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ETHYL-ACETATE SYNTHESIS IN GAS PHASE BY IMMOBILISED LIPASE

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Gas-solid phase biocatalytic reactions offer economic and environmentally sound ways to produce ester compounds, which can be used as natural flavour components, and other types of value-added products. Therefore, the aim of this work was first to study the continuous gas-solid phase manufacture of ethyl-acetate (EtAc), which is an important fruit flavour compound, from ethanol (EtOH) and acetic acid (AcAc) applying immobilised *Candida antarctica* lipase B enzyme in a self-constructed bioreactor and then to determine the effects of initial substrate composition, applied temperature, and the amount of used enzyme on the yield. It can be concluded that there was a well-defined connection between the yield of the ethyl-acetate product, the temperature and the amount of used enzyme, while the correlation between the initial substrate composition and the product yield could not be described so easily. The activation energy of the esterification was found to be much lower in our system than that of the same enzymatic reaction carried out in other reaction media, such as organic solvent system, ionic liquid, etc.

Keywords: solid/gas phase, enzymatic reaction, lipase, ethyl-acetate

Introduction

Organic flavour compounds can be defined as natural when they are produced either from natural sources [1] or in natural processes, such as physical treatment, fermentation [2], or enzymatic reaction [3]. Although these natural compounds are healthier and more attractive to the consumers [4], their extraction from natural sources, e.g. various plants and fruits, is not only expensive but also results in extensive waste production.

Therefore, the production of natural aroma compounds, especially esters by fermentation and enzymatic reactions in aqueous [5], organic solvent [6], or novel environmentally friendly solvent media [7] has become a widely-studied field of research in the last few years.

Solid-gas phase biocatalysis, where biocatalysts are in solid form while the substrates are in gaseous state or can be easily vaporized [8], offers the following advantages over solid-liquid systems and organic liquid medium [9–11]:

- higher biocatalyst stability
- thermo-denaturation of the (partially) dehydrated biocatalyst is limited
- mass transfer of components is more efficient in the gas phase
- diffusion limitation is reduced due to the low viscosity in the gas phase
- production of by-products is reduced or avoided
- very high conversion yields can be achieved
- products and unconverted substrates can be easily recovered with condensation
- risk of microbial contamination is lower

Recently, both enzymes (mainly lipases in esterification reactions [12, 13]) and whole cells (e.g. baker's yeast) have been studied in solid-gas systems. The experiments were carried out either by immobilized or soluble biocatalyst [14, 15].

Barzana [16] studied the gas-phase oxidation of ethanol vapour with molecular oxygen and dehydrated, immobilized alcohol dehydrogenase from *Pichia pastoris* cells and they found that dry alcohol oxidase was more thermostable in gas-phase than in aqueous solution.

In the works of Mikolajek [17] and Spiess [18] immobilized enzyme preparations were used successfully in carboligation reactions, where benzaldehyde was converted to benzoin using thiamine diphosphate-dependent enzymes.

Gas phase ethyl acetate production was studied by Hwang and Park [19] in a batch bioreactor applying porcine pancreatic lipase in a powder form. In the experiments, effects of reactant concentration, amount of enzyme, and reaction temperature on the performance of the bioreactor were investigated.

Letisse and co-workers [20] used a continuous gas phase reactor to study the effect of organic molecules on the kinetic parameters of the alcoholysis of methyl propionate by 1-propanol catalyzed by immobilized *Candida antarctica* lipase B.

The gas phase continuous production of acetaldehyde from ethanol and the production of hexanal from hexanol using dried baker's yeast were studied in a continuous operational system and it was found that after 20 hours long experiments the hexanal conversion was as high as 32% without a decrease in enzyme

activity [21]. The results suggested that dehydrated enzymes may have potential advantages in solid-gas phase bioreactors.

The aim of this work was first to construct a solid-gas phase laboratory scale experimental set-up and then to carry out experiments on the continuous esterification of ethyl-acetate from acetic acid and ethanol applying *Candida antarctica* lipase B enzyme in this bioreactor and to determine the effects of initial substrate composition, applied temperature, and amount of used enzyme on the esterification reaction. The synthesis of ethyl acetate is considered to be a quite important process since it can be extended to the gas-solid phase synthesis of other flavour esters, such as isopropyl acetate, isobutyl acetate, ethyl propionate, and butyrate. Furthermore, it was planned to determine the activation energy of the reaction and to compare the results to the data in the literature concerning the same esterification reaction carried out in other types of reaction media.

Material and methods

Materials

The immobilized biocatalyst, Novozym 435 *Candida antarctica* lipase B was purchased from Novozysmes (Bagsvaerd, Denmark).

Acetic acid (≥99.7%), ethanol (≥99.7%), and ethyl-acetate were of the highest purity and provided by Sigma-Aldrich, Germany.

Nitrogen (N_2) gas was provided by Linde, Hungary. All other chemicals were analytical grade and purchased from Sigma-Aldrich, Germany.

Experimental set-up

The experimental set-up, shown in *Figure 1*, is composed of three main functional units; I. substrate saturation module, II. bioreactor, III. product and remaining substrate recovery module. It is built up of the following parts; two thermostates (1), flow meter (2), substrate separation unit (3), septums for gas sampling (4), bioreactor with the immobilized biocatalyst (5), two vapour condensation units (6,7), condensated sample reservoir (8), N_2 gas outlet (9), and a cryostat (10).

Substrate was continuously fed to the bioreactor via the flow meter by passing N_2 carrier gas through the mixed ethanol and acetic acid substrate solution with a rate of 2 $dm^3(N_2)h^{-1}$. The carrier gas first was saturated by the substrates in the substrate saturation unit then was passing through the thermostated glass spiral tube bioreactor packed with approximately 11 g of immobilized lipase operating under atmospheric pressure and with a mean residence time of 72 s.

After the enzymatic synthesis of ethyl-acetate took place in the bioreactor, the remaining substrates and products were first condensated and trapped into cold ethanol solution (-18°C), then removed from the N_2 stream by cooling, while N_2 was recycled.

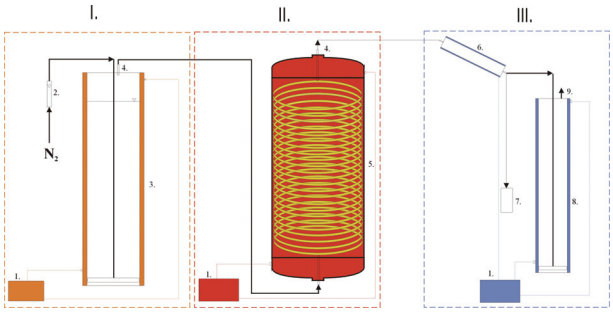

Figure 1: Scheme of the experimental set-up
1. thermostate, 2. flow meter, 3. substrate saturation unit, 4. septum for gaseous sampling, 5. bioreactor, 6.,7. vapour condensation units, 8. condensated sample reservoir, 9. gas outlet, 10. cryostat

In the first two functional units the constant temperature (which was lower in the case of unit I than that of unit II in order to avoid occasional condensation) was maintained by thermostates. Gas phase samples were taken both from the substrate separation unit and the bioreactor through the septums.

Analytical method

The gas samples were analysed by gas chromatography using a HP4890 type gas chromatograph equipped with a FID detector and a FFAP fused silica capillary column (Macherey Nagel, Germany). The temperature program was the following: 3 min isothermal period at 60°C, then temperature was raised to 250°C at 10°Cmin^{-1}, while the injector and detector were maintained at 250°C.

Optimization of carrier gas amount

Optimal flow rate, hence optimal amount of the carrier N_2 gas - which affects the residence time of the gaseous reactant mixture and productivity of the whole system - was determined in the experimental set-up by measuring the conversion of the acetic acid substrate and the yield of ethyl-acetate product at various carrier gas flow rates (2, 3, 4, 5 dm^3h^{-1}). In all cases the ratio of acetic acid and ethanol was 80:20 cm^3cm^{-3} (3.86 gg^{-1}), the amount of used enzyme was 11 g, and the temperature of the bioreactor and unit I were 50°C and 30°C, respectively.

Effect of temperature on the esterification reaction

Selection of the optimal reaction temperature and the investigation of the effect of temperature were realized by the determination of the acetic acid substrate conversion and ethyl-acetate product yield at different bioreactor temperatures (30°C, 40°C, 50°C, and 60°C).

The temperature of unit I was always higher than that of unit II in order to avoid occasional condensation, and the temperature of unit I and III were continuously detected. The ratio of acetic acid and ethanol was 80:20 cm^3cm^{-3} (3.86 gg^{-1}), the amount of used enzyme was 11 g, the carrier gas flow rate was 2 dm^3h^{-1} and the experiments were 9 hours long, in all cases.

Effect of initial substrate composition on the esterification reaction

Effect of initial substrate composition was studied and the optimal substrate composition was selected by measuring the acetic acid conversion and the ethyl-acetate yield applying various acetic acid and ethanol ratios (80:20, 75:25, 65:35, 50:50, 25:75 in cm^3cm^{-3}). The amount of enzyme was 11 g, the carrier gas flow rate was 2 dm^3h^{-1}, the bioreactor and the substrate saturation temperatures were 50°C and 30°C, respectively.

Effect of the amount of enzyme on the esterification reaction

Experiments were carried out applying different amounts of Novozym 435 lipase enzyme (3.7 g, 6.7 g, 11 g) to determine the effect of enzyme quantity on the ethyl-acetate production. Temperature of the bioreactor was kept at 50°C, the N_2 carrier gas flow rate was 2 dm^3h^{-1}, and the acetic acid and ethanol ratio was 80:20 cm^3cm^{-3} (3.86 gg^{-1}).

Calculation of the activation energy

The activation energy of the reaction was calculated from the Arrhenius-equation (Eq. 1), and the logarithmic Arrhenius-equation (Eq. 2), where reaction rate is proportional to the activation energy.

$$v = A \times \exp(-E_a/RT), \quad (1)$$

$$\ln v = \ln A - E_a/RT. \quad (2)$$

where:

A – Arrhenius constant
R – 8.314 ($Jmol^{-1}K^{-1}$)
E_a – activation energy ($Jmol^{-1}$)
T – temperature (K)

Results and discussion

Optimization of carrier gas amount

Ethyl-acetate yields and acetic acid conversions as a function of carrier N_2 gas flow rate are represented in *Figure 2* and *Figure 3*, respectively.

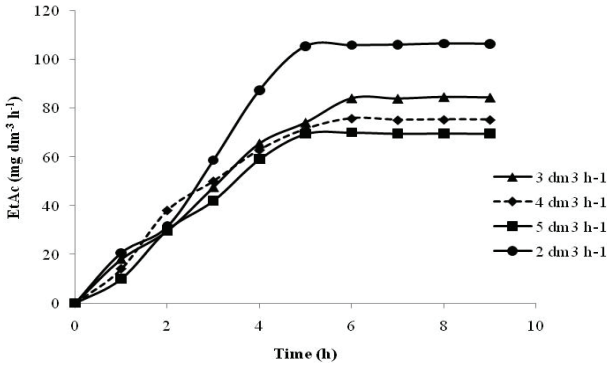

Figure 2: Effect of carrier gas flow rate on ethyl-acetate yield as a function of time

It can be seen that both higher conversion and yield can be achieved at lower flow rate, hence longer reaction time provided for the production of ethyl-acetate can be obtained. Therefore, 2 dm^3h^{-1} carrier gas flow rate was selected for further experiments.

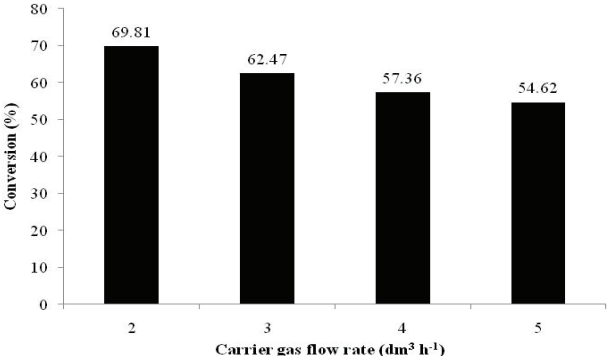

Figure 3: Effect of carrier gas flow rate on acetic acid conversion

Effect of temperature

Results of the experiments concerning the effect of temperature on the enzymatic esterification and the selection of the optimal reaction temperature are shown in *Figure 4* and *Figure 5*.

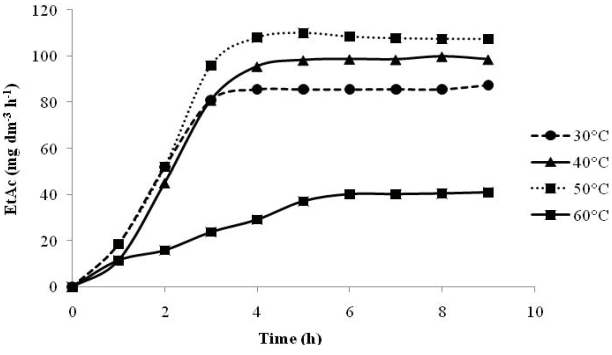

Figure 4: Effect of temperature on ethyl-acetate yield as a function of time

Fig. 3 shows that the amount of produced ethyl-acetate increases relatively fast at the beginning of the esterification until it reaches equilibrium, hence, the

curves follow the trend of enzymatic reactions. The system can be considered continuous after it reaches equilibrium, which happens approximately after 3–6 hours.

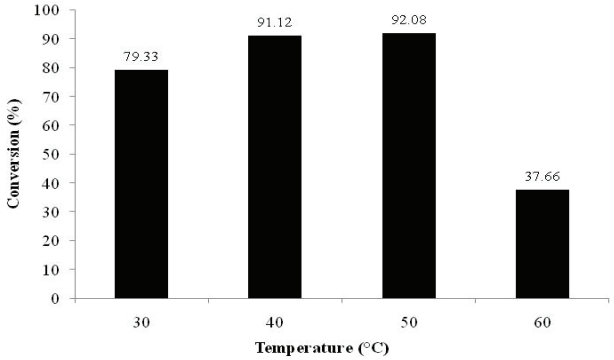

Figure 5: Effect of temperature on acetic acid conversion

It is reasonable that higher coversion and yield values belong to higher temperatures; the highest values were measured at 50°C, up till 60°C, where the enzyme activity decreased due to the temperature sensitivity of enzyme proteins. At the selected optimal temperature of 50°C quite high conversion was achieved and the yield of ethyl-acetate was higher than 110 $mgdm^{-3}h^{-1}$.

Effect of initial substrate composition

The values of ethyl-acetate yield and acetic acid conversion in the case of different substrate mixture compositions are shown in *Figure 6* and *Figure 7*. It is important to mention that due to the difference in the vapour pressure of the two substrate components (b.p. acetic acid = 11.8°C, b.p. ethanol = 78.4°C), higher amount of liquid acetic acid in the mixture would mean lower amount of acetic acid vapour and higher amount of ethanol vapour and vica versa. The exact values for the substrate composition in liquid and gaseous phases are shown in *Table 1*.

Table 1: Comparison of the initial substrate composition in liquid and gaseous phase

Ratio of substrates in liquid phase AcAc:EtOH (cm^3cm^{-3})	Ratio of substrates in gaseous phase EtOH:AcAc (gg^{-1})
80:20	2.0 ± 0.04
75:25	3.5 ± 0.05
65:35	1.0 ± 0.03
50:50	0.9 ± 0.05
25:75	0.2 ± 0.06

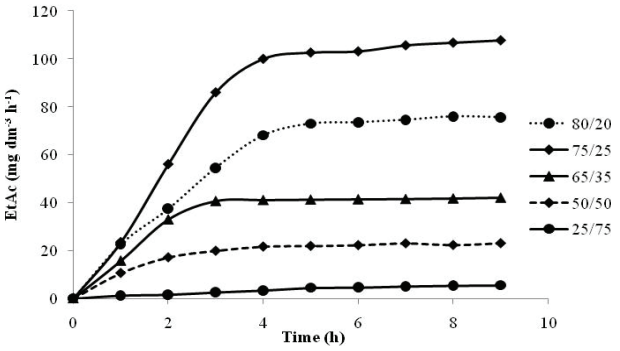

Figure 6: Effect of initial substrate composition on ethyl-acetate yield as a function of time

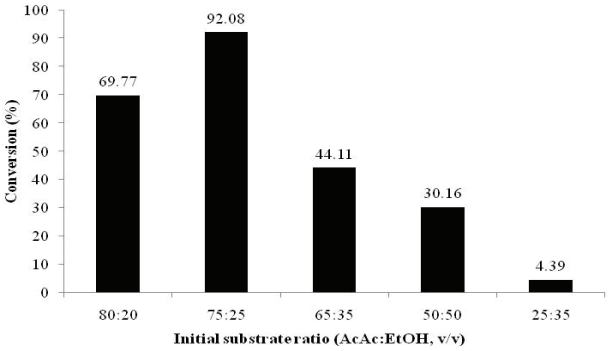

Figure 7: Effect of initial substrate composition on acetic acid conversion

In *Fig. 6* it can be seen that the enzymatic esterification has reached equilibrium independent of the initial substrate composition. In the case of 25:75 acetic acid and ethanol ratio, there was so small amount of acetic acid vapour in unit I that the reaction started only after one hour reaction time. The highest conversion and yield values were obtained by the experiment in which the acetic acid and ethanol ratio of 75:25 $mlml^{-1}$ was used, hence this value is considered to be the optimal initial substrate ratio.

The highest conversion values are obtained for 75:25 liquid acetic acid and ethanol ratio, while the lowest value belongs to its opposite, the ratio of 25:75. This can be explained by the fact that the substrate mixture has inhibition effect on the enzymatic reaction mainly caused by acetic acid. Therefore, the lower is the acetic acid vapour proportion in the bioreactor is, the less significant of inhibition and more efficient the enzymatic reaction will be.

Effect of the amount of enzyme

The effect of the used enzyme quantity on the conversion is shown in *Figure 8* and on the yield of ethyl-acetate product is represented in *Figure 9*.

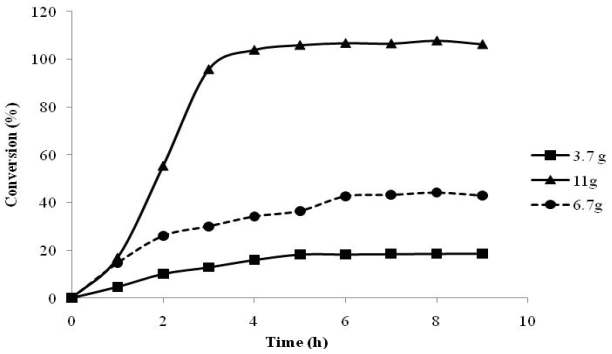

Figure 8: Effect of the amount of enzyme on ethyl-acetate yield as a function of time

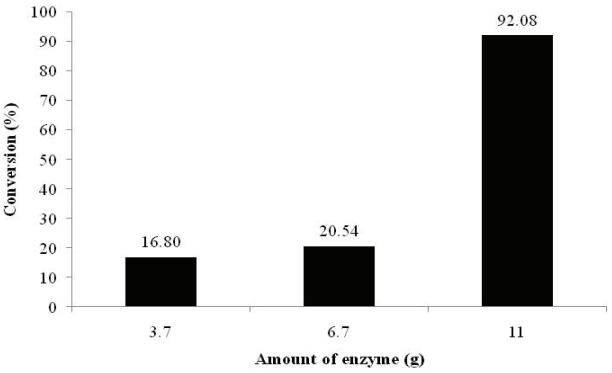

Figure 9: Effect of the amount of enzyme on acetic acid conversion

Although both the conversion and yield results showed an increase with the increase in the amount of enzyme, it is quite surprising to note that there is only a small difference between the conversion values for 3.7 g and 6.7 g of enzymes, while the conversion was more than four times higher when the amount of enzyme was increased from 6.7 g to 11 g. Probably, further enzyme addition would further increase the conversion and the product yield but taking the relatively high price of the enzyme into account, further enzyme addition was not studied due economical considerations.

Calculation of the activation energy

The calculated activation energy of the gas-solid phase ethyl-acetate production reaction was $E_a = 9.2$ kJmol^{-1}, which is in good agreement with the value reported by Perez [12]. Our result and activation energies determined for the same esterification reaction in other reaction media are summarized in *Table 2*. It can be seen that our result is lower than the ones found in the corresponding literature. Therefore, it can be stated that the studied enzyme catalyzed esterification reaction can be carried out more easily and efficiently in gas phase than it other reaction media due to the much more effective mass transfer, low viscosities, and high diffusion coefficients in the gas phase.

Table 2: Activation energy of esterification in different reaction media

Reaction media	Activation energy (kJmol^{-1})
solid-gas phase	9.2
[bmim][PF_6] ionic liquid [22]	21.7
n-hexane [23]	30.6
solvent-free system [23]	52.9

Conclusion

In this work, first the *Candida antarctica* lipase B enzyme catalyzed esterification of ethyl-acetate from acetic acid and ethanol was investigated in a self-designed and self-constructed continuous gas-solid phase system. Then, the effect of temperature, enzyme quantity, and initial substrate composition on the conversion of acetic acid and on the yield of ethyl-acetate was determined and the activation energy of the biocatalytic reaction was calculated.

It was found that both conversion of acetic acid substrate and yield of ethyl-acetate increased with the increase of temperature and the amount of enzyme and that there was no such linear correlation between these values and the initial substrate ratio. The optimal reaction conditions were found to be described by the following parameters:

- flow rate of N_2 carrier gas: 2 dm^{3}h^{-1},
- temperature in the bioreactor: 50°C,
- amount of used enzyme: 11 g,
- initial acetic acid and ethanol substrate ratio: 75:25 mlml^{-1}.

The activation energy was found to be $E_a = 9.2$ kJmol^{-1}, which is much lower than that of the same reaction carried out in other reaction media. Therefore, it can be stated that it is easier and much more effective to carry out this type of enzymatic esterification in the gas-solid phase bioreactor constructed and studied by our research group.

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