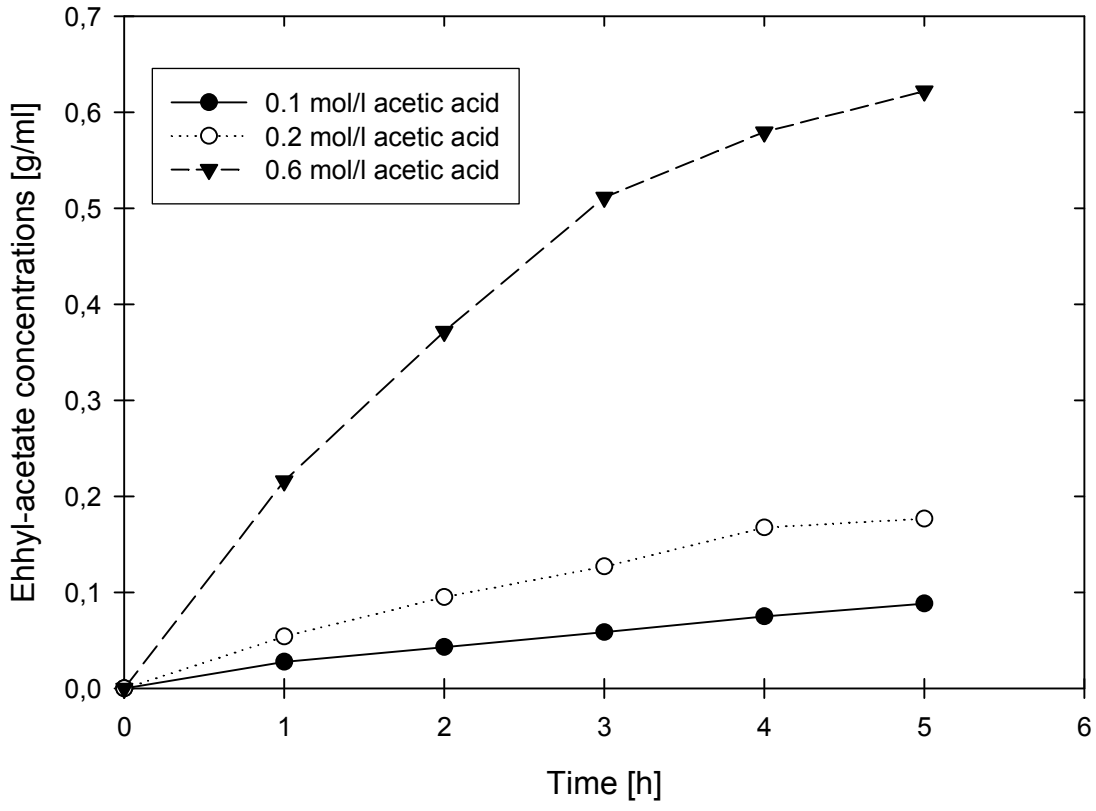


Figure 4.1.: Time curves of producing ethyl-acetate

at 4.4 mol/l initial ethanol concentration

(40° C, 150 rpm, 0,5 w/w % initial water content)

The initial reaction rates (v_o) for the ester production were calculated from the first part of the progress curves where conversions were always below 10 %, (in higher initial concentrations – it was below 2 %). The calculations were performed in a way that water concentration - initially 0.50 w/w% - never reached 0.55 w/w%, otherwise it would have influenced the reaction rate and should have been removed continuously. Thus effects of products present (especially water) could be eliminated.

The reaction rates as a function of initial substrate (acid) concentrations are presented in Figure 4.2.

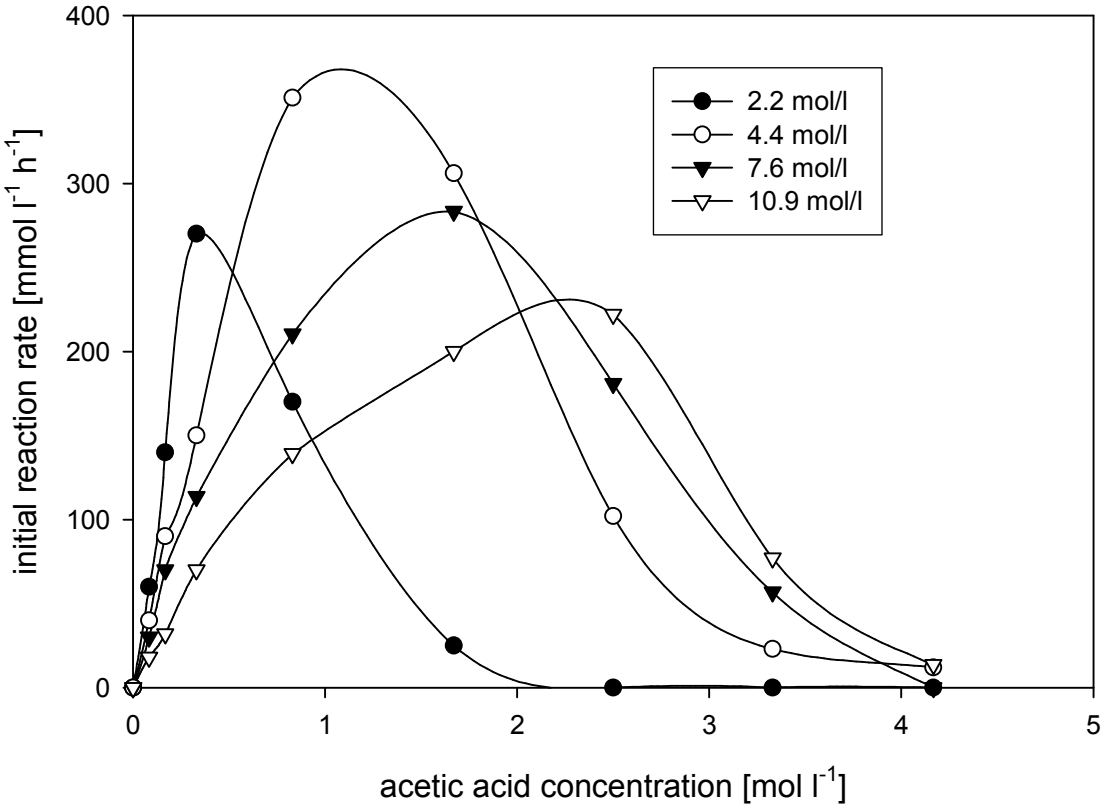


Figure 4.2: Initial reaction rates versus substrate (acid) concentrations

in n-heptane organic solvent

(40° C, 150 rpm, 0,5 w/w % initial water content)

As it can be seen, all of the curves on reaction rate versus acid concentration have a maximum, but their heights and positions are different. Thus the effect of different initial ethanol concentrations used is considered two-fold. The maximal initial reaction rates determined from the progress curves are shifted towards higher initial acid concentrations as the ethanol content increases, on one hand.

Among the summits of the highest maximum is observed at 4.4 mol/l ethanol concentration, on the other hand. It means that the increasing ethanol content seems to „defend" the enzyme from the „harmful" acid, thus the lipase preparation is able to work more effectively in higher and higher acid concentrations.

However, acid inhibition effect can be observed in every reaction rate versus substrate concentration curves, although it occurred at higher and higher acids concentrations as the amount of ethanol present increased. It seemed, that the acid concentration - where the highest initial rate was observed - increased proportionally with the ethanol concentrations. Therefore a table was compiled to compare the data (table 4.1.).

Table 4.1.: Acid – alcohol molar ratios at the highest reaction rate

Highest reaction rate observed mmol/l.h	Initial alcohol concentration mol/l	Acid conc. at the highest reaction rate mol/l	Acid – alcohol molar ratio
275	2.2	0.5	1:4.4
370	4.4	1.2	1:3.7
280	7.6	2.0	1:3.8
210	10.9	3.0	1:4.2

In table 4.1 the acid-alcohol molar ratio resulting in the highest reaction rates were calculated from the data of figure 4.2. The acid-alcohol ratios obtained are quite similar, their average is 1:4.

From these results it can be concluded, that one of the most important parameters in this reaction is the ***initial acid-alcohol molar ratio***. This should be taken into account designing the technology.

Based on the data obtained, now it is clear that *acid* inhibition occurs in the particular reaction. According to our experimental results for esterification of short chain acids and alcohols, the effect of acid is stronger and more harmful towards the enzyme than that of the alcohol.

As a consequence of these results, it seemed reasonable to perform more experiments where ethanol concentration is increased further on, reaching finally a point where no organic solvent present at the mixture. In other words, excess of ethyl alcohol "substituted" for the organic solvent.

4.1.3 Solvent-free system

Experiments were carried out with various initial acid concentrations (in the range of 0.05 and 3.5 mol/l) in solvent-free media. In the two-component system (acetic acid and ethanol are present) the initial alcohol concentration varies when the value of acid concentrations varied. Thus there is a fundamental problem to give the exact initial alcohol concentration as it was presented in case of the solvent using systems.

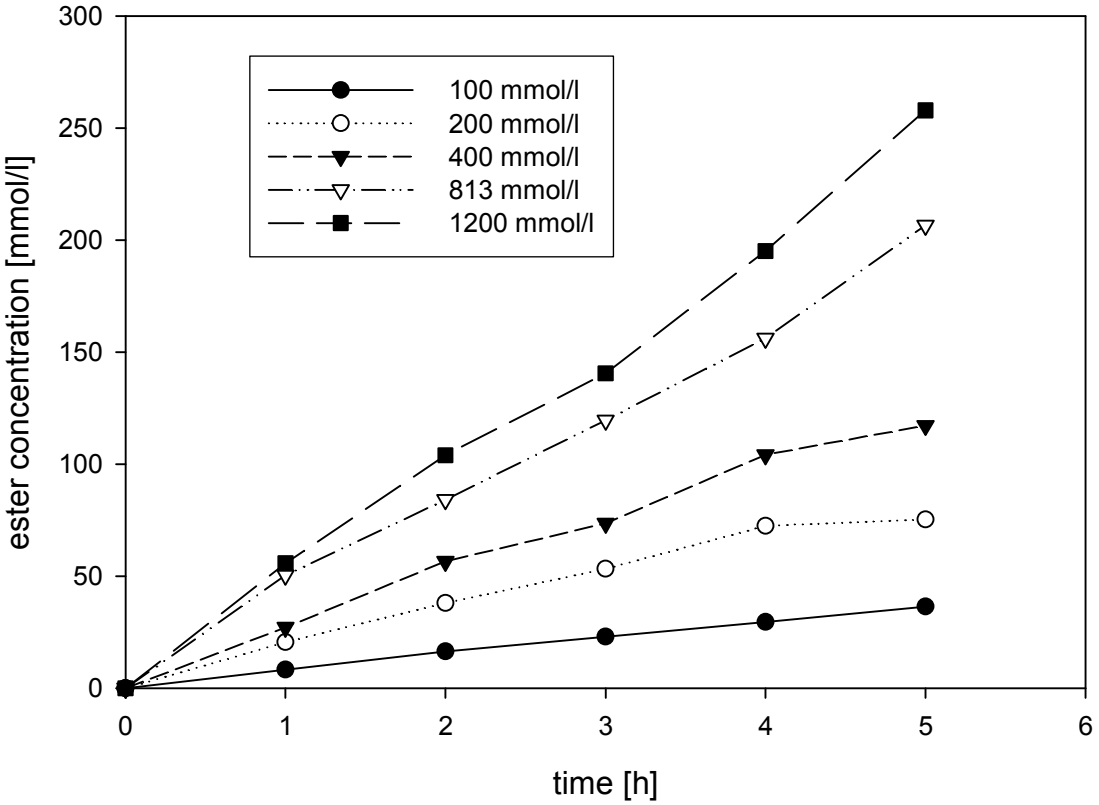


Figure 4.3.: Progress curves in solvent-free system

(40° C, 150 rpm, 0,5 w/w % initial water content)

From the progress curves (concentrations of ester formed as a function of reaction time, figure 4.3) the initial reaction rates were determined. The values obtained were plotted against initial acid (substrate) concentration (Figure 4.4).

It can be seen, that beyond 0.8 mol/l substrate concentration the reaction rate has reached a maximal value, a plateau. However, the rate has not remained constant, but started to decrease approximately at 2.8 mol/l acid concentration. It means that substrate (acid) inhibition has occurred here, as well.

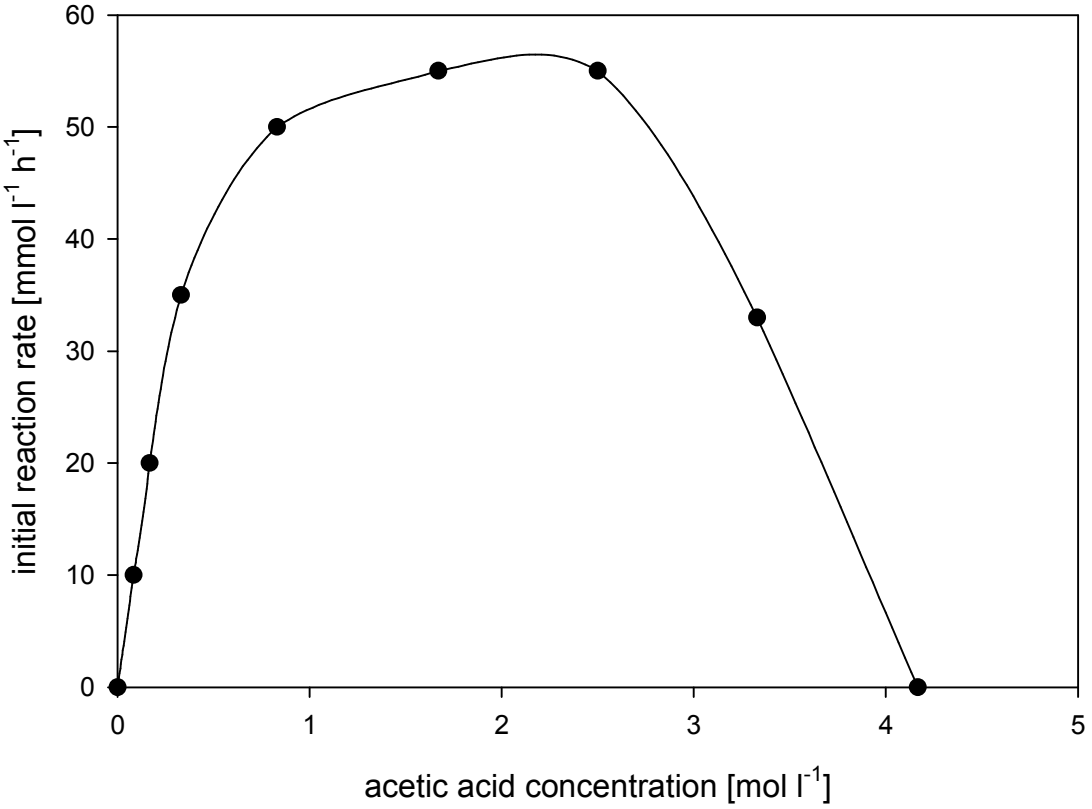


Figure 4.4.: Initial reaction rates versus substrate (acid) concentrations in solvent-free media.
(40° C, 150 rpm, 0,5 w/w % initial water content)

Comparing the data obtained in solvent-free system and n-heptane, the reaction rates determined in the organic solvent were found much higher than those measured in the solvent-free system. Moreover, the acid concentration values, where maximal reaction rates were observed in n-heptane, cover the range where the initial reaction rate values in the solvent-free system have formed the plateau.

4.1.4. Kinetical analysis

For the description of the reaction kinetics of ethyl acetate synthesis by lipase, we have to consider the fact, that it is two-substrate, two-product enzymatic reaction (bi-bi) which makes the kinetical analysis quite complicated. To decide the mechanism, a graphical method (double reciprocal) was used firstly: reciproc values of reaction rate were ploted against 1/acid (Figure 4.5.) and 1/alcohol (Figure 4.6.) concentrations. These lines should give information on the possible reaction mechanism [Laszlo, E., 2004; Keleti, T., 1985].

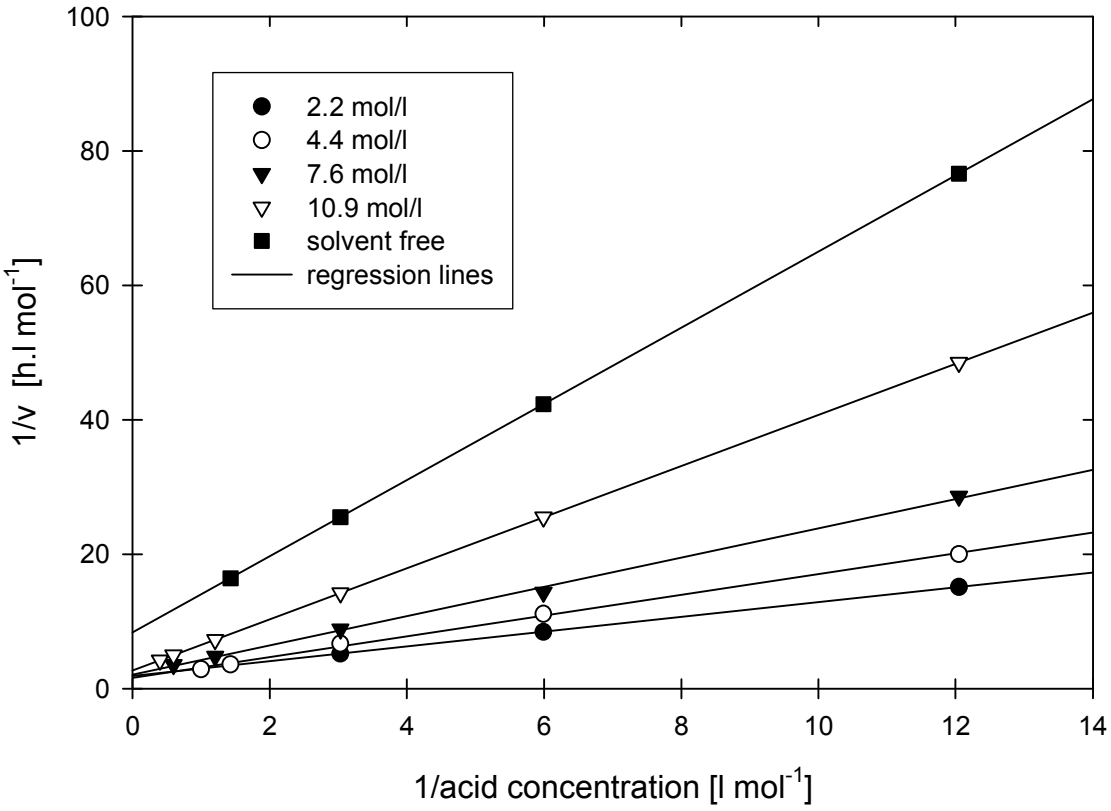


Figure 4.5.: Lineweaver-Burk linearizations for determination of the kinetic constants by acid concentration

From Figure 4.5. it can be seen the lines are linearly increasing and they cross each other in the second quarter, implying an ordered mechanism.

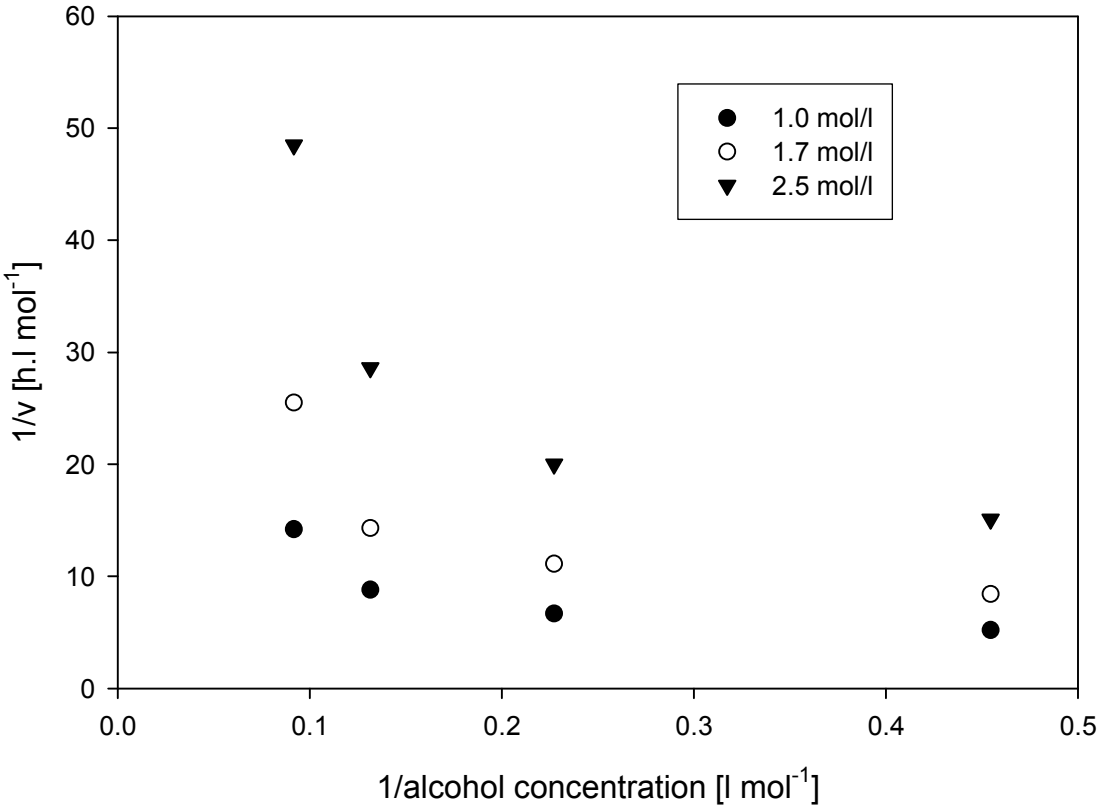


Figure 4.6.: Lineweaver-Burk linearizations for determination of the kinetic constants by alcohol concentration

The data shown in Figure 4.6., however do not flow a similar trend. They seen to be experimentally declining as function of 1/ alcohol concentration. Thus the diagram distincts from the previous one in both the linearity and the direction (negative slope !). Therefore no regression lines were plotted on the data.

This strange phenomenon is probably the result of the strong inhibition effects, which were clearly observed in the initial reaction rate versus substrate concentrations diagrams (Figure 4.2. and 4.4.). Due to this peculiar behavior, kinetic analysis for the enzymatic reaction in n-heptane solvent was not carried out, it needs further investigations.

For the description of the reaction kinetics in solvent-free system the modified, apparent one substrate Michaelis-Menten equation [Segel, I. H., 1975] was used, as follows:

$$v_o = \frac{v_{max} S}{K_M + S + S^2 / K_I} \qquad (12)$$

where K_M – Michaelis-Menten constant

v_{max} – maximal reaction rate

K_I - the inhibition constant.

This equation contains three parameters, which can be determined for the particular initial reaction rate versus substrate concentration curve either by graphical methods (e.g. Lineweaver-Burk linearisation) or by numerical methods.

Applying the apparent one substrate model (12), using the increasing sections of the rate versus acid concentration curve (up to the maximum), the values of K_M and v_{max} were determined by graphical method (double reciprocal transformation). Then values of K_I constants were determined by a numerical method (parameter estimation by Nelder-Mead type simplex search) applying these data as initials. The results of the determination for the solvent-free system are summarised in Table 4.2.

Table 4.2.: Kinetic constants calculated for the solvent-free system

v_{max}	(mol/l.h)	0.12
K_M	(mol/l)	0.68
K_I	(mol/l)	1.73

Since the enzyme used for the experiments was an immobilized preparation, all the parameters calculated in the Michaelis-Menten model can be accepted as apparent values (because diffusion process as were not considered in the system).

As a consequence to draw based on our results, it seems that, lipase catalysed solvent-free esterifications of short chain alcohols and acids are worth realizing in *alcohol* excess (to substitute the organic solvent). The data obtained in this work help to choose the most suitable reactor system (including the coupled continuous water removal) and to determine the proper reaction parameters, hence this study was useful to elaborate a technology for pilot or semi-pilot production of natural ethyl acetate.

4.1.5. The effect of temperature

Temperature has a significant influence on the enzyme catalytic reactions; therefore it is extremely important to determine its effect on the esterification by lipase.

The reaction between the absolute temperature (T) and the reaction rate constant (k) is described by the Arrhenius equation:

$$k = k_o \, e^{-E_A/RT} \qquad (13)$$

where k_o- pre-exponential factor

E_A- activation energy

R- gas-law constant (8.31 J/mol K)

Another form of the equation is

$$\ln k = \ln k_o - E_A/R * 1/T \qquad (14)$$

which is suitable for the determination of the E_A activation energy. Plotting ln k against 1/T results in a straight line, where the slope of the line is $-E_A/R$, thus E_A can be calculated. This graphical method can be applied for the reaction rates, as well, since only the position of the line is changed in this way, not the slope itself.

Experiments were planed for determination of activation energy of esterification reaction in solvent-free system. In the reaction mixture the ethanol/acetic acid molar ratio was 20:1. Temperature in the range of 25-80°C was controlled by a thermostatic bath. The duration of experiments was 5 hours; the reactions were followed by GC. The experimental data presented in Figure 4.7.

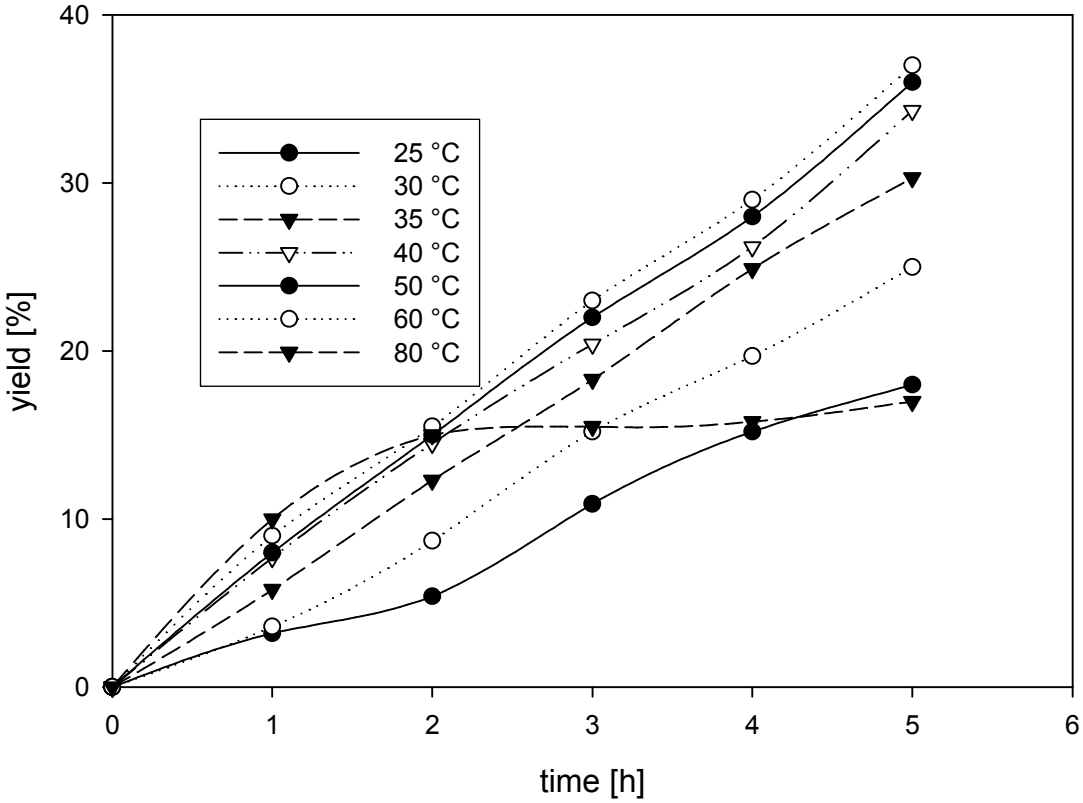


Figure 4.7.: Effect of temperature on the reaction

(Ethyl acetate production as a function of time)

From the results shown in Figure 4.7., the highest production rate of ethyl acetate was reached at 60°C. The reaction rates were calculated from the yield-reaction time data and summarized in a rate – temperature diagram. The calculated data are presented in Figure 4.8.

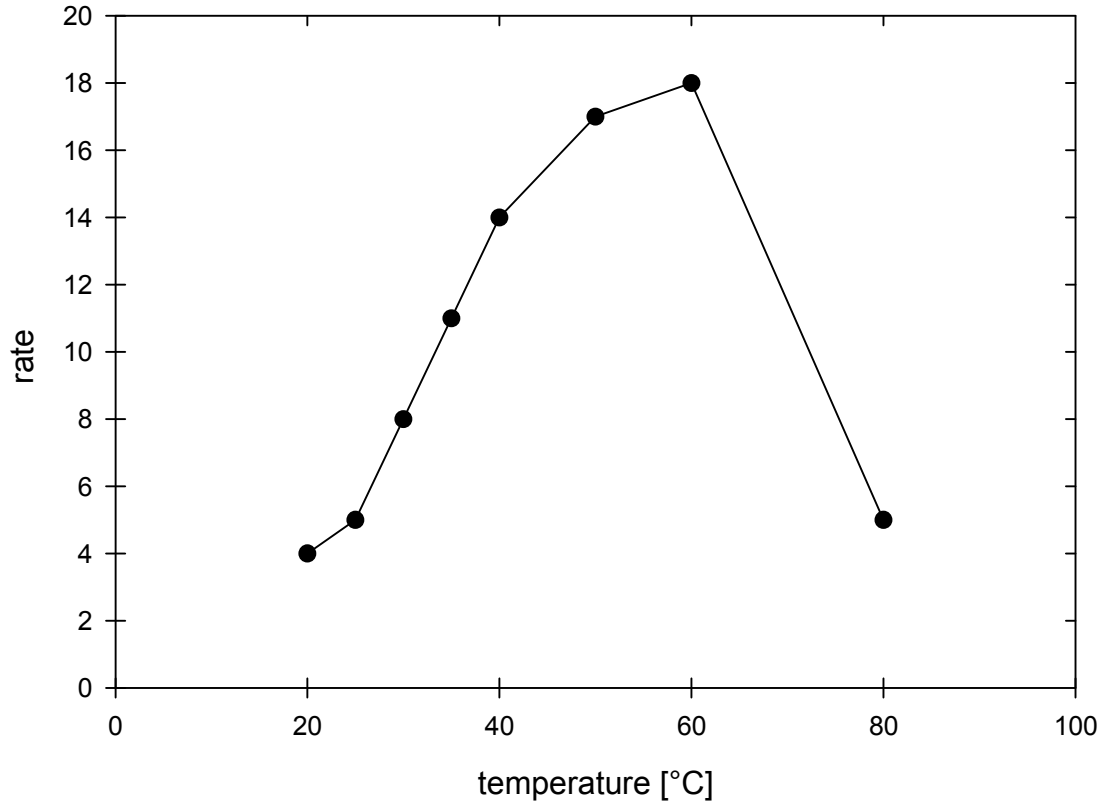


Figure 4.8.: Reaction rate versus temperature

Finally the values of 1/T and ln k were calculated from the increasing section (between 25 ^{o}C and 50 ^{o}C) of the data in rate-temperature curve and summarized in table 4.3. Then the values of ln k obtained were presented against 1/T in Figure 4.9. Straight line was plotted on the dots by linear regression using the least squares method. The equation obtained by the regression is shown in the figure, as well. The regression coefficient (R^2) was 0.9816.

Table 4.3.: Data for determination of activation energy

Temperature (°C)	Reaction rate (yield/2 hours)	1/Temperature (1/K)	ln k (-)
25	5.0	0.003356	1.61
30	8.0	0.003300	2.08
35	10.9	0.003247	2.39
40	14.1	0.003195	2.65

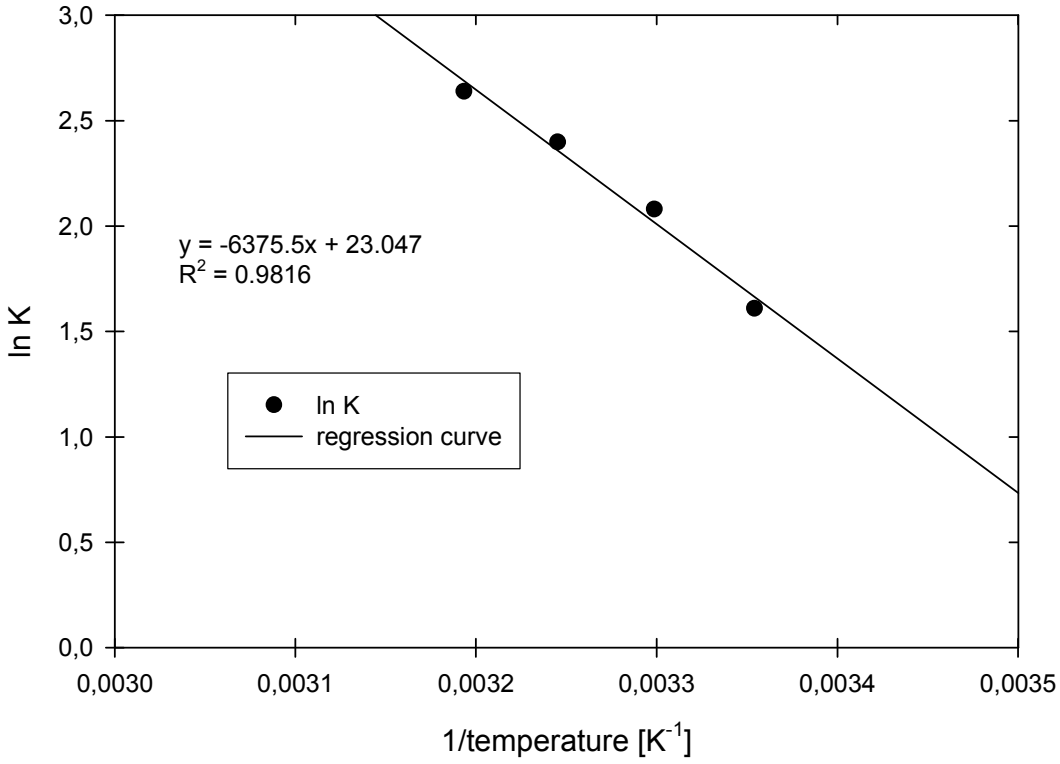


Figure 4.9.: Plot of ln k versus 1/ T

The activation energy (E_A) of the reaction can be calculated from the slope of the regression line.

$$\text{Slope} = - E_A/R$$

Thus $E_A = 52.9\ \text{kJmol}^{-1}$

According to our calculation above for solvent–free system the value of activation energy was found 52.9 kJ/mol, while it was 30.6 kJ/mol for using n-hexane as solvent. As it was expected, the energy barrier is higher in solvent-free system. The values for activation energy compared with literature data are summarized in Table 4.4. It can be seen that the values are quite similar, in the same order of magnitude, in spite of the different enzyme sources, solvents and substrates used.

Table 4.4.: Values of activation energies

Substrate (alcohol, acid)	Solvent	Enzyme source	E_A (kJ/mol)	Data from
ethanol, caproic acid	n-heptane	Esterase from *Bac. licheniforms*	15.5	Alvarez-Macail, E. 1999
i-propyl-alcohol, palmitic acid	No data	*Mucor miehei* (Lipozyme IM)	43.7	Kee, C. X. 1999
i-propyl-alcohol, palmitic acid	solvent-free	*Candida antarctica* (Novozyme 435)	11.7	Garcia, T. 1999
2-Cl butyric acid 1,2-epoxy-hexane	solvent-free	*Mucor miehei* (Lipozyme IM)	33.6	Garcia, T. 2000
ethanol, acetic acid	n-hexane	*Candida antarctica* (Novozyme 435)	30.6	Gubicza, L. 1994
ethanol, acetic acid	solvent free	*Candida antarctica* (Novozyme 435)	52.8	This work

4.2. Water removal possibilities

Statement:

> *Semi-pilot scale enzymatic esterification of ethanol and acetic acid with continuous water removal was realised to produce a natural flavour compound. The process in organic solvent was coupled with hetero-azeotropic distillation, while in solvent-free system it was integrated with adsorption resulting in more than 100% increase in conversion [2, 8].*

A/ Esterification in organic solvent coupled with water removal by hetero-azeotropic distillation

Enzymatic manufacture of natural ethyl acetate can be carried out in organic solvent. During the reaction water is produced having strong inhibition effect. Therefore water produced should be removed continuously to keep its level constant thus the yield can be enhanced. One of the most interesting opportunities for water removal is hetero-azeotropic distillation.

4.2.1. Azeotropic distillation

Azeotropes are mixtures of two liquids that boils at constant composition; i.e. the composition of the vapour is the same as that of the liquid [Oxford Dictionary of Chemistry, 2000]. Azeotropes occur because of deviations in Raoult's law leading to a maximum or minimum in the boiling point - composition diagram. When the mixture is boiled, the vapour initially has a higher proportion of one component than is present in the liquid, so the proportion of this in the liquid falls with the time. Eventually, the maximum or minimum point is reached, at which the two liquids distil together without change in composition. The composition of an azeotrope depends on the pressure.

The azeotropic distillation is a technique for separating components of an azeotrope by adding a third liquid to form a new azeotrope with one of the original components. It is most commonly used to separate ethanol from water, adding benzene to associate with the ethanol.

To carry out such an experiment, firstly the proper solvent should be selected based on the special properties of the solvents (Table 4.5.) [Handbook of Chemistry and Physics, 1977]. From the table it can be seen, that all the solvents form ***low-boiling point azeotropic mixture*** with water. For our purposes, however *n*-pentane was selected as a solvent since it has a lower boiling point (the boiling point of n-pentane – water mixture is 34.6 °C) than the enzyme's optimal temperature (60 °C), so lipase will keep its activity for longer periods at this temperature and also traces of n-pentane will easily evaporated from the final product.

Table 4.5.: Data of solvents for azeotropic distillation

Solvents	Boiling point (C°)	Toxicity	Azeotrope formation	Azeotropic boiling point (C°)
n-heptane	98.4	-	yes	72.0
n-hexane	69.0	-	yes	61.6
n-pentane	36.1	-	yes	34.6
toluene	110.6	X	yes	85.0
benzene	80.1	Highly carcinogenic	yes	69.4

4.2.2. Study on the effect of initial composition of the reaction mixture

As it turned out during our experiments in n-heptane, the yield of the reaction is very much influenced by the ***initial acid-alcohol ratio***. In Thesis I it was shown that 1:4

average acid-alcohol initial molar ratio resulted in the highest reaction rate. Here, however, n-pentane was selected for the particular experiments. Therefore firstly this effect was studied at laboratory scale using n-pentane as solvent. In Figure 4.10. the effect of acid-alcohol molar ratio is shown for ethyl acetate production.

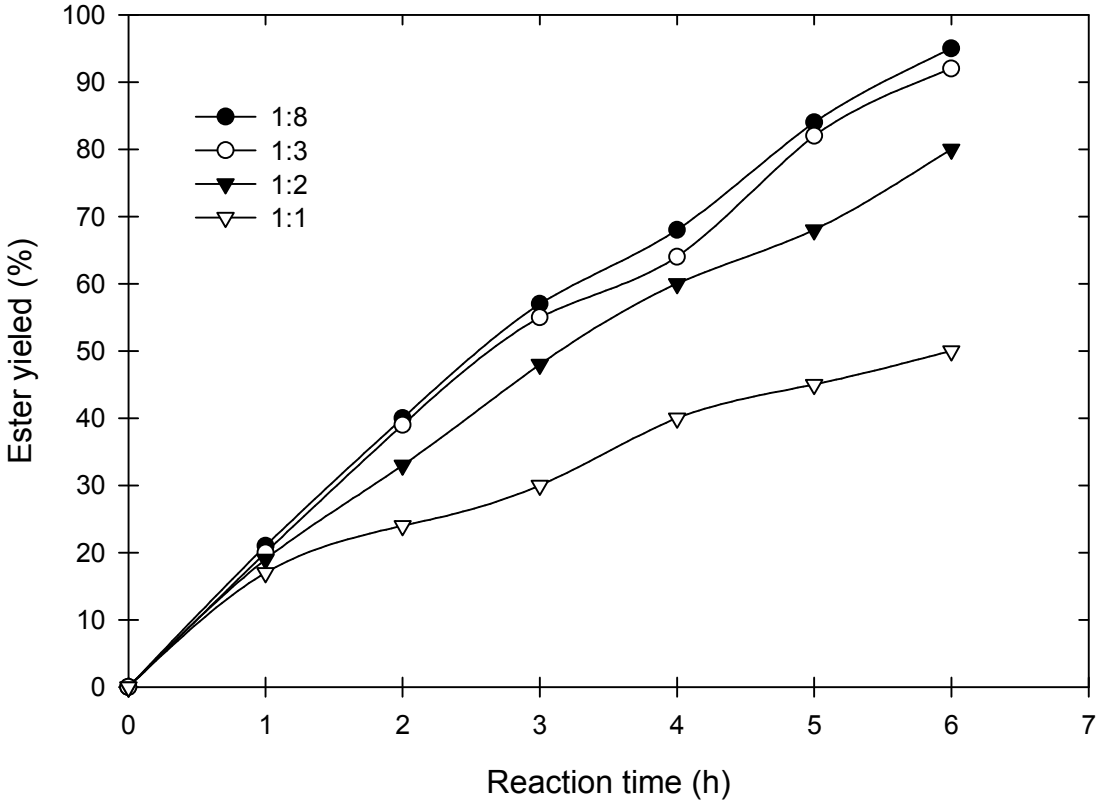


Figure 4.10.: Ester yield at different initial acetic acid: ethanol molar ratios

(Reaction conditions: 0.01 mol acetic acid, 50 ml solvent (n-pentane), 0.1 g enzyme (Novozyme 435™, NOVO Nordisk, Denmark), initial water content 0.4 w/w%, reaction temperature: 35 ^{o}C, shaken flaks: 250 rpm)

It can be seen that beyond 1:3 acid-alcohol ratio the yield does not increase. Based on these experimental results it can be concluded, that the n-pentane solvent selected for the water removal by the hetero-azeotropic distillation behaved similarly to n-heptane. Thus our further experiments were carried out with this ratio.

4.2.3. Enzymatic esterification coupled with hetero-azeotropic distillation

As it is shown in Figure 4.11. semi-continuous natural flavour ester production was performed in a 50 l nominal capacity stirred (40-1400 rpm) batch reactor (glass vessel,) containing an inner spiral heating (60W) system (1). It was equipped with a manometer and thermometer. A glass vapour tube (ID 35 mm) was connected to the reactor, attached to a rectification column (length 500 mm, ID 100 mm) filled with Pro-Pak™ protruded metal packing (Scientific Development, PA) (2). The theoretical plate number of the rectification column was 10. The reflux flowing backwards runs to the reactor via a regulating siphon. The vapours from the upper part of the column come to the azeotropic reflux head (3). Above it, a condensator with water cooling system is placed (4). In the reflux head a bell-shaped plate is located, thus all the vapour condensed then flowed into a Florentini vessel (5) (cooled with water) under the head. In the Florentini vessel the hetero-azeotropic mixture separated into two phases. The upper phase (solvent) flowed back to the column, while the lower part (water) was collected.

The acetic acid-ethanol mixture (in container 6) was pumped to the reactor by a Teflon membrane pump (7.7 l/h) (fed batch made of operation).

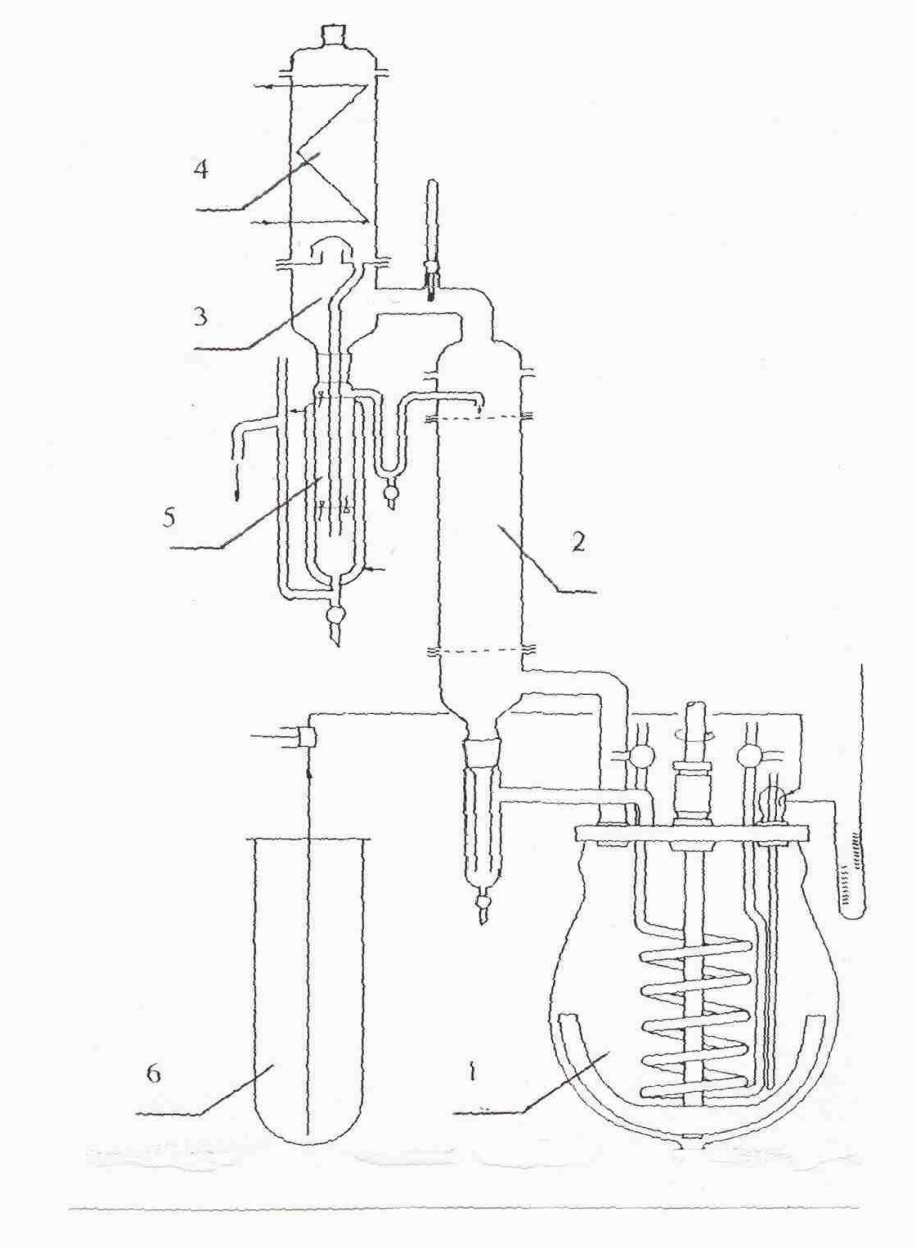


Figure 4.11.: Semi-pilot scale enzymatic esterification reactor

Procedure: Firstly 14 l pentane and 1 kg Novozyme lipase enzyme were poured into the reactor. Then the substrates: absolute alcohol (3 l, 52 mol) and acetic acid (0.8 l, i.e. 14 mol) were added in 1:3 optimal molar ratio.

After starting the stirring (75 rpm) and the heating, the temperature of the reaction mixture increased up to 35-36 C° and the esterification began. The vapours were leaving the reactor continuously.

In the vapour phase not only pentane and water were present, but ethanol, as well. Thus 3-component hetero-azeotropic mixture was formed, having a boiling point at 32.5 C° and the phase-separation resulted in an upper phase (containing mainly n-pentane) and lower phase containing 30-35 w/w% water and 65-70 w/w% ethanol. The upper phase was recycled into the reactor continuously, while the lower phase was collected.

After 2 hours reaction time substrates (acetic acid – ethanol mixture 1:3 molar ratio) were started to add from the container filled with the particular mixture earlier. The rate was 0.6-0.8 l/h, taken into account the rate of the substrate consumption. The water produced was continuously removed by the hetero-azeotropic distillation, its content was kept at 0.4-0.5 w/w% and was checked Karl-Fischer titration. The ester, ethanol and acetic acid content of the reaction mixture were determined regularly by GC. As the amount of ester increased (Figure 4.12.), the boiling point of the mixture was growing, as well (after 100-110 hours reaction time, it was 55-56 C°).

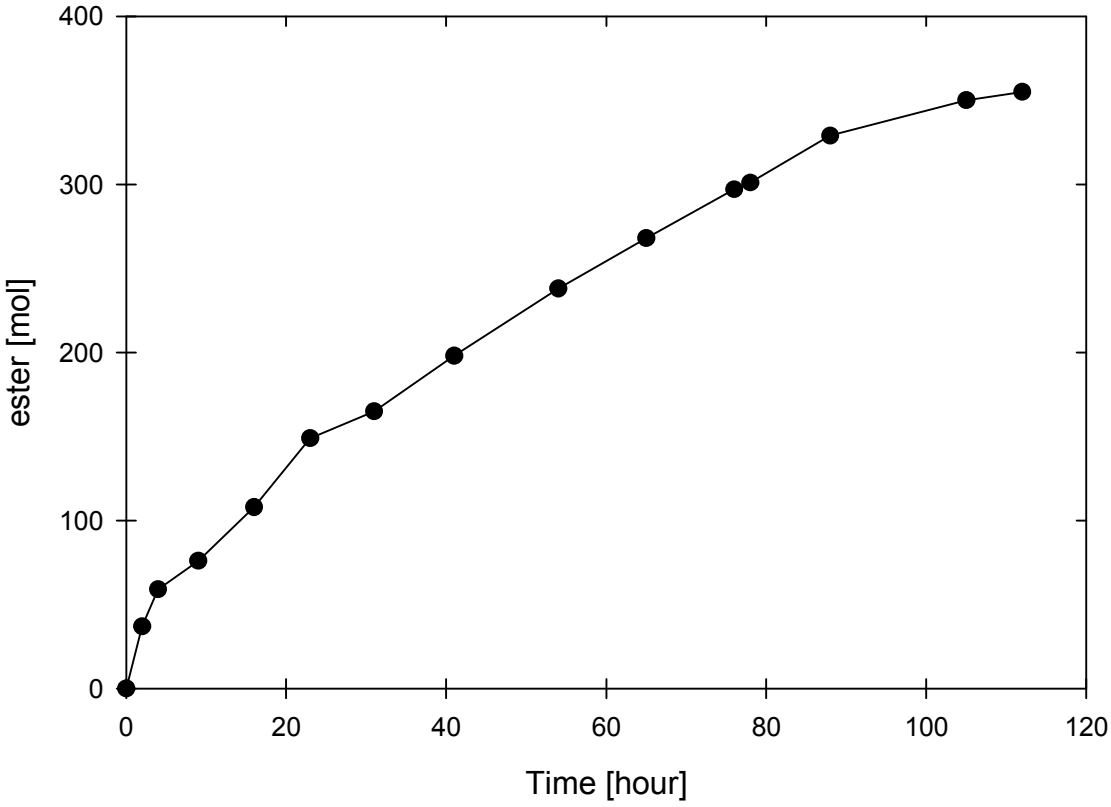


Figure 4.12.: Amount of ester produced during reaction

Having consumed all the substrates in the container, the concentration of ethanol was measured carefully, then stoichiometric amount of acetic acid was added into the reaction mixture. Finally the reaction was stopped, the reaction mixture was cooled down at room temperature. The final composition was 1.5-2.0 mol acetic acid, 5-8 mol ethanol, 0.4 w/w% water.

Altogether 28 kg ethyl acetate was produced after 120 h reaction time, which corresponds to a 93.2 % yield. The enzyme sank down to the bottom of the reactor and was easily separated. After separation and concentration steps, the purity of the end product obtained was 99.9 w/w% and its water content was less then 0.01 w/w%. The enzyme was recycled and kept its activity at the same level over more then ten cycles.

The large-scale experiments have proven that hetero-azeotropic distillation is a suitable method to maintain the water content of a reaction mixture at a constant level during enzymatic esterification in organic solvents.

B/ Water removal by adsorption

If the esterification reaction is carried out in solvent-free system, hetero-azeotropic distillation cannot be used for water removal. Therefore another method, adsorption was tested, where A4 zeolite was used.

Zeolites are natural or synthetic hydrated alumino-silicates with an open three-dimensional crystal structure, in which water molecules are held in cavities in the lattice. The water can be driven off by heating and the zeolites can be regenerated in this way.

The adsorption capacity of A4 zeolite was tested by test solutions (containing water and ethanol). It was found that the water adsorption capacity was 20% based on the weight of the zeolite.

Water was removed in this system by a semi-batch method, because the continuous way was not possible. The acetic acid has an aggressive character, damaging the fine structure of zeolite. To compare the esterification in solvent-free method with and without water removal, parallel experiments in laboratory scale were carried out. The ethanol-acetic acid ratio was 1:5, initial water content 0.5 w/w%, temperature 40 °C, and 0.5 g Novozyme 435 lipase enzyme was applied.

In the experiment with water removal, a short (5 cm long) column filled with zeolite A4 was connected to the stirred reactor. The reaction mixture was pumped through the column regularly, which was controlled by a timer (the pump worked 1.5 minutes long, in every 15 minutes). The parallel experiments in Figure (4.13) show the efficiency of the

water removal. It can be seen that without water removal only 20 % conversion was achieved after 6 hours reaction time, which in the experiment with water removal the conversion was more than 40 %.

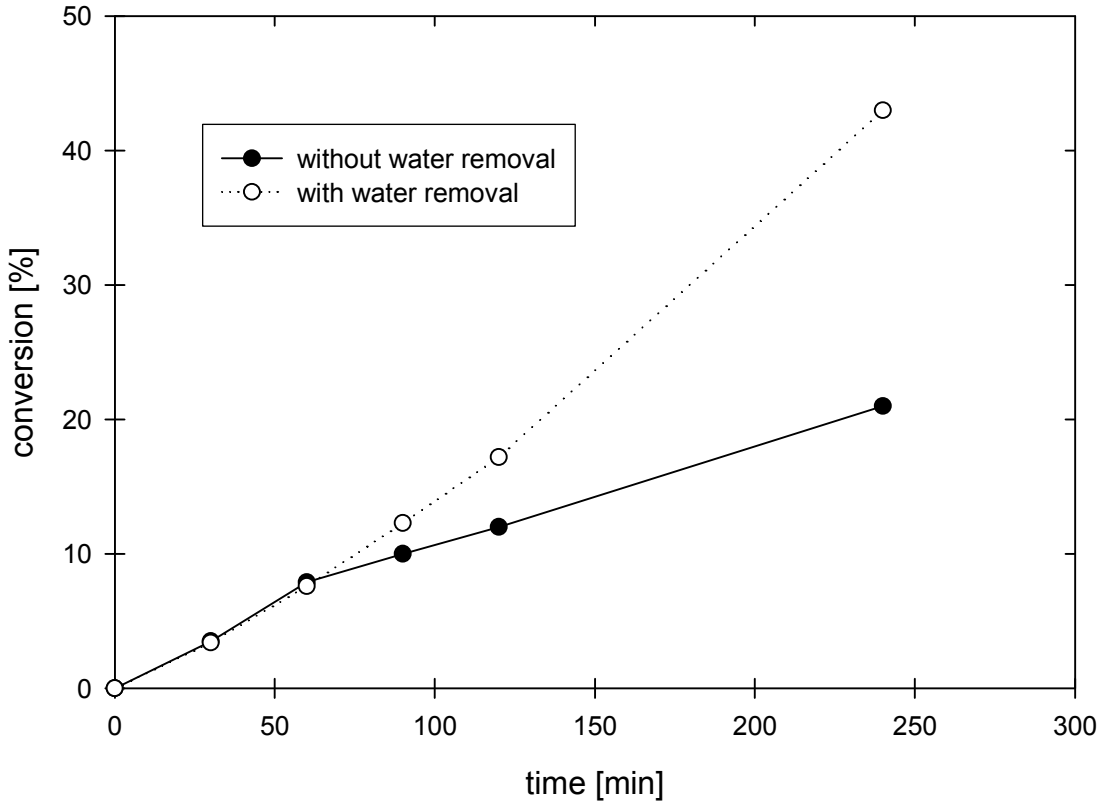


Figure 4.13.: Enzymatic ethyl acetate production in solvent-free system with and without water removal

During the reaction with water removal by adsorption the water level could be controlled. Its level was kept around 0.5% (m/m) (Figure 4.14).

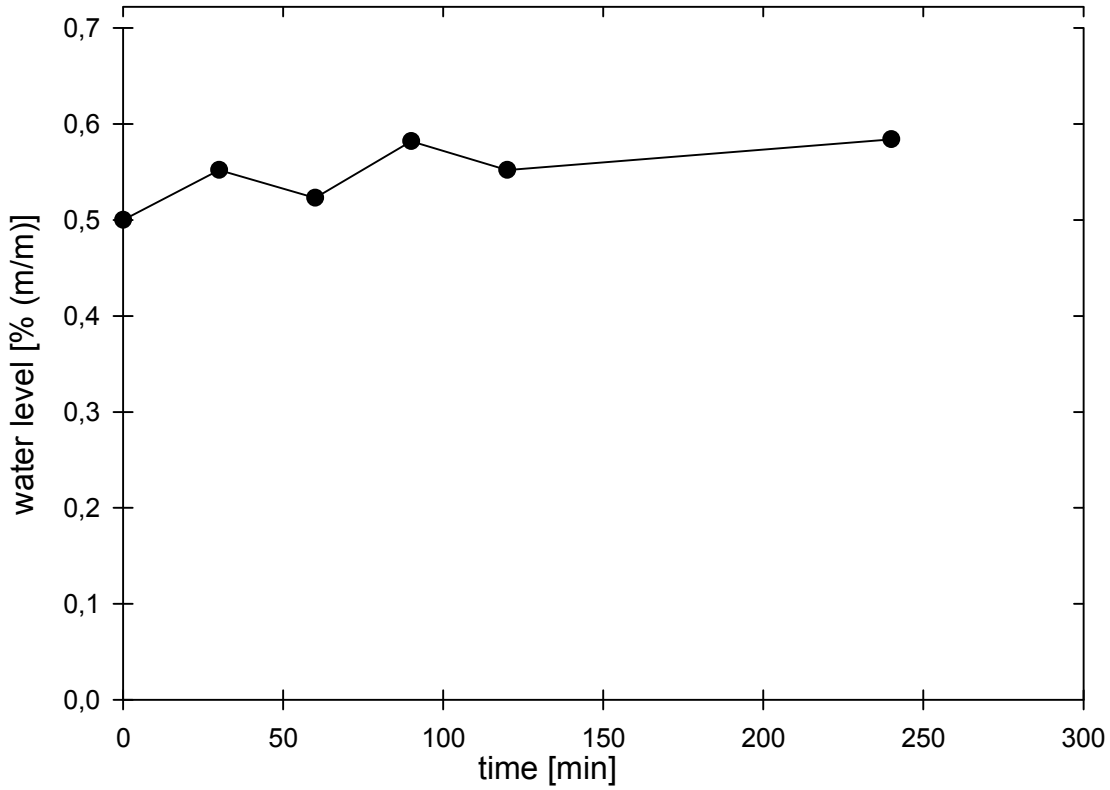


Figure 4.14.: Water level during the esterification experiment with water removal by adsorption

From our experiments it can be concluded that adsorption with zeolite is a reliable water removal method. Although acetic acid present in the system may damage the structure of the zeolite, but the method itself was proven as a simple and effective technique. Moreover it can be easily integrated to the continuous enzymatic reactions, which is definitely advantageous.

4.3. Pervaporation for ester recovery

Statement:

The construction of the flat sheet GFT pervaporation test cell was improved and an organophilic PV membrane for removal of product in esterification was tested [3, 12, 14, 15, 18].

4.3.1. Pervaporation

Pervaporation is a membrane process in which a pure liquid or liquid mixture is in contact with the membrane on the feed or upstream side at atmospheric pressure and where the permeate is removed as a vapour because of a low vapour pressure existing on the permeate or downstream side. This low (partial) vapour pressure can be achieved by employing a carrier gas or using a vacuum pump. The (partial) downstream pressure must be lower than saturation pressure at least. A schematic drawing of this process is shown in Figure 4.15.

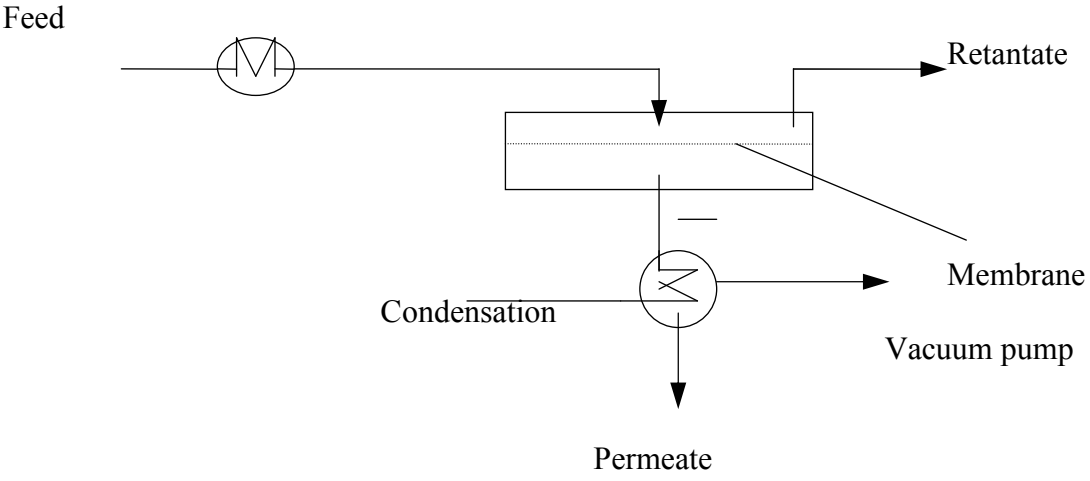


Figure 4.15.: Scheme of the experimental test system

Essentially, the pervaporation process involves a sequence of three steps:

- Selective sorption into the membrane on the feed side
- Selective diffusion trough the membrane
- Desorption into a vapour phase on the permeate side.

4.3.2. Description of the original module

Figure 4.16. shows the structure of a pervaporation unit (GFT, Sulzer, Germany), made of stainless steel. The membrane sheet is secured inside the unit using a perforated plate (with a diameter of 16 cm) with an o-ring. Installing the membrane divides the unit into two parts. Upper part, where the inlet and retantate outlet are located, and a lower part, where the permeate is taken. The feed in liquid phase is recirculated in the upper part, while the permeate in vapour phase is withdrawn from the down part outlet by a vacuum pump.

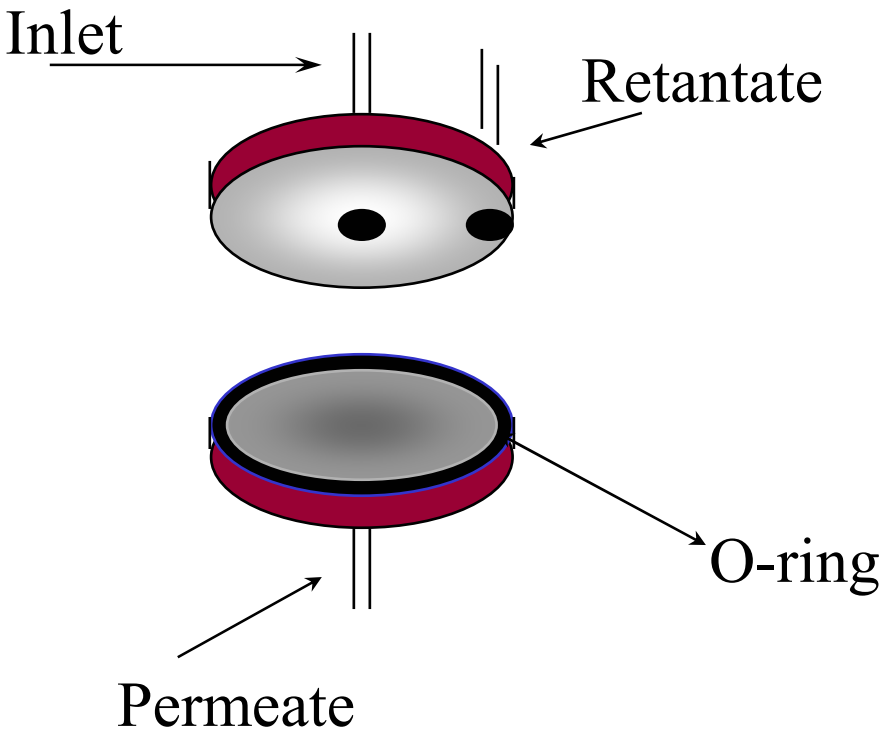


Figure 4.16.: Scheme of the pervaporation unit

4.3.3. Modifications

Jacketing of PV unit to keep temperature constant

Due to heat lost and effect of it on selectivity, the unit was jacketed at workshop of institute and later it was connected to the thermostat to keep the temperature constant at 40°C (Figure 4.17.).

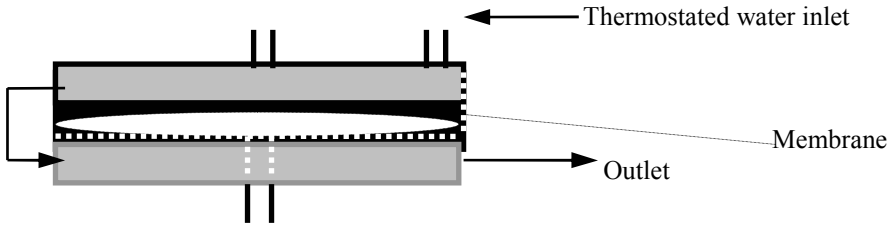


Figure 4.17.: Representation of heat system of the experimental equipment.

Inlet modification of PV unit

Since the inlet and outlet of the PV unite was close to each other, some modifications were done to increase the flux. Firstly the inlet was moved from centre of PV unit to the left side and the previous inlet was sealed (Figure 14.18.). However the result was still not satisfying.

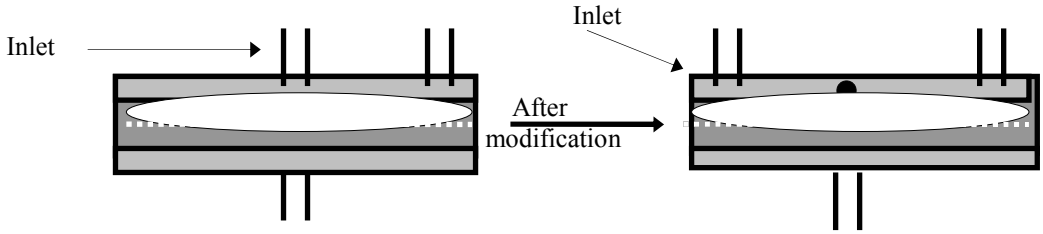


Figure 4.18.: The first modification of the membrane unit

Secondly the inlet was introduced to PV unit tangentially and previous inlet sealed (Figure 4.19.).

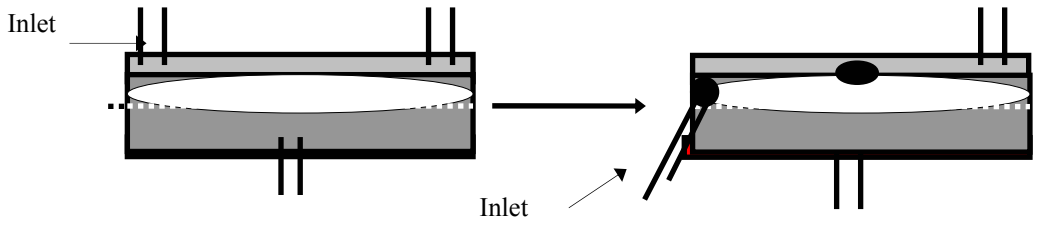


Figure 4.19.: The second modification of the membrane unit

In this way the effective membrane surface area was enlarged considerably, as it can be seen in Figure 4.20.

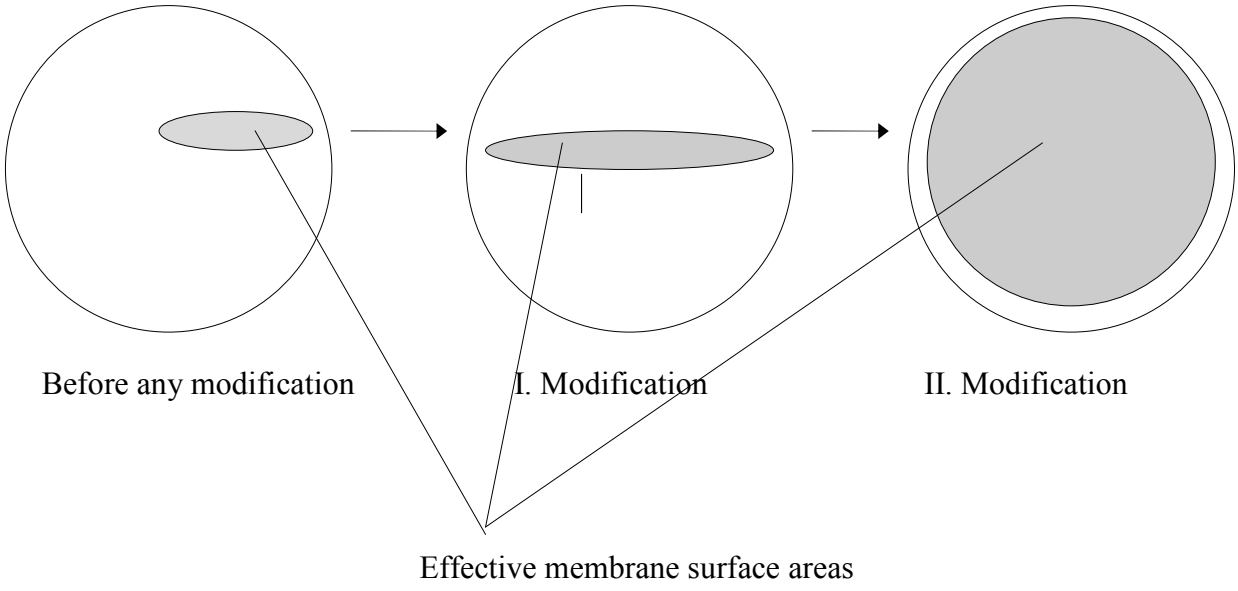


Figure 4.20.: The effect of the modifications on the membrane test cell

It can be considered in Figure 4.20., that after the final modification the effective area of the membrane was ten time larger than before any modification. Thus almost all the area of the membrane can be used in the further applications.

4.3.4. Characterisation of the pervaporation membrane

Applying pervaporation for a given separation, the most important thing is to find a proper membrane. Then it has to be characterised experimentally. The two main parameters for characterisation are the flux and the selectivity of the membrane.

Pervaporation can be used for separation of both products in the esterification reactions. However, in this section recovery of only the ester component from the reaction mixture having no solvent (solvent free system) is aimed. The ester compound, ethyl acetate – having hydrophobic feature – can be separated from the reaction mixture by hydrophobic (organophilic) membranes. Unfortunately only one type of orghanophilic membrane available commercially was suggested to apply for our purposes: the PV 1060 type membrane from GFT (now Sulzer, Germany). So our experiments were carried out with this membrane, using model solutions (ethyl acetate – ethanol, containing no water, acetic acid or enzyme)

Procedure of the experiments. Prior to the characterisation of the PV membrane, firstly its compatibility should be checked. Therefore it was immersed into the particular reaction mixture, containing ethyl acetate and ethanol. It was found that the compounds present did not damage the membrane, they were not harmful. Moreover, the gasket material has to be stable against the components of the feed mixture, as well (which was also checked) and must be free from apparent damages.

Then the membrane was cut into a disc form suitable for the test cell (diameter 158 mm). The membrane must not handle with naked hands, it is necessary to use gloves when holding the membrane. Folding, dirt, bending and punching should be avoided to keep the membrane unharmed, intact, defect-free.

The PV membranes have to be conditioned before using, this was kept in the reaction mixture to be separated for a couple of hours. After the permeate condensers were

installed under the test cell, the disc from PV membrane was adjusted carefully onto the top of the lower part. The o-ring must fit tightly into the test cell. Then the cell was closed by fixing and fastening the bolts and nuts.

As a next step the cell was connected to the vacuum pump and the vacuum of the system was checked, to study if any leakage occurs. Similarly the thermostat operation was checked and the jacketed parts of the test cell were filled with the heat carrier liquid (water). Between the permeate side of the unit and the vacuum pump, there are three dry ice acetone traps to collect the vapours that permeate through the membrane. Those cold traps are joint parallel to condense more permeate if necessary. During the experiment those traps were collected and heated it up to the room temperature to measure the weight. The vacuum pump was inline with columns to avoid the vapour getting into pump. To measure the pressure manometer was used. The scheme of the system is presented in Figure 4.21.

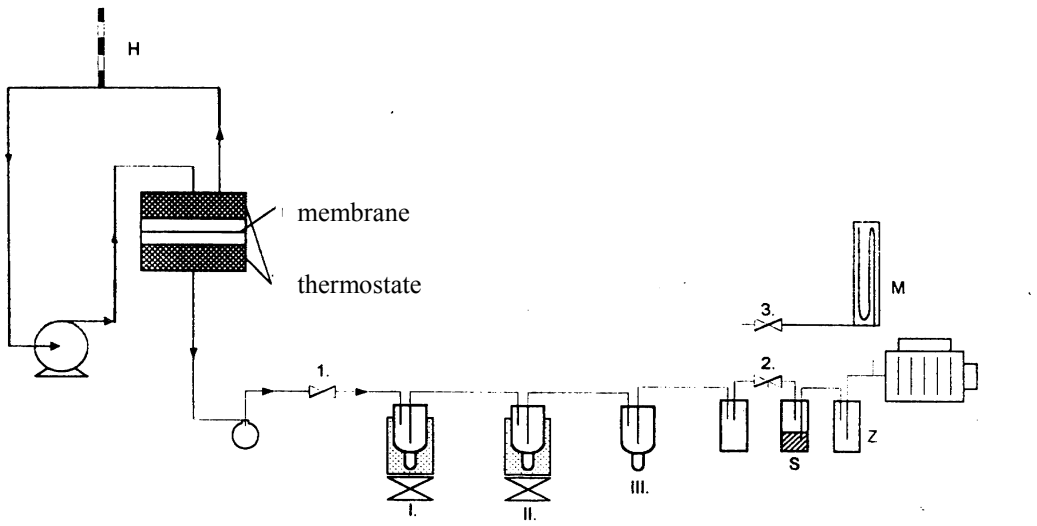


Figure 4.21.: Scheme of the system

During the tests 90 w/w% ethanol and 10 w/w% ethyl acetate mixture (200 ml) was circulated by a peristaltic pump in the primary (upper) side of the cell at 40 °C temperature and the vacuum used was 8 kPa. The effective membrane surface area was 178 cm^2. Permeate samples were collected in the traps and their weights and compositions were determined for the calculations of selectivity and flux, respectively. The composition of the permeates as well as the retentates was measured by a GC method. The tests were repeated five times, and the average was calculated.

Experimental results. The results of one representative experiment (as an example) are summarised in Table 4.6. The selectivity and the flux data were calculated from the composition (by GC analysis) and weight of the permeate samples, respectively.

Table 4.6.: The result of the GFT PV 1060 membrane test experiment

Time (min)	Sample amount (g)	Ethanol (g)	Ethyl-acetate (g)	Selectivity (-)	Flux ($kg\ h^{-1}m^{-2}$)
20	10.07	6.40	3.67	5.16	1.71
40	7.67	4.96	2.71	4.92	1.36
60	7.98	5.32	2.66	4.50	1.30
80	5.97	4.14	1.83	4.00	1.02
Altogether	31.69	20.82	10.87		

The selectivity and the flux as they were defined in chapter 1.6., page 20, obtained are shown in Figures 4.22. and 4.23. It can be seen that ethyl acetate was concentrated in the permeate (compared to the feed) so the PV membrane is suitable to enrich the mixture in ethyl acetate. It can be seen that the selectivity and flux were changed as a function of the measurement time. Its reason is the changing in composition of the feed, thus the driving force has changed, as well. Therefore only the first data were used in our further calculations.

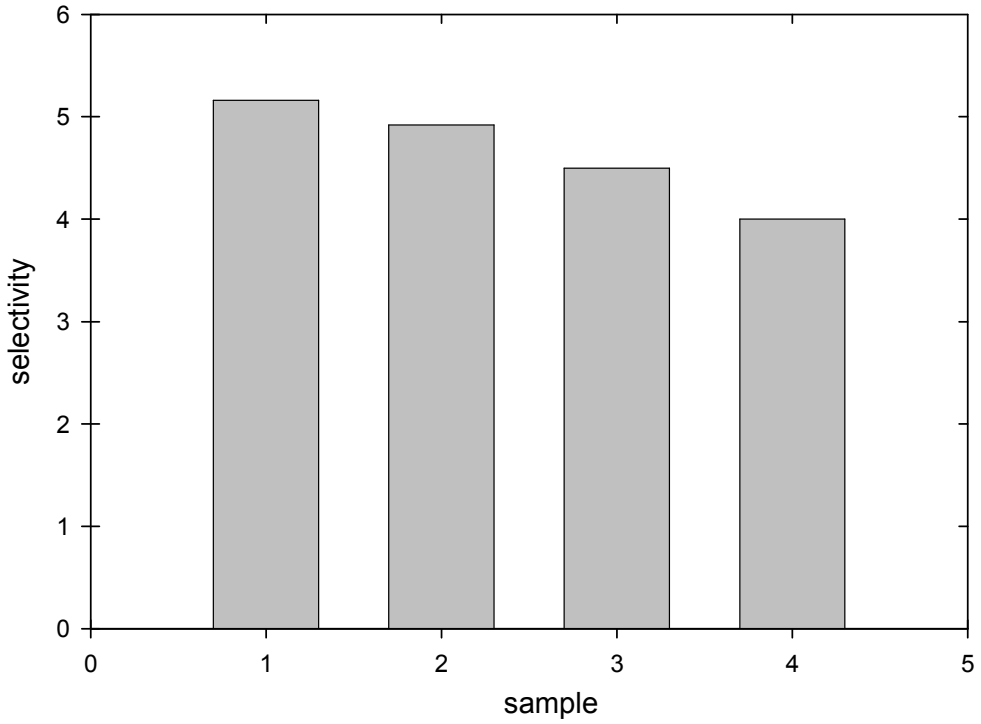


Figure 4.22.: The selectivity of the pervaporation membrane

As a result of our investigations it was found, that the average selectivity of the PV 1060 membrane was 5.1, while its flux can be characterised as 1.6 kg/hm^2 (as an average).

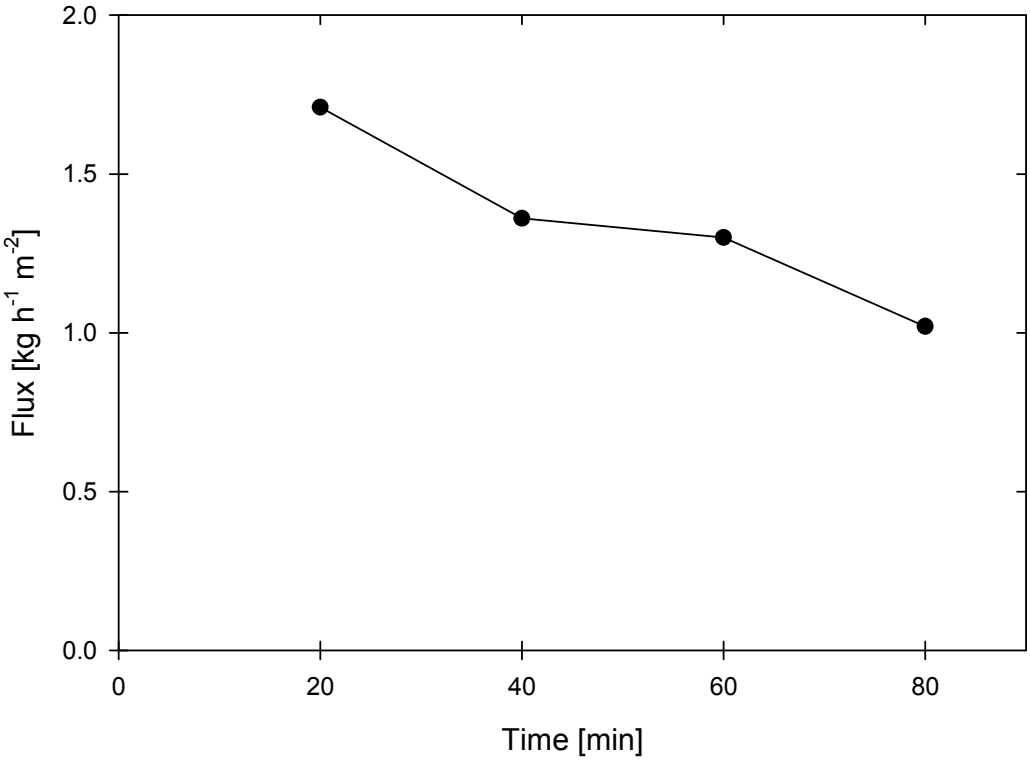


Figure 4.23.: The flux of the pervaporation membrane

4.4. Enzymatic esterification in integrated system

Statement:

In solvent-free esterification of acetic acid and ethyl alcohol by lipase carried out in an integrated system, where simultaneous ester removal by pervaporation and water removal by adsorption were coupled to the reaction, 20 % higher conversion was reached than in the traditional reaction [1, 6, 7, 9, 13, 16, 17, 19].

4.4.1. Introduction

Ester removal from the reaction mixture has not been investigated earlier (before my work), since its effect is not so crucial if the reaction is carried out in organic solvent. Since the esterification is a typical equilibrium reaction, its equilibrium can be shifted towards the synthesis by removing the products. Moreover, in a solvent-free system the conversion and reaction rates are expected much lower than in organic solvent, thus more efforts should be done to enhance the effectiveness of the reaction. Therefore simultaneous removing of the ester produced, beyond the water removal, is considered as an effective tool to promote the synthesis. Since esters of short chain acids are volatile compounds, pervaporation seemed to be an attractive method for their removal, too.

In this project our aim was to build an integrated system where an esterification reaction in solvent-free system by lipase can be carried out. In this way, natural flavour esters free from any solvent traces can be obtained, which are considered as the next generation of flavour compounds.

4.4.2. Bioreactor selection

During the study of the esterification reaction by lipase, experiments were carried out in shacking flasks. In the integrated system complete mixing had to be maintained by another method. Since the enzyme is an immobilized preparation, vigorous stirring, high shear stress would damage it easily. Therefore a gentle process should be selected: the recirculation flow rate of the reaction mixture was adjusted at a certain level (80 ml/min) to assume the proper mixing of the reactants.

Unfortunately, the relatively high flow rate was not enough for the complete mixing of the system. Therefore a stirrer with two blades was built and used in the bioreactor, with low rpm.

4.4.3. Structure of the integrated system

After the previous investigations on the enzymatic reaction, and the water as well as ester removal possibilities, an integrated esterification system was designed. The scheme of the system is presented in Figure 4.24.

The central unit of the system is a stirred tank reactor. It is equipped with a heating and thermostatic system and a reflux condenser. The reaction mixture placed in the reactor was circulated by a pump through one of the two columns filled with zeolite for water removal, on one hand; and through the primary side of the pervaporation cell, on the other hand. The secondary side of the membrane module was under vacuum, and cooled traps, vessels were built in the system between the module and the vacuum pump. The used membrane was the GFT PV 1060. For the continuous operation two parallel zeolit columns were built in. The mixing was maintained in the reactor by the circulating the reaction mixture and the enzyme preparation was trapped inside the reactor by glass filter (fibres).

4.4.4. Procedure

Firstly the reaction mixture containing the substrates and the immobilised enzyme preparation (Novozym 435) were placed into the reactor.

The total amount of the reaction mixture was 200 ml with 4 g enzyme, this mixture contained 148 g ethanol and 9.73 g acetic acid. The earlier determined optimal reaction conditions were used during the test experiments (initial acetic acid : ethanol ratio 1:20). Each columns were filled with 12 g zeolites. The temperature in the reactor was 40 °C and in the secondary side of the module the pressure was 8 kPa.

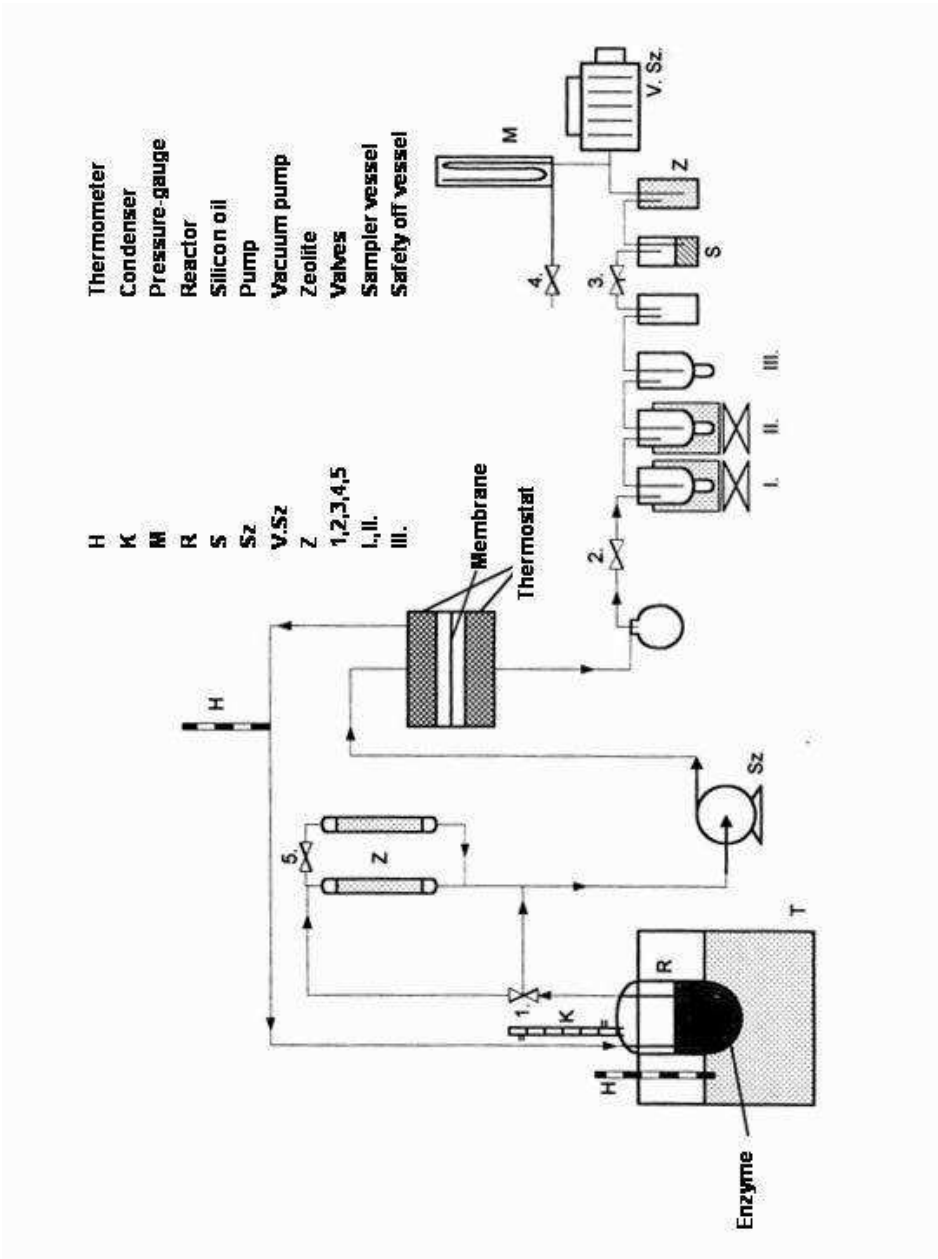


Figure 4.24.: Scheme of integrated system

After the reaction was initiated, the reaction mixture was started to circulate. The membrane module was used continuously, while one of the columns was used only in regular periods. Samples were taken from the reactor and from the permeate, regularly..

4.4.5. Experimental results

The results of the solvent-free esterification experiment in integrated system are presented in the Figures 4.25. and 4.26.

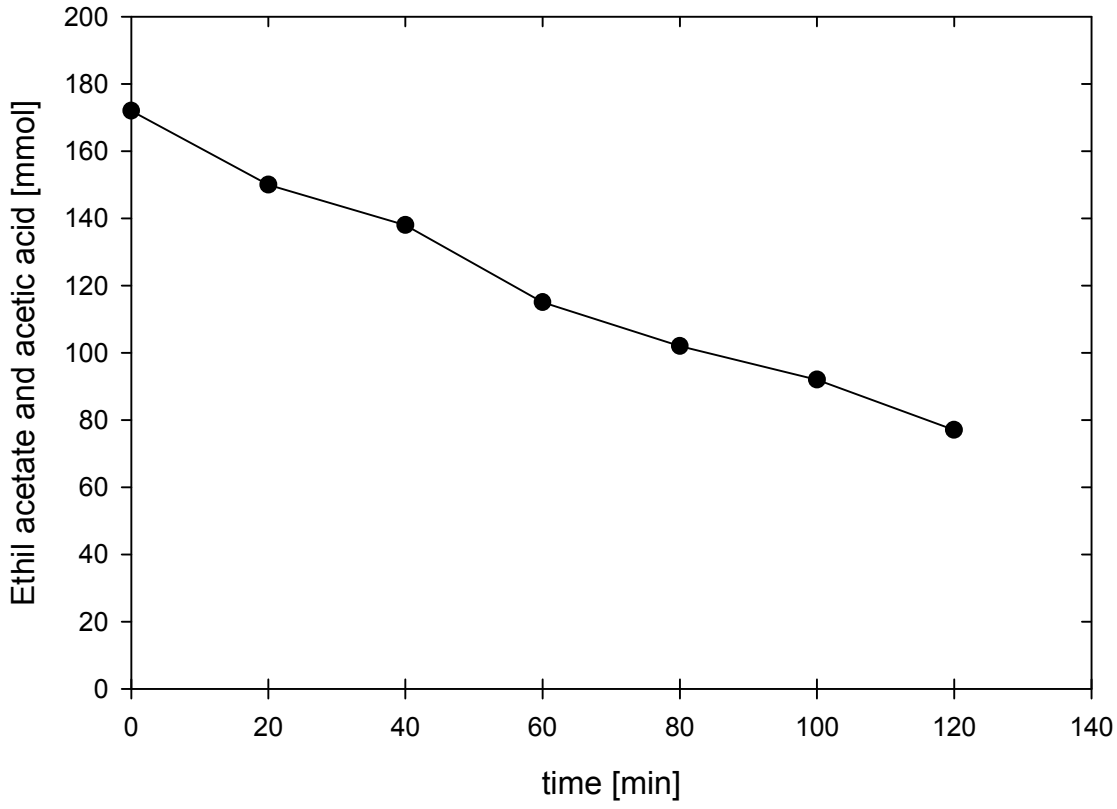


Figure 4.25.: Amount of acetic acid in the reactor

It was found (Figure 4.25.) that the amount of acetic acid (substrate) decreased monotonously as a function of time. Altogether 60% of the initial acetic acid was transformed to ester after 2 hours reaction time.

In Figure 4.26. the water level in the reactor can be seen. As it is shown the water level was managed to keep around 60 mmol in the reactor, so the process developed was appropriate to maintain the optimal water content for the enzymatic reaction.

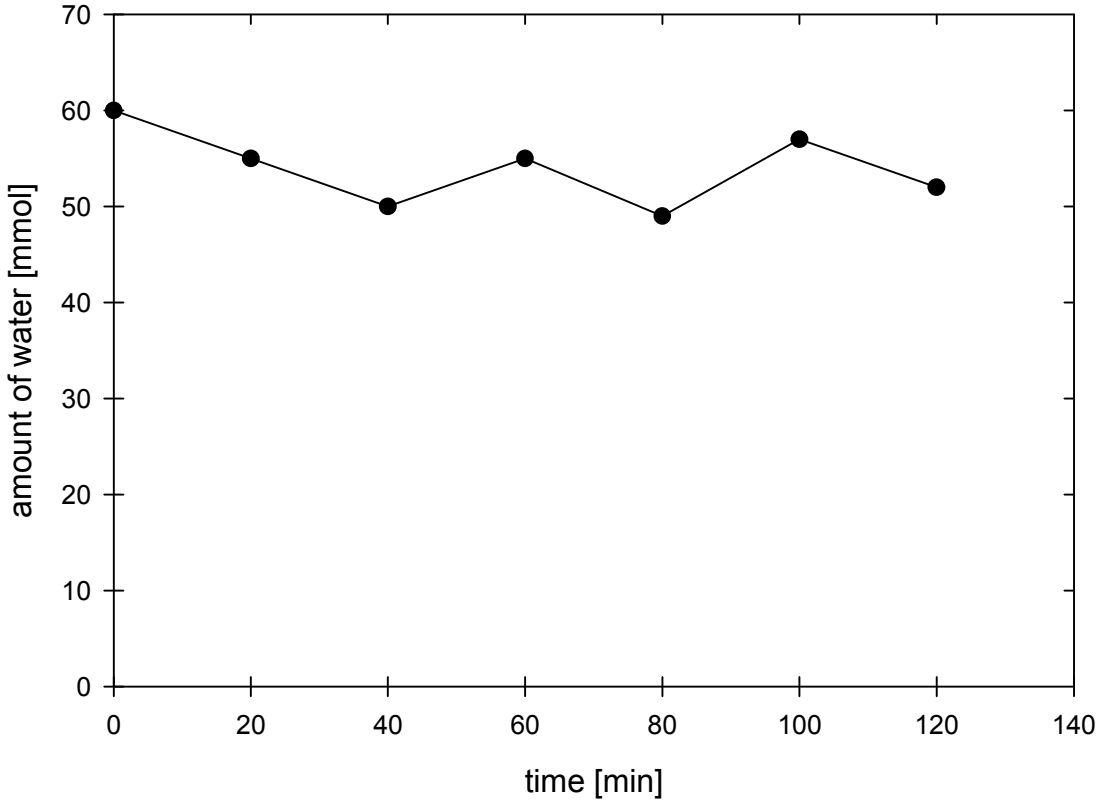


Figure 4.26.: The amount of water in the reactor

The experimental data of pervaporation are summarised in Table 4.7., where the amounts of permeated compounds during the reaction was calculated based on the data obtained in the experiments.

Table 4.7.: The amount of the permeates and their composition during the experiment

Time (min)	Sample (g)	Ethanol (g)	Ethyl acetate (g)
20	15.70	15.50	0.19
40	10.07	9.82	0.25
60	10.14	9.56	0.57
80	9.81	9.14	0.66
100	10.39	9.57	0.81
120	10.11	9.22	0.88
Sum.	56.11	53.6	2.50

It was found that the amount of the permeate after the first period was similar, so the flux was constant during the two hour long experiments. The amount of ethyl acetate was growing in the permeate phase as it was produced in the reaction.

The total amount of the ester produced in the reaction was calculated in Table 4.8. as the sum of its amount in the permeate and in the reactor. It can be seen that altogether more than 100 mmol ester was manufactured during the two-hour reaction time.

Table 4.8.: Total amount of ester produced in the integrated system

Time (min)	Ester in the reactor (mmol)	Ester in the permeate (mmol)	Sum (mmol)
0	0.00	0.00	0.00
20	6.18	2.22	8.40
40	13.90	2.84	18.97
60	20.38	6.52	31.96
80	31.49	7.55	50.63
100	56.34	9.30	84.77
120	63.63	10.02	102.08

In Figure 4.27. the total amount of ester produced is presented with and without products removal. It can be seen, that higher conversion was achieved in integrated system and the difference between the two modes of operation was formed more than 20 %. So the integrated system has really worked with higher effectiveness.

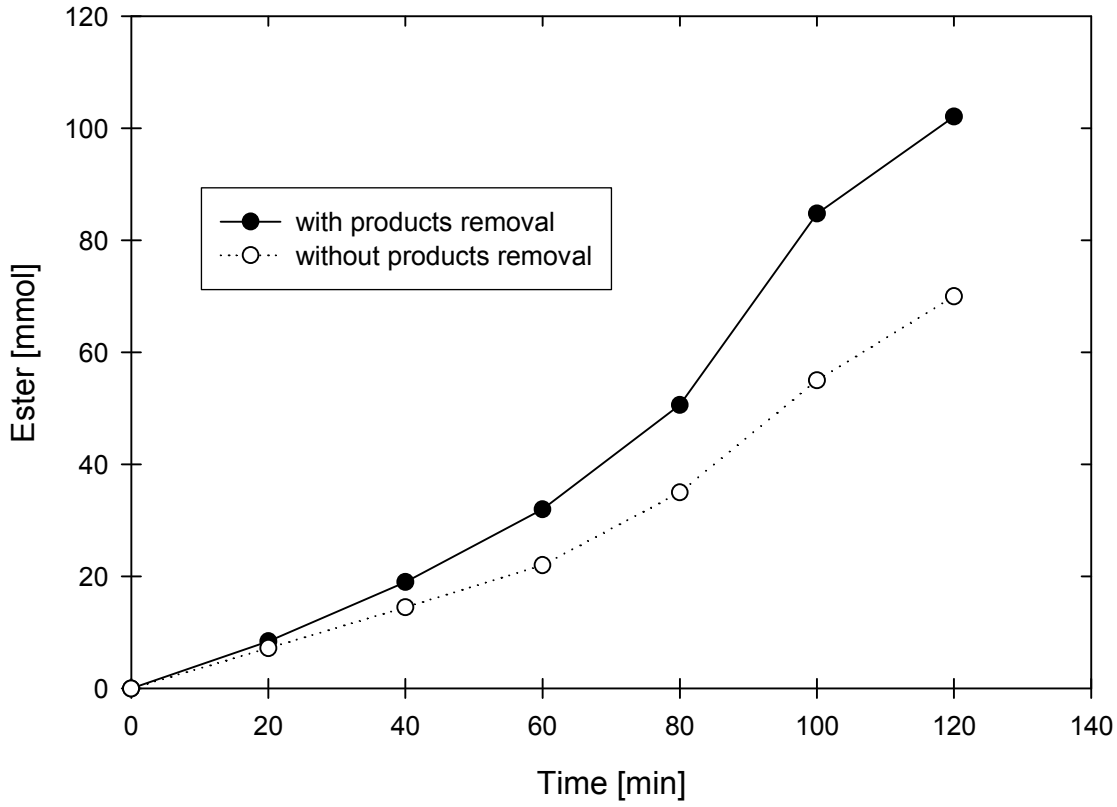


Figure 4.27.: Comparison of the total amount of ester produced with and without products removal

4.4.6. Evaluation

The experimental data presented here were the results of the successful attempt to realize a solvent-free esterification process by lipase immobilized in integrated system.

Before this successful lab scale reaction, several experiments were failed in the integrated system, due to the numerous difficult parts built in the apparatus, though each section (the reaction itself, bioreactor, ester removal, and water removal) as investigated and characterized in details earlier.

Since the system is quite complicated, many experiments should still be carried out in lab-scale, before building a pilot system. Moreover it is extremely important to evaluate

the results critically to face the weaknesses of the system to be able to carried and improve it later.

Evaluating the experimental results it should be noted that the productivity of the system can be enhanced by applying a better strategy for feeding the bioreactor, based on the substrate inhibition kinetics, described in Thesis 1. Fed-batch mode of operation seems promising in this case. For water removal, adsorption is a good method, water content was possible to keep at a constant level during the experiment. However a better type of zeolite should be found, having better resistance against acid traces.

Ester recovery by pervaporation is again a promising process. According to a preliminary economical calculation, the coast determining step here is the effectivity of the product recovery and the ester purity obtained as a permeate. As the used hydrophobic pervaporation membrane selectivity and flux are the not so high (since it was developed for other purposes) integrated system's productivity can not be expected high enough for a profitable process. This is the "weakest" part of the system; therefore further search for better pervaporation membranes must be done.

Although these problems seem difficult to solve, the findings presented here have proven clearly the usefulness of these types of complicated reaction systems, and hopefully will serve as an example for similar solvent-free enzymatic reactions in future.

5. Summary

In this work the aim was to study the enzymatic esterification of acetic acid and ethanol to manufacture natural ethyl acetate, an important flavour ester compound. The experimental and theoretical results obtained during the investigations are summarised in the following findings:

Thesis I:

Kinetics and the effect of temperature were studied in the esterification of ethanol and acetic acid by lipase both in organic solvent and in solvent–free system. It was found that strong acid inhibition occurs during the reaction, and Michaelis-Menten parameters (v_{max}, K_M, K_I) were determined for the solvent-free system. The activation energy of the system was calculated, as well.

Thesis II:

Semi-pilot scale enzymatic esterification of ethanol and acetic acid with continuous water removal was realised to produce a natural flavour compound. The process in organic solvent was coupled with hetero-azeotropic distillation, while in solvent-free system it was integrated with adsorption, resulting in more than 100% increase in conversion [2, 8].

Thesis III:

The construction of the flat sheet GFT pervaporation test cell was improved and an organophilic PV membrane for removal of product in esterification was tested.

Thesis IV:

Solvent free esterification of acetic acid and ethyl alcohol by lipase was carried out in integrated system, where simultaneous ester removal by pervaporation and water removal by adsorption were coupled to the reaction

The results summarized here can hopefully contribute to the better understanding of the phenomena appeared in enzymatic esterifications and in the applications of integrated systems, as well as to the elaboration of a technology to produce a new generation of natural flavour esters in solvent-free system.

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