TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

Diplomarbeit

Study of an enzymatic packed bed reactor for production of butyl acetate under liquid and supercritical conditions

ausgeführt am

Institut Européen des Membranes
der Ecole Nationale Supérieure de Chimie de Montpellier – Frankreich

und am

Institut für Verfahrenstechnik, Umwelttechnik und Technische Biowissenschaften
der Technischen Universität Wien

unter der Anleitung von

Ao.Univ.Prof. Univ.Prof. Dipl.-Ing. Dr.techn. Anton **Friedl** (TU Wien)

Ass.Prof. Dipl.-Ing. Dr.techn. Michael **Harasek** (TU Wien)

und

Prof. Dr. Delphine **Paolucci** (ENSCM Montpellier)

durch

David Wurm

Matr.Nr.: 0728354

Hubertusgasse 2, 7350 Oberpullendorf

Wien, am 04. Juni 2013

Acknowledgement

I would like to thank Prof. Anton Friedl for his support and for giving me the opportunity to undertake my master thesis at the Institute of Chemical Engineering at the Vienna University of Technology.

A special thank you goes to Michael Harasek who made this project possible and who always supported the idea of carrying out the practical work for this master thesis at an institute in a foreign country. Thanks for helping me with the planning, execution and completion of my thesis and defence.

In addition I want to extend my thanks to my colleagues at the institute of chemical engineering in Vienna who supported me throughout, especially when I had technical difficulties with the pilot plant in France.

I would like to express my deepest gratitude to Delphine Paolucci who gave me the chance to work in a lab at her institute for five months. The guidance and support received from her and Marie-Pierre Belleville was indispensable for the success of this project and I am grateful for their constant guidance.

Furthermore I would like to acknowledge the technical support and advice of Christoph and Loubna.

I think it is important to also say thank you to all of my colleagues at the Institut Européen des Membranes, Montpellier who welcomed me into their team right from the first day and who always helped me out. Special thanks to Matthias, Jennifer and Damien who I could always count on and who made my stay in France really special and I cannot thank them enough for this tremendous kindness.

Last but not least I would like to recognise the importance of the support I have received from my loved ones, throughout the entirety of my studies. I want to thank my parents Anita and Martin who made all this possible and who have constantly been extremely supportive of my endeavours. I also want to thank my sisters Sigrid and Isabel and all my friends for making my years of study in Vienna a very pleasant time and an experience which I will never forget. Finally I want to thank my girlfriend Lisa for her endless patience with me when I was swamped with work and her ability to always cheer me up.

Abstract

Esters of short chained acids and alcohols which are known for their aroma and flavour quality are widely used in food, cosmetic and pharmaceutical industries. The synthesis of those substances is conventionally carried out in organic solvents with acid catalysers.

Recent studied showed that non-conventional solvents such as supercritical fluids offer many advantages compared to conventional solvents. In addition *Candida antarctica* Lipase B (CALB) is known to catalyse the synthesis of short chained esters also under supercritical conditions and then allow to carry out a biological catalysis thus avoiding problems induced by chemical ones.

A green solvent such as supercritical Carbondioxide combined with lipases as biocatalysts is a very attractive approach towards an environmentally friendly and sustainable process.

The goal of this master thesis was to develop a process to synthesise a flavour that can be used for food industry with an enzymatic packed bed reactor under supercritical conditions.

A way of immobilising the lipase CALB on a ceramic support was found and the model reaction synthesis of butyl acetate was carried out in a pilot plant of industrial scale with sc-CO_2 as solvent. After literature revue and preliminary experiments in aqueous and organic solvent optimised reaction parameters were found regarding substrate ratio, concentration, temperature and pressure. The effect of water activity was found to play an important role on the enzymes activity. Finally the activity of the immobilised enzymes regarding the synthesis of butyl acetate was measured in an organic solvent and in under supercritical conditions. The results showed equal yield and even higher activities when carrying out the reaction in supercritical CO_2 compared to an organic solvent making this new concept very attractive for further studies and industrial applications.

Kurzfassung

Ester von kurzkettigen Säuren und Alkoholen, die als Aroma- und Geschmacksstoffe bekannt sind, werden in der Lebensmittel-, Kosmetik- und Pharmazeutischen Industrie eingesetzt. Herkömmlicherweise wird die Synthese dieser Substanzen in organischen Lösungsmitteln mit sauren Katalysatoren ausgeführt.

Aktuelle Studien zeigen, dass nicht konventionelle Lösungsmittel wie überkritische Fluide viele Vorteile im Vergleich zu klassischen Lösungsmitteln bieten. Zusätzlich ist das Enzym *Candida antarctica* Lipase B (CALB) dafür bekannt, die Synthese von kurzkettigen Estern auch unter überkritischen Bedingungen zu katalysieren. Somit wird eine biologische Katalyse ermöglicht, wodurch Probleme, die durch chemische Synthese verursacht werden, verhindert werden können.

Ein „grünes“ Lösungsmittel wie überkritisches Kohlendioxid kombiniert mit Lipasen als Biokatalysatoren bereitet einen sehr attraktiven Ansatz zu einem umweltfreundlichen und nachhaltigen Prozess.

Das Ziel dieser Diplomarbeit war die Entwicklung eines Prozesses zur Synthese eines Aromastoffes, der in der Lebensmittelindustrie eingesetzt werden kann, in einem Festbettreaktor und unter überkritischen Bedingungen. Eine Methode zur Immobilisierung der Lipase CALB auf einem keramischen Träger wurde entwickelt und die Modellreaktion der Synthese von Butylacetat wurde in einer Pilotanlage mit überkritischem Kohlendioxid als Lösungsmittel durchgeführt. Nach einer Literaturrecherche und Vorversuchen in wässrigem und organischem Medium konnten Reaktionsparameter wie Substratverhältnis, Konzentration, Temperatur und Druck optimiert werden. Es wurde auch festgestellt, dass die Wasseraktivität einen entscheidenden Einfluss auf die Enzymaktivität hat. Schließlich wurde die Aktivität der Enzyme bezüglich der Synthese von Butylacetat in organischem Lösungsmittel und unter überkritischen Bedingungen ermittelt. Verglichen mit organischen Lösungsmitteln wurden ähnliche Umsätze und sogar höhere Aktivitäten für die Reaktion in überkritischem Kohlendioxid beobachtet, was dieses neue Konzept sehr interessant für zukünftige Studien und industrielle Anwendungen macht.

Nomenclatures and Abbreviations

CALA	*Candida antarctica* lipase A
CALB	*Candida antarctica* lipase B
CALB L	Commercially available solution of CALB
Novozym	CALB immobilised on macroporous resin
DCM	Dichloromethane
Phosphate buffer	If not stated otherwise 10mM phosphate buffer prepared as described in the appendix F.2
IEM	Institut Européen des Membranes, Montpellier - France
GC	Gas chromatography
sc-CO_2	Supercritical carbon dioxide
FDA	US Food and Drug Administration
FID	Flame ionisation detector

Table of contents

A.Introduction

A.1.General Introduction

Traditionally esters of short chained acids and alcohols were produced by chemical synthesis. They are known for their flavour and aroma quality and are used in food, beverage, cosmetic and pharmaceutical industries. (Gandhi 1997, Dijkstra, Merchant et al. 2007, Berger 2009)

Nowadays the sensitivity of ecological systems is increasing and people are looking for environmentally friendly processes. By using enzymes acid catalysts and high temperatures can be avoided and the selectivity is much higher, which reduces the amount of side products. As US and EU legislation considers flavours resulting from enzymatic synthesis as 'natural' and consumers demand 'natural' products biocatalysts will be used in future a lot for producing flavours as well as for other fine chemicals. (Serra, Fuganti et al. 2005, Habulin, Sabeder et al. 2007, Oliveira, Rebocho et al. 2009)

The enzymes can be immobilised on a carrier prior to usage as it brings many advantages such as easy isolation of biocatalyst from the reactants, improved stability of the enzymes and the ability to reuse the enzymes several times as the cost for enzymes are relatively high (Christensen, Andersen et al. 2003).

In this work Lipase B from *Candida antarctica* (CALB) produced by the basidiomyceteous yeast *Candida antarctica* was used as enzyme to synthesis butyl acetate from vinyl acetate and n-butanol. (Kirk and Christensen 2002)

Butyl acetate is a liquid with a strong fruity odour that occurs in many fruits such as apples, strawberries and pears and has a pineapple flavour. (Fahlbusch, Hammerschmidt et al. 2003, Martins, Graebin et al. 2011, Radzi, Mustafa et al. 2011)

As hydrolysis of butyl acetate occurs in aqueous solvents traditionally organic solvents are used for its synthesis. Due to the toxicity of organic solvents they are progressively banned

for use in production of food additives and health products. During the last few decades supercritical fluids and especially supercritical Carbon Dioxide (sc-CO_2) as solvents for enzymatic reactions have been studied intensively. There are numerous advantages using sc-CO_2 amongst others having low toxicity, near ambient critical temperature and moderate critical pressure, easy product-solvent separation and natural abundance. (Oliveira, Rebocho et al. 2009) Therefore the aim of this work was to use sc-CO_2 as solvent after several preliminary experiments with an organic solvent.

Previously Sawsen Ben Ameur (Ben Ameur 2012) studied the synthesis of an ester both in an enzymatic membrane reactor and in a packed bed reactor working with sc-CO2 as solvent at the "Institut Européen des Membranes" (IEM) in 2012. She proved that both reactors lead to attractive conversions. The packed bed reactor gave better reaction performances but a high pressure drop was observed. In addition productivity was limited due to the low solubilities of the substrates involved in the chosen reaction. In this work the interest of an enzymatic packed bed reactor working under supercritical conditions was investigated. In a first step another reacting involving substrates with a higher solubility had to be chosen. It is worth noting that the product of interest which can be used as a flavour and is permitted as food additive can be obtained with the chosen reaction. Furthermore a different support for the enzymes and a new strategy of immobilisation had to be found to limit hydrodynamic problems. After characterising the immobilised enzymes in liquid conditions (hydrolysis of butyl acetate in buffer, synthesis of butyl acetate in hexane) synthesis of butyl acetate was carried out in the pilot plant with sc-CO_2 as solvent.

A.2.Enzymes

Lipases are known for their ability to convert a broad range of substrates mainly in hydrolysis, esterification and transesterification reactions. Thanks to their stability they can also be used at moderate temperatures and high pressures. (Dijkstra, Merchant et al. 2007)

Using enzymes offers important advantages compared to classical chemical synthesis.
Producing esters enzymatically is considered as an environmentally friendly process and consumers prefer products which are referred as 'natural'. Enzymes allow carrying out

reactions at lower temperatures and without the use of acid catalysts which results in lower energy costs and an easier waste stream treatment as no acid catalysts are present. Some reactions are not even possible without the help of enzymes. Another advantage compared to conventional chemical methods is a higher specificity resulting in a reduced amount of by-products. (de Castro, de Oliveira et al. 1999, Pirozzi and Greco 2004)

A.2.1.CALB

As in previous works at the "Institut Européen des Membranes" (IEM) also in this work Lipase *Candida antarctica* (CALB) was used as biocatalyst. Several characteristics of this lipase are summarised in Table 1.

Table 1: Characteristics of CALB

Molecular weight [kD]	33
Isoelectric point [pI]	6,0
pH optimum	7-8
pH stability	5-9
Temp max. [°C]	75
Temp recommended [°C]	40-60

CALB is isolated from the basidiomyceteous yeast *Candida Antarctica* which produces also another Lipase called *Candida Antarctica* Lipase A (CALA). CALB is a very well characterised lipase and is used for many different organic reactions also in processes that have been scaled up to commercial scale. CALB shows a high degree of substrate selectivity and can be used for region-selective and enantio-selective products which are difficult to be obtained by chemical synthesis.

In aqueous solutions CALB can hydrolyse a broad range of substrates whereas under organic conditions in the presence of very little water it is used for the synthesis of esters.

CALB can be used as free enzyme in solution but is a lot more stable in its immobilised form where it can be used at elevated temperature for thousands of hours without significantly losing its activity. CALB is commercially available in aqueous solution as well as in immobilised form the second being relatively expensive.

(Heldthansen, Ishii et al. 1989, Kirk and Christensen 2002, Ben Ameur 2012)

A.3.Immobilisation of Enzymes

Immobilisation of enzymes brings many advantages. They are easier to handle and product-enzyme separation is simple. In addition they can be reused several times, which plays an important role when working with expensive enzymes. Another convenience is enhanced stability under both storage and operational conditions and also an increased thermodynamic stability due to stabilisation of the tertiary structure of the protein. There are several ways of immobilising enzymes (immobilisation on a support by physical, ionic or covalent binding; entrapment (encapsulation) in organic or inorganic polymer matrices; cross-linking of enzyme molecules).

A.3.1. Immobilisation by adsorption

Non covalent carrier-bound enzyme immobilisation can methologically be classified into the following categories:

- Non-specific physical adsorption

Enzyme is adsorbed via non-specific forces such as van der Waals forces, hydrogen bonds and hydrophilic interaction.

- Bio-specific adsorption

Immobilised ligands are used for adsorption of the enzymes making it a bio-specific method.

- Electrostatic interaction

This method is also called ionic binding and is based on the charge-charge interaction between the carrier and the enzyme.

- Hydrophobic interaction

Enzymes are immobilised by interaction of hydrophobic regions of the enzyme and the carrier.

For non-specific physical adsorption no corresponding functionalities have to be available on the support and basically any type of carrier can be used such as synthetic, naturally occuring, and insoluble organic or inorganic materials.

In this work non-specific physical adsorption was chosen as immobilisation technique being one of the easiest, most inexpensive and fastest way of immobilising enzymes, though this technique is reported to be very effective.

Gamma alumina pellets were chosen as support for CALB offering a large surface area ($255 m^2/g$), high mechanical resistance and being inert.

A protocol to immobilise enzymes by adsorption on gamma alumina pellets was elaborated in this work.

In her previous work Ben Ameur (Ben Ameur 2012) used Novozym 435 (commercially available CALB immobilised on acrylic resin) for ester synthesis. When working under supercritical conditions in a packed bed reactor problems concerning hydrodynamics were observed. In the course of time the pressure drop across the packed bed increased immensely due to swelling of the beads. After three days the experiment had to be stopped as the pressure difference was too high and the flux decreased. CALB was also immobilised on ceramic membranes. Enzymes were stable over a long period of time (three day experiment) and no hydrodynamic problems occurred though with this process conversions did not exceed 50% which is not sufficient for industrial applications. The aim of this work was to find a different strategy to use immobilised enzymes for ester synthesis (Almeida, Ruivo et al. 1998, Kirk and Christensen 2002, Christensen, Andersen et al. 2003, Cao 2005, Sheldon 2007, Ben Ameur 2012).

A.4.Supercritical fluids

Synthesis of short chained esters is conventionally carried out in organic solvent as substrates are mostly poorly water soluble. In addition, degradation and side reactions can occur when using certain substrates in water. In 1985 first attempts were made by Randolph et al. to use supercritical fluids as solvent for enzymatic reactions (Randolph, Blanch et al. 1985).

Supercritical fluids and especially sc-CO_2 offer various benefits over organic solvents and exhibit similar solubility properties that can even be tuned by varying temperature and pressure.

Organic solvents are progressively prohibited as solvents for food and health products because of their toxicity while sc-CO_2 is considered as GRAS (Generally regarded as safe) and can be used for food and pharmaceutical processes without major regulatory issues. Due to the natural abundance and low toxicity sc-CO_2 is very attractive as “green designer” solvent and reduces the amount of organic waste.

As CO_2 has near ambient critical temperature (31,1°C), moderate critical pressure (73,8 bar) and is non-flammable it is convenient and safe to work with.

Another major advantage is the solvent-product separation which can be achieved by simple venting. CO_2 is commercially available at high purity and is not expensive (Garcia, Lourenco et al. 2004, Lozano, Villora et al. 2004, Oliveira, Rebocho et al. 2009).

A.5.Butyl acetate

Butyl acetate is a liquid with a strong fruity odour. It occurs in many fruits such as apples, strawberries and pears and is a constituent of apple aromas. Butyl acetate has a fruity banana odour, a pineapple, banana, tropical flavour and is permitted as food additive (http://www.thegoodscentscompany.com , Fahlbusch, Hammerschmidt et al. 2003, Martins, Graebin et al. 2011, Radzi, Mustafa et al. 2011).

Producing butyl acetate by esterification of n-butanol and acetic acid with sulphuric acid is state of the art. The reaction can be forced to completion by removing water through azeotropic separation. After neutralising the acid catalyst the ester is purified by distillation. (Cheung, Tanke et al. 2011)

B.Materials and Methods

B.1.Materials

The enzyme lipase B was purchased in its liquid form at Novozym (Denmark).

Octanol (99,5%), n-butanol (99,5%), vinyl acetate (99%), butyl acetate (99%), dichloromethane (99,9%) and n-hexane (97%) were all purchased from Sigma Aldrich (France). The substrates for the preparation of buffer are of analytical grade and were as well purchased from Sigma Aldrich.

Carbon dioxide (99,98%; 10ppm water) was supplied from Air Liquide (France).

Gamma alumina beads were purchased at Alfa Aesar (aluminium oxide, gamma-phase, catalyst support, high surface area, bimodal)

B.2.Analysis

B.2.1.Gas chromatography

For tracing the yield of butyl acetate synthesis, samples were analysed by gas chromatography.

A gas chromatograph (GC Agilent 6850) with a flame ionisation detector (FID) was used for the detection and quantification of alcohols and esters. The compounds were separated in a non-polar DB-1 column (Agilent Technologies; 30m long; 0,25mm inner diameter; 0,25µm film). Hydrogen was used as carrier gas at a flow of 20 mL/min. A constant split ratio of 60 was maintained for better resolution of analytes.

Octanol was used as internal standard and samples were prepared at a concentration of about 1g/L with dichloromethane as solvent. When the amounts of the compounds in the reaction solution to be analysed were in a different order in magnitude GC samples with different amounts of internal standard had to be prepared to analyse the yield/productivity. 1µL of the prepared samples was injected and analysed by the gas chromatograph.

The temperature of the injector was 250°C and that of the FID detector was 250°C. The column oven temperature was initially maintained at 50°C for 2 min, then ramped at a rate

of 10°C per minute to 70°C, then increased to 230°C at a rate of 20°C per minute and kept at that temperature for 9,5 min.

The retention times of the different reactants and the coefficients of the calibration are summarized in Table 2. Chromatograms can be found in chapter C.2.

Table 2: Retention time and coefficient of reactants for GC-calibration

Reactant	Retention time [min]	$\frac{m(Oct)}{m(n-But)}$	R^2
n-Butanol	1,41	1,0053	0,9988
Butyl acetate	2,70	1,3320	0,9985
Octanol	6,29		

The exact data of the calibration can be found in the appendix (see chapter F.1).

B.3.Immobilisation of enzymes on alumina beads

The preparation of the solutions used in the following chapters can be found in the appendix F.2.

200g of beads were captured in a net and submerged in an agitated phosphate buffer. The beads were not directly put in the buffer to prevent disruption of the beads caused by the agitator as the beads are sensitive towards shear stress. The buffer solution was exchanged three to five times until the solution stayed clear. The beads were left in the buffer overnight to assure that the beads were well hydrated.

The next days the beads were submerged under agitation in an enzymatic solution (300mL CALB solution pure, 1200mL phosphate buffer) at 25°C for four hours.

The beads were rinsed with buffer three times and dried in a desiccator over phosphorus pentoxide (P_2O_5) until their weight remained constant.

B.4.Determination of activity of enzymes concerning hydrolysis of butyl acetate

To determine the activity of enzymes (in solution or immobilised on beads) regarding their ability to hydrolyse butyl acetate in phosphate buffer a pH-Stat was used to follow acetic acid production. For analysing of the activity of enzymes reactions were carried out as described below.

B.4.1.Hydrolysis of butyl acetate with pure CALB-solution

50mL of 50mM butyl acetate substrate solution were heated to 37°C and agitated magnetically. To determine the activity of the free enzymes 1mL of diluted solution of enzymes was added to the substrate solution and the reaction was monitored by pH-Stat for five minutes. The pH was measured with an electrode and kept constant by neutralising the formed acetic acid by adding 30mM solution of NaOH. With the amount of added NaOH-solution as a function of time and their concentration one can calculate the production rate of acetic acid in [µMol/min] as well as the specific activity of the free enzymes in [$\mu Mol_{butyl\ acetate\ formed}/(mL_{CALB\ L}*min)$].

B.4.2.Hydrolysis of butyl acetate with immobilised enzymes – preliminary experiment (batch)

To quickly screen the activity of enzymes immobilised on alumina beads preliminary experiments were carried out in batch mode with beads under agitation. As described in chapter B.4.1 50mL of 50mM butyl acetate substrate solution were heated to 37°C and agitated magnetically. Then a certain amount of beads (about 1g) was added and the reaction was monitored by pH-Stat for five minutes. Similarly as described in B.4.1 the production rate of acetic acid in [µMol/min] and the specific activity of the enzymes immobilised on alumina beads in [$\mu Mol_{acid\ generated}. min^{-1}. g^{-1}_{beads}$] were calculated.

B.4.3.Hydrolysis of butyl acetate with immobilised enzymes - packed bed

2,17 g of beads with immobilised enzymes were put in a packed bed reactor. The feed bottle equipped with a magnetic stirrer and the packed bed reactor were immerged into a water bath at 40°C. To hydrate the beads phosphate buffer was pumped through the packed bed reactor at low flux (0,5-1mL/min) for three hours. Then feed solution (50mM butyl acetate) was pumped through the packed bed at a flow rate between 1 and 20 $mL_{substrate\ solution}$/min i.e. 23-136 $\mu Mol_{substrate\ solution}/(g_{beads}*min)$. The first approx. 10 mL were collected and used to determine the flow rate of the feed pump and to eliminate the dead volume. Then a beaker with 50 mL phosphate buffer equipped with a magnetic agitator and the pH-electrode were placed at the exit of the packed bed reactor to collect the solution to be analysed by pH-Stat for about 10-20 minutes.

After the experiments the beads were rinsed and left in phosphate buffer overnight and reused for similar experiments the following days.

A simplified scheme of the experimental setup is shown in Figure 1.

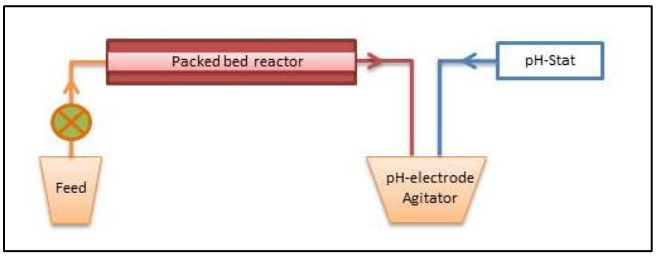

Figure 1: Simplified scheme of experimental setup

B.5.Determination of activity of enzymes concerning synthesis of butyl acetate with organic solvent

B.5.1. Synthesis of butyl acetate with immobilised enzymes – packed bed

A packed bed reactor filled with 12g of alumina beads with immobilised enzymes was used for the determination of their activity concerning synthesis of butyl acetate with hexane as solvent. The substrates were vinyl acetate and n-butanol and the reaction temperature was 40°C. The beads were conditioned by pumping hexane at a low flux (1-2 mL/min) through the packed bed reactor for 10 minutes. A feed solution (0,5M n-butanol, 1,5M vinyl acetate in hexane) was prepared and heated to 40°C. Experiments were carried out at different fluxes (0,5-10mL/min i.e. 20-400µMol$_{n\text{-}butanol}$/(g$_{beads}$*min). About 50 mL of reaction solution was collected to determine the flux of the pump and to eliminate the dead volume. Afterwards several samples were taken, octanol as internal standard was added and the samples were diluted with dichloromethane before being analysed by GC.

B.6. Determination of activity of enzymes concerning synthesis of butyl acetate with sc-CO_2 as solvent

B.6.1.Description of pilot plant supercritical carbon dioxide

The pilot plant is shown in Figure 2. Carbon dioxide enters the pilot plant via valve MV100 and is cooled to 0°C by CE1000. CO_2 pump (P200) increases the pressure in the system. The temperature of the sc-CO_2 is controlled by heating (HE2100) and the pressure is controlled by valve MV200. With the help of pump P330 a split stream can be recirculated. The feed pump (P300) is a HPLC-pump and adds reactants to the sc-CO_2. After the reactor (TR31)

separation between solvent and reactants takes place in separator S51 and S52 and the CO_2 is recirculated. Samples can be taken by opening valve DMV510/520 where liquid products and gaseous carbon dioxide exits at a pressure of 50 bar.

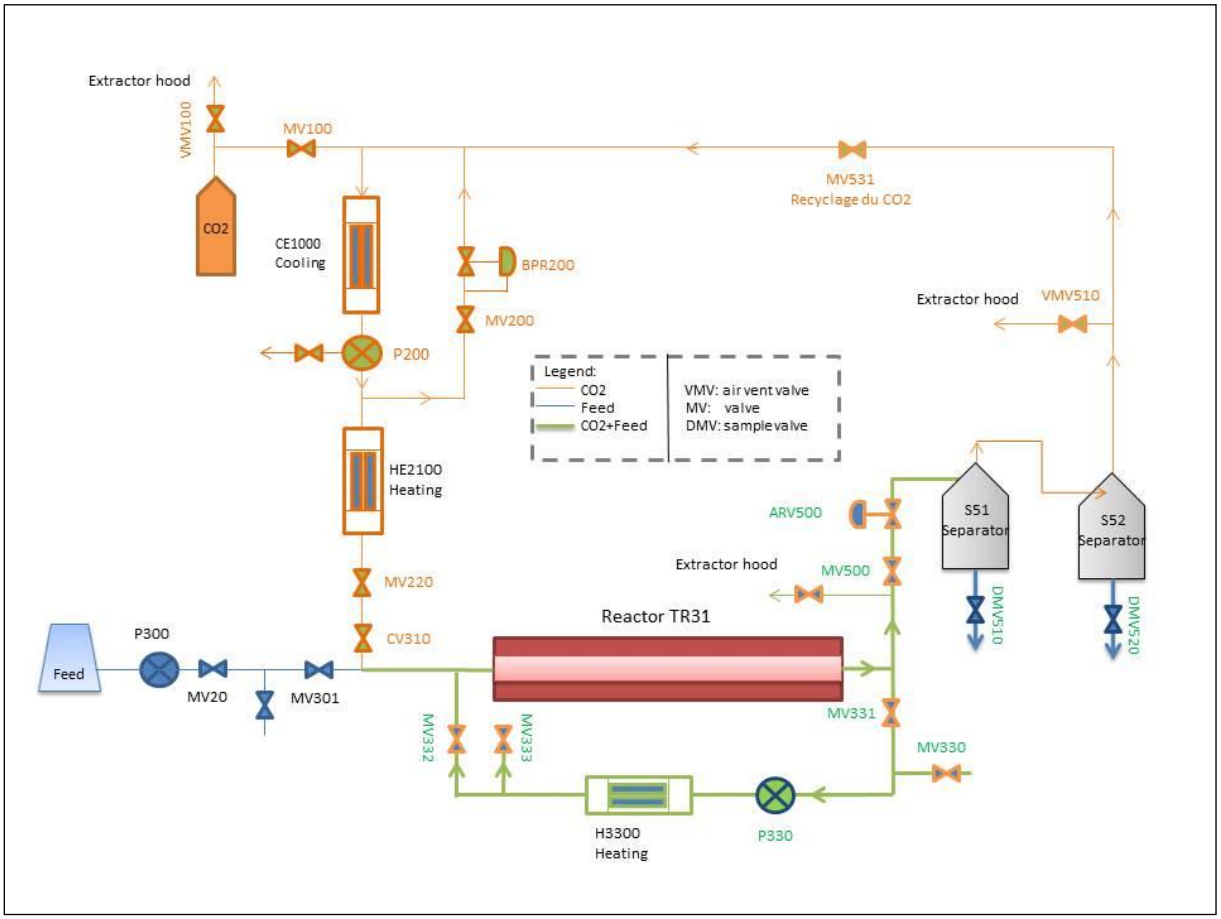

Figure 2: Simplified flow sheet pilot plant sc-CO_2

Figure 3: Photo pilot plant Sc-CO_2

There were two packed bed reactors available for the pilot plant with sc-CO_2 as solvent: a small one (13cm length, 8 mm diameter) and a big one (120cm length, 2cm diameter). The small packed bed reactor could be filled with about 2,2 g beads and the big reactor with about 150 g beads but there were also experiments to be carried out at a scale in between that range through the reactors. At both ends of the reactor the beads were retained by frits. When using less than 150 gram ceramic beads with immobilised enzymes the remaining volume was filled with glass beads (6mm diameter). To inhibit a mixing of the glass beads and the ceramic beads they were separated with mineral wool (see Figure 4).

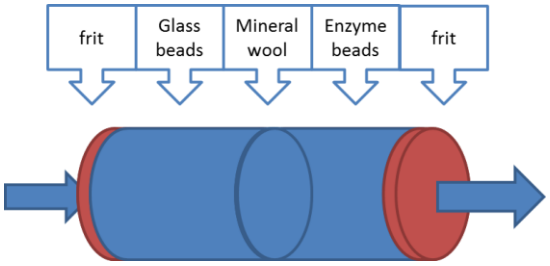

Figure 4: Schema big packed bed reactor of pilot plant

B.6.2.Synthesis of butyl acetate – small packed bed reactor

A packed bed reactor filled with 2g beads with immobilised enzymes for synthesis of butyl acetate in supercritical conditions. The system was kept at 50°C and slowly pressurised to 100 bar at a flux of 1,8kg CO_2/min.

A feed solution with a molar ratio 3:1 (vinyl acetate:n-butanol) was prepared. As soon as flux, pressure and temperature were constant the feed pump was set to a value between 0,2 and 1$mL_{feed\ solution}$/min i.e. 280-1300 µ$Mol_{n\text{-}butnaol}$/(g_{beads}*min).

B.6.3.Synthesis of butyl acetate - big packed bed reactor

The big packed bed reactor can be filled with up to about 150g beads. In order to carry out experiments with a lower amount of beads the reactor can be partially filled with ceramic beads with immobilised enzymes. The rest of the packed bed can be filled with glass beads (diameter of used beads 6mm) separating the two different beads with mineral wool.

C.Results and discussion

C.1.Choosing a reaction for synthesis of flavours

In previous works Ben Ameur (Ben Ameur 2012) tried to produce an ester from an alcohol and vinyl acetate at the IEM. One of her main problems was low solubility of substrates and reactants. Thus for this work another product had to be chosen.

The main idea was to produce an ester that can be used as food additive. For a bibliographic research the following criteria were taken in account.

- The reaction should be catalysed by a Lipase of the type *Candida antarctica*.
- All substrates and products should be sufficiently soluble in supercritical CO_2 without the use of toxic co-solvents and if possible without any co-solvents at all.
- Reaction conditions should be realisable in the pilot plant at the IEM (80°C and 200bar at maximum)
- The produced flavour has to be permitted as food additive.
- The reactants and products can be analysed with GC.

Several different reactions can be found in literature. The results of this bibliographic research are summarised in Table 3.

The following assumptions and abbreviations were used in Table 3:

- If not otherwise indicated the default reactor type is a batch reactor. Only if a continuous reactor was used it is stated with the abbreviation “conti”.
- N.435 stands for Novozym 435: This is CALB immobilised covalently on a macroporous acrylic resin and is commercially available from Novo Nordisk.
- CLEA-CALB is a cross-linked enzyme aggregate of *Candida antarctica* lipase B
- The given yield is always indicated as mol product per mol alcohol or ester.

Sources	Substrates	Products	Temperature / pressure of CO2-Sc	Immobilised enzyme	Yield	Type of GC-column
(Oliveira, Rebocho et al. 2009)	decanol + vinyl acetate	decyl acetate $C_{12}H_{24}O_2$	35°C/100bar	N.435	1	Varian CP-3800, capillary column
(Lozano, Villora et al. 2004)	butanol + vinyl butyrate	butyl butyrate $C_3H_{16}O_2$	50°C/90bar	N.435	1	Shimazu GC-17A Nukol, capillary column
			60°C/80bar	CALB on ceramic α-alumina membrane	-	
(Knez, Kavcic et al. 2012)	butanol + lactic acid	butyl lactate $C_7H_{14}O_3$	55°C/400bar + n-heptane	N.435	0,99	HP6890 : HP-Ultra 2, capillary column
			55°C/ 300bar		0,756	
(Varma and Madras 2007)	butyl butyrate + geraniol	geranyl butyrate $C_{14}H_{24}O_2$	50°C/213 bar	N.435	100min : 0,25 1600min : 0,45	Varian CP-3800, capillary column
(Romero, Calvo et al. 2005, Dijkstra, Merchant et al. 2007)	isoamyl alcohol + acetic anhydride	isoamyl acetate $C_7H_{14}O_2$	40°C/140bar	N.435	0,93	TR-FFAP, capillary column
	isoamyl alcohol + Acetic acid		conti : 40°C/110bar	CLEA-CALB	0,7	Rtx-5, capillary column
			40°C/110bar	CLEA-CALB	0,9	
(Garcia, Lourenco et al. 2004)	2-phenyl-1-propanol + vinyl butyrate	**1-phenylethyl butyrate** $C_{12}H_{16}O_2$	conti : 35°C/100bar + ionic liquid	N.435	-	capillary column
(Olsen, Kerton et al. 2006)	lavandulol + acetic acid	lavandulol acetate $C_{12}H_{20}O_2$	60°C/100bar	N.435	0,62	Agilent HP-5, capillary column
(Liaw and Liu 2010, Liu and Huang 2010)	α- terpineol + acetic anhydride	terpinyl acetate $C_{12}H_{20}O_2$	50°C/100bar	*Candida rugosa* type VII	0,53	Hitachi G-3000, capillary column
			50°C/100bar + n-heptane			

C.1.1. Final criteria for the choice of reaction for synthesis of flavours

After a literature review the final criteria for choosing a reaction were defined as described below.

- All of the products of the reactions stated in Table 3 are permitted as food additives by the US Food and Drug Administration (FDA) but a few of them are not really interesting due to their rather specific odour (e.g. butyl lactate: creamy fruity fermented mushroom).
- Reaction conditions must not exceed 80°C (denaturation of enzyme) and 200 bar (max. pressure of pilot plant).
- In order to use the advantage of simple product solvent separation when working with sc-CO_2 a reaction without co-solvent is to be chosen.
- As CALB had been successfully used in previous works at the IEM a reaction catalysed by this lipase is to be chosen.
- Ben Ameur showed that big quantities of acetic acid can inhibit the enzyme. Therefore reactions without acetic acid as reactant were preferred.
- Using vinyl acetate as reactant is very convenient as it reacts with an alcohol and forms vinyl alcohol. Vinyl alcohol tautomerises to acetaldehyde thus being removed from the reaction medium (see Figure 5) and reaction equilibrium is shifted towards synthesis resulting in higher yields. In addition this compound shows good solubility properties in sc-CO_2.

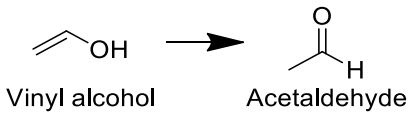

Figure 5: Schematic pathway of tautomerisation of vinyl alcohol to acetaldehyde

With those criteria two reactions were taken in account:

- Decanol + vinyl acetate → decyl acetate (Oliveira, Rebocho et al. 2009)
- Butanol + vinyl butyrate → butyl butyrate (Lozano, Villora et al. 2004)

In addition for both reactions the solubilities of most of the substrates (decanol, butanol, vinyl acetate) are known and the products are interesting as food additives and the yields are almost 100% (Mol product/Mol alcohol).

For this work the two reactions were combined: For the reasons already stated vinyl acetate was chosen as one substrate. Due to higher solubility in sc-CO_2 (solubility see chapter C.6.1) compared to decanol, butanol was preferred as the second substrate (alcohol). The schematic pathway of the reaction is thus presented in Figure 6.

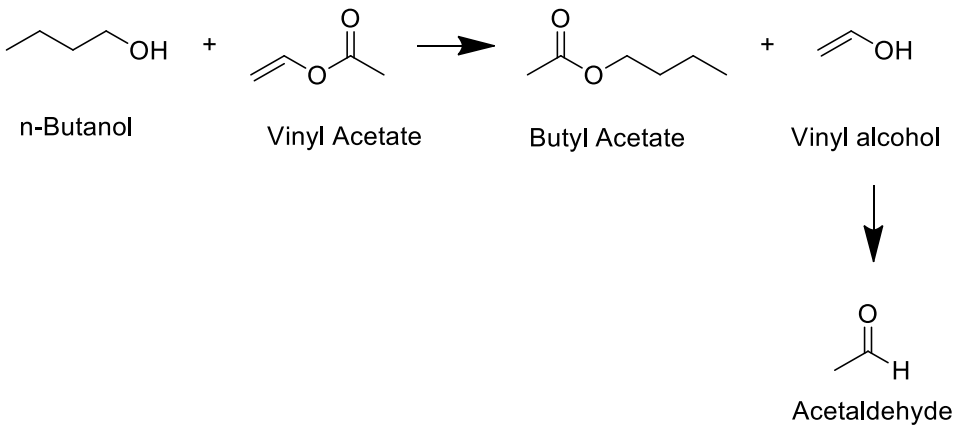

Figure 6: Schematic pathway of transesterification reaction

The final product butyl acetate is also permitted and interesting as food additive having a strong fruity odour and a tropical flavour.

C.2. Analysis with Gas chromatography

In order to be able to trace the yield of the chosen reaction (see chapter C.1.1) at least one of the substrates and one of the products has to be quantifiable.

As vinyl alcohol is transformed to acetaldehyde and acetaldehyde has a boiling point of 20,2°C is almost impossible to measure that component.

Octanol was chosen as internal standard and its peak is nicely separable from the solvent peak and the other reactants and products.

Different solvents to dilute the samples were tested such as hexane, pentane, cyclohexane and dichloromethane. As the chromatograms with pentane as solvent were not significantly better resolved and pentane is - due to its low boiling point - difficult to handle no further experiments were carried out with this solvent.

When using hexane as solvent only the butyl acetate and the octanol peak were nicely separated from the solvent (see Figure 7).

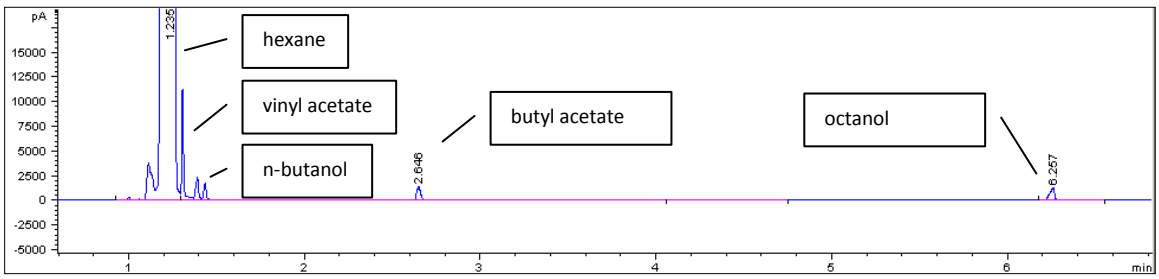

Figure 7: Chromatogram of different compounds with hexane as solvent

n-Butanol is not visible when using cyclohexane as solvent (see Figure 8)

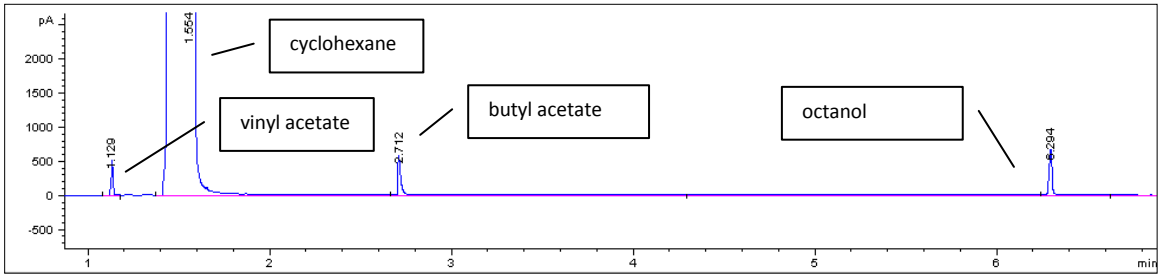

Figure 8: Chromatogram of different compounds with cyclohexane as solvent

Dichloromethane showed best results concerning peak separation of solvent and reactants. All peaks except for the vinyl acetate were nicely separated (see Figure 9).

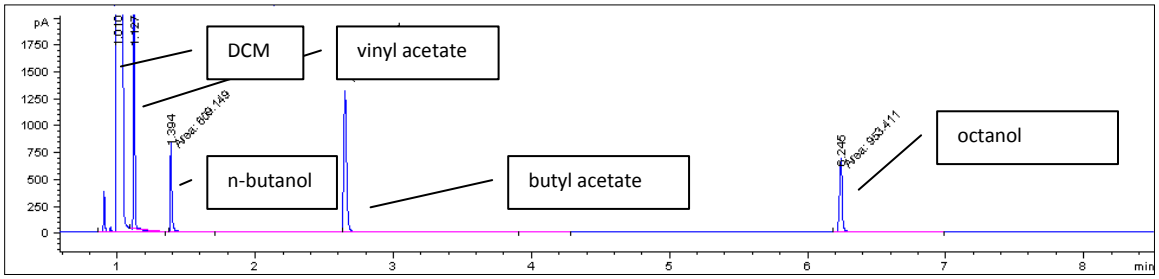

Figure 9: Chromatogram of different compounds with dichloromethane as solvent

As one product and one substrate could be quantified dichloromethane was chosen as solvent and no further solvents were tested due to the limited time of that project. After choosing the solvent the temperature program was optimized to improve peak separation and minimize analysis time. The temperature program, retention times and coefficients for the quantitative calibration are summarized in Table 2. The exact data for each calibration can be found in the appendix F.1.

C.3.Immobilisation

As stated in chapter A.3 working with immobilised enzymes brings many advantages compared to free enzymes.

C.3.1.Support

CALB is commercially available as an aqueous solution and can also be purchased immobilised on a macroporous acrylic resin (Novozym 435) at Novo Nordisk. When working with Novozym 435 under supercritical conditions Ben Ameur observed an increasing

pressure drop after two days due to swelling and compacting of the beads and the experiment had to be stopped as the CO_2 flux decreased.

Due to higher mechanical resistance of ceramic beads, it was thus decided to immobilise CALB on gamma alumina beads.

C.3.2.Bibliographic review about immobilisation technique absorption with inorganic support

Ben Ameur found out that adsorption leads to immobilised biocatalysts as effective and stable under supercritical conditions as when immobilisation is achieved through covalent bounds. Due to the faster and easier process adsorption was used for immobilising enzymes on the inorganic support.

To establish a protocol for immobilisation literature was reviewed and the publications which resembled our process the most were summarised in Table 4.

Table 4: Summary of literature review about immobilisation of enzymes by adsorption

Source	(Gaffar, Kermasha et al. 1999)	(de Castro, de Oliveira et al. 1999)	(Minovska, Winkelhausen et al. 2005)	(Mori, Garcia et al. 2005)
Support	alumina/silica	celite	alumina	alpha alumina (membrane)
Enzyme	chlorophylase	*Porcine Pancreatic Lipase*	*Candida rugosa*	*CALB*
Preparation of enzymatic solution	tris-buffer 20mM pH 8	0,1M Na_2PO_4 buffer pH 7	water/buffer	10mM buffer phosphate pH 8
Solvent for precipitation	-	0,1 M Na_2PO_4 buffer + 0,1M hexane	acetone	dichloromethane
Quantity of enzyme	1mg (=0,35mg protein)	500 mg	3mL (4g lipase in 100mL water or buffer)	150 µL
Quantity of diluent for adsorption	1mL water or buffer	10 mL buffer for 2h	-	5mL dichloromethane from the beginning
Quantity of solvent for precipitation	-	after 2h 10mL for 30 min	(ad.+ prec.) after 1h 10mL, immediate filtration	
Quantity of support	4 mg	0,5-5g	1g	membrane tube 130mm
Duration of immobilisation	60 min	2h + 30min	3h (ad.); 1h (ad.+prec.)	until all solvent is evaporated
Temperature	4°C	room temperature	25°C	room temperature
Immobilisation efficiency	69 %	79 %	96 %	50 %
Final treatment	filtration, lyophilisation	filtration	desiccator (4h)	dia-filtration

The best immobilisation activity was obtained by Minovska et al. (Minovska, Winkelhausen et al. 2005) with *Candida rugosa* using adsorption followed by precipitation of the enzyme. The temperature at which immobilisation was carried out was in most cases room temperature and the time the support was left in contact with the enzymatic solution was at least one hour but mostly longer. As described in literature the ceramic beads were left in contact with the enzymatic solution for two hours at room temperature. At first the effect of adsorption follow by precipitation was studied in comparison to simple adsorption.

C.3.3.Analysis of commercially available enzymatic solution regarding hydrolysis

Before using the commercially available enzymatic solution (CALB L) for immobilisation it was characterised regarding their activity to hydrolyse butyl acetate in aqueous milieu

(phosphate buffer). Based on the protocol of Ben Ameur (Ben Ameur 2012) five samples with different amounts of CALB L (1-5µL) were analysed in batch experiments as described in chapter B.4.1. Changes were made concerning the analysis time when calculating the activity. Figure 10 shows the data of a typical analysis. With the slope of the linear regression of the graph added volume of NaOH vs. time and the concentration of the NaOH the productivity (amount of acetic acid produced per minute [µMol/min]) can be calculated. When taking in account the data obtain over a period of 10 min the effect of substrate limitation can be observed and the estimated activity do not correspond to initial reaction rate. For this reason the analysing time was changed to five minutes to achieve measured values which are closer to the actual activity.

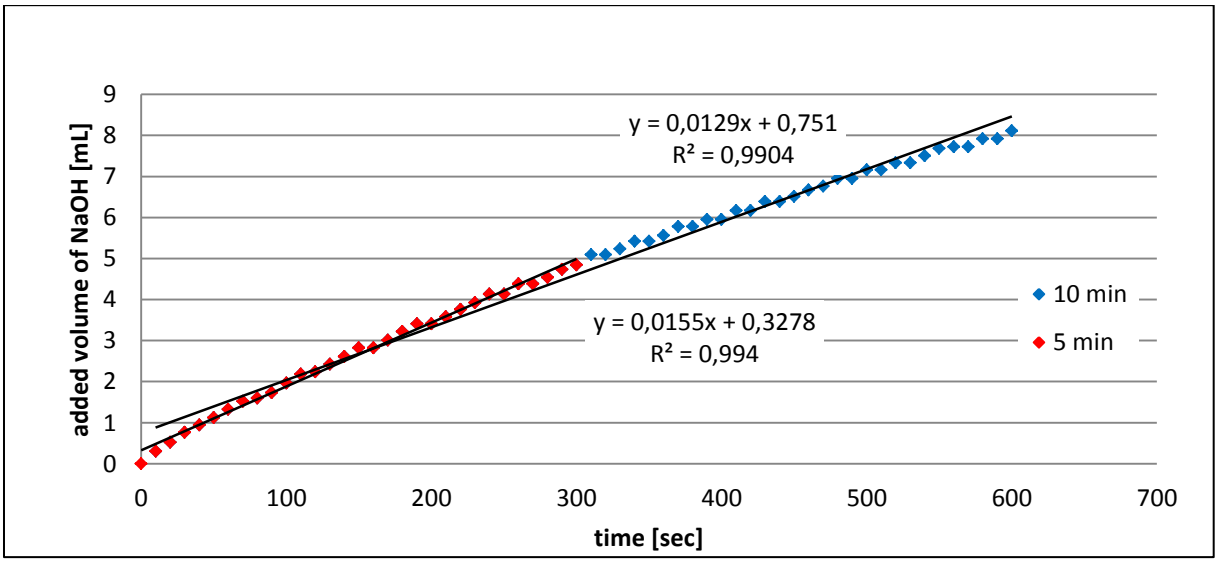

Figure 10: pH-Stat analysis of CALB L 5µL (37°C; solvent: phosphate buffer)

With the values from the experiments with different amounts of CALB L the activity can be obtained by calculating the slope of the graph "productivity vs. amount of CALB L" Figure 11. By measuring the activity of immobilised enzymes on ceramic beads the amount of free enzymes that has the same activity as 1 g beads can be evaluated with Figure 11. When measuring the productivity or activity of a solution with free enzymes its unity is [$\mu Mol_{acetic\ acid\ formed}/(mL_{CALB\ L}*min)$] while the unity of productivity or activity of immobilised enzymes on ceramic beads is [$\mu Mol_{acetic\ acid\ formed}/(g_{beads}*min)$].

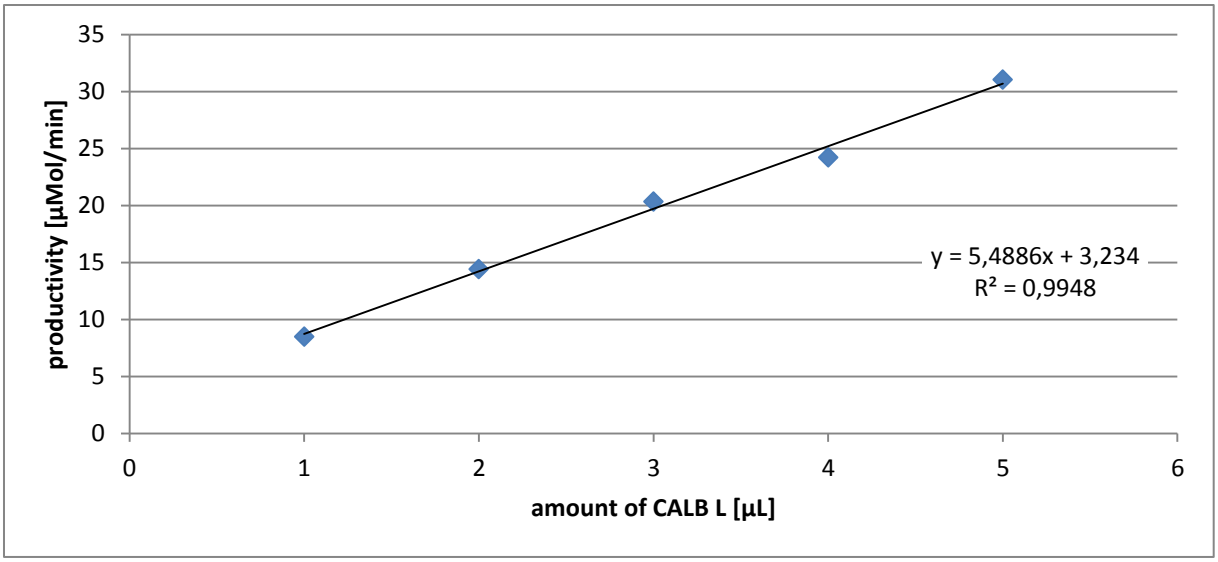

Figure 11: Activity of hydrolysis of butyl acetate-in aqueous medium with CALB L in aqueous conditions as a function of time (37°C, solvent phosphate buffer)

The obtained activity of the CALB L solution in aqueous conditions regarding the hydrolysis of butyl acetate is 6370 µMol/(mL*min).

C.3.4.Analysis of immobilised enzymes - batch

To analyse the activity of the immobilised enzymes on ceramic beads and to quantify the immobilisation efficiency preliminary experiments were carried out similar to analysing the pure CALB solution but instead of adding a certain amount of CALB L 1g of beads was analysed. This analysing method is not very precise though quick and allows to qualitatively compare the different immobilising essays. There are many parameters concerning the reaction conditions that cannot be controlled as precisely as in continuous mode (reaction temperature, condition of the beads, agitation, etc.).

C.3.5.Analysis of desorption of immobilised enzymes

It was also tested whether the immobilised enzymes desorb when using them in aqueous solution for hydrolysis. The activity of 1g of beads was analysed . The supernatant and the beads were separated and the same beads were re-analysed. To verify that the reason for the decrease in activity is because of desorption and not for other reasons (e.g. denaturation) the activity of the supernatant was also analysed.

C.3.6.Analysis of effect of precipitation agent on activity of enzymatic solution

To determine whether the precipitation agent hexane has an impact on the activity of the enzymatic solution used to immobilise the enzymes on the ceramic support two enzymatic solutions (10mL, 5%) were prepared equally. To one of them 10mL of hexane was added and the solutions were stirred vigorously before analysing the aqueous solutions with the pH-Stat.

C.3.7.Comparison of simple adsorption vs. adsorption with precipitation

Simple adsorption

10g of beads were immersed in 40mL of an agitated solution of CALB L diluted with phosphate buffer (5% (m/m); 320 µMol/(mL*min)) for two hours at 25°C.

For the simple adsorption the beads were separated from the enzymatic solution and rinsed several times with phosphate buffer. Afterwards the activity of one part of the beads was measured in aqueous conditions immediately. The other part of the beads was dried in a desiccator over P_2O_5 and analysed afterwards.

Adsorption with precipitation

For the adsorption with precipitation the same amount of beads was used, the enzymatic solution was prepared equally and the beads were left in the solution at the same temperature for two hours. Then 40mL of hexane were added and the solution was agitated vigorously as hexane is not soluble in water. The liquid phase was separated from the beads and the beads were rinsed with hexane several times before drying in a desiccator.

C.3.8. Results and interpretation simple adsorption vs. adsorption with precipitation

As stated in chapter C.3.6 the effect of the precipitation agent hexane on the enzymatic solution was tested and the experiment showed no difference of activity between the enzymatic solution which was in contact with hexane and original solution. This shows that adding hexane doesn't affect the activity of an enzymatic solution.

After drying 1g of beads with enzymes immobilised by simple adsorption analysing them showed an activity that equals the activity of 0,72 µL CALB L. For the beads immobilised with adsorption and precipitation an activity equivalent to 0,53 µL CALB L was measured. Taking

in account the error in measurement both methods results in the about the same immobilising efficiency (same order in magnitude).

With the experiment stated in chapter C.3.5 it could be proven that desorption of enzymes occurs neither when immobilising enzymes by simple adsorption nor when immobilising them by adsorption and precipitation.

As simple adsorption is an easier and faster process as adsorption followed by precipitation and both methods have the same immobilisation efficiency all further experiments were carried out without precipitation.

C.3.9. Improving the yield of immobilisation

To study the effect of the concentration of the enzymatic solution used for immobilisation experiments were carried out with different concentrations of CALB L diluted with phosphate buffer (5-20 % (m/m)). The results obtained through those experiments are summarized in Table 5.

Table 5: Immobilisation of enzymes – comparison of different concentration of enzymatic solution

40mL enzymatic solution			10 g support		
Conc. of enzymatic solution	**Conditions**	**Activity of solution [µMol/(mL*min)]**	**Productivity of beads in batch mode [µMol/(g*min)]**		**Amount of CALB L [µL] with equal activity as 1g beads**
5% (m/m)	agitation, 2h, 25°C	320,0	before drying	52,7	8,3
			after drying	4,8	0,7
10% (m/m)	rinse then agitation, 2h, 25°C	637,9	before drying	26,2	4,1
			after drying	8,9	1,4
20% (m/m)	rinse then agitation, 2h, 25°C	1116,3	before drying	58,0	9,1
			after drying	28,6	4,5

As the enzymatic solution was found to be cloudy after the first immobilisation experiment with a 5% solution due to ceramic powder that emerged from the pores, in the following experiments the beads were rinsed several times with phosphate buffer before being used.

After immobilising the enzymes, the activity of the beads was tested twice: once immediately after the immobilisation step and before and once after drying the beads.

It is also noticeable that after drying the beads, an enormous loss of activity can be observed. An increased concentration of the enzymatic solution results in a higher activity of the beads. When comparing the results of 10% and 20% enzymatic solution the activity doubles. The activity of the beads immobilised with a 5% enzymatic solution is probably overestimated due to insufficient rinsing.

To test the effect of agitation during immobilisation tests were carried out with and without a magnetic stirrer (see Table 6). Furthermore it was tried to minimise the observed effect of loss of activity after drying the beads by immerging the beads in phosphate buffer for 2h before analysis. The results of those experiments can be found in Table 6.

Table 6: Immobilisation of enzymes – Comparison of different conditions

40mL enzymatic solution			10 g support		
Conc. of enzymatic solution	**Conditions**	**Activity of solution [µMol/(mL*min)]**	**Productivity of beads in batch mode [µMol/(g*min)]**		**Amount of CALB L [µL] with equal activity as 1g beads**
10% (m/m)	Rinse then agitation 2h, 25°C	637,9	Before drying	26,2	4,1
			After drying	8,9	1,4
10% (m/m)	Rinse then no agitation 2h, 25°C	688,2	Before drying	18,5	2,9
			After drying + 2h in buffer	18,4	2,9
20% (m/m)	Rinse then agitation 2h, 25°C	1116,3	Before drying	58,0	9,1
			After drying	28,6	4,5
20% (m/m)	Rinse then no agitation 2h, 25°C	1053,5	Before drying	45,9	7,2
			After drying + 2h in buffer	45,4	7,1

When immerging the beads into phosphate buffer for two hours before analysing them an activity almost equal to that before drying was measured. The reason for the loss of activity after drying is that the enzymes get denaturised when being dry and need time to rehydrate

and to get used to the reaction medium. That shows that the enzymes do not get irreversibly denatured but just need some time to get activated again.

The activity of the beads immobilised with and without agitation is in the same order in magnitude though a little augmentation of activity can be observed when immobilising with agitation (20-30% see Table 6).
After immobilising with agitation the enzymatic solution was found to be cloudy again. This is due to the agitator hitting the beads which are not very resistant against mechanical impact.
In order to agitate the solution without hitting the beads two attempts were made. The first attempt was to agitate the solution with a pump. In a second attempt the beads were captured in a net before being immerged in the enzymatic solution with the magnetic stirrer underneath the net assuring sufficient agitation in order to prevent the beads from being hit by the stirrer.
As the second method is easier and uses smaller amounts of enzymatic solution (dead volume of tubes) with same immobilisation efficiency this method was used for further immobilisation.

Furthermore a decrease of activity was observed when comparing the sum of measured activity of the immobilised enzymes and the enzymatic solution after immobilisation to the measured activity of the solution before immobilisation. That can be due to several reasons. Either the reactive site of some of the immobilised enzymes is not accessible as enzymes are immobilised in small pores or they form a multi-layer at the ceramic surface and the ones at the bottom are not accessible.

C.3.10.Conclusions of preliminary experiments of immobilisation

From the preliminary experiments of immobilisation the following conclusions can be made.

- It was found that increasing the concentration of the enzymatic solution used for immobilisation increases the activity of the obtained beads. However, it has to be considered that the concentration is not too high due to high costs of the enzymatic solution.
- Before using the beads they have to be rinsed several times with phosphate buffer to remove powder that emerges from the pores.

- Keeping the enzymatic solution agitated shows also an improvement of the immobilisation efficiency though the beads mustn't be in direct contact with the stirrer to prevent the formation of powder by mechanical abrasion.
- Before using the dried beads for hydrolysis in aqueous medium the beads have to be rehydrated by putting them in phosphate buffer for at least two hours.
- No desorption of enzymes takes place in aqueous medium when immobilising beads with adsorption.

According to all the results obtained through the preliminary experiments a protocol of immobilisation was established (see chapter B.3).

C.4.Packed bed hydrolysis in aqueous conditions with immobilised enzymes

To characterise the enzymes immobilised on ceramic beads regarding their activity to hydrolyse butyl acetate experiments were carried out in a packed bed reactor in continuous mode and not in batch mode. This method allows to run experiments for a longer time and to control reaction parameters more accurately.

To start off and to get an idea of the order of magnitude a small packed bed reactor with 2,17g beads was chosen and the flux was controlled with a HPLC pump (0,10-10mL/min). To rehydrate the beads phosphate buffer was pumped through the reactor at a low flux (0,5-1mL/min). Then substrate solution was pumped at 37°C through the reactor at four different fluxes (1, 2, 4, 6 mL/min; 23, 44, 90, 138 $\mu Mol_{butyl\ acetate}/(g_{beads}*min)$).

Figure 12 shows a typical diagram obtained from packed bed hydrolysis experiment in aqueous conditions with a flux of 1mL/min; 23µMol/(g*min).

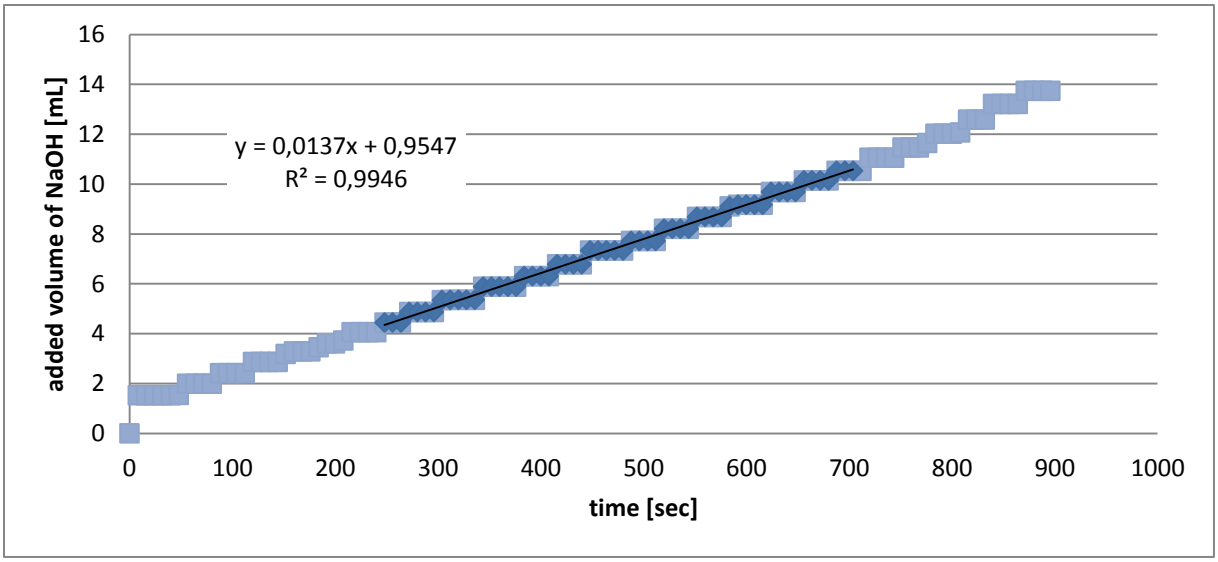

Figure 12: Packed bed hydrolysis in aqueous conditions with immobilised enzymes (37°C; 8mL/min; 182 µMol/(g*min), 2,17g beads)

The amount of acetic acid produced per minute was in a range that could be very well analysed by pH-Stat with the same solutions and parameters as in batch experiments.

Figure 13 shows the productivity of the beads after being three hours in phosphate buffer as a function of the specific substrate flux.

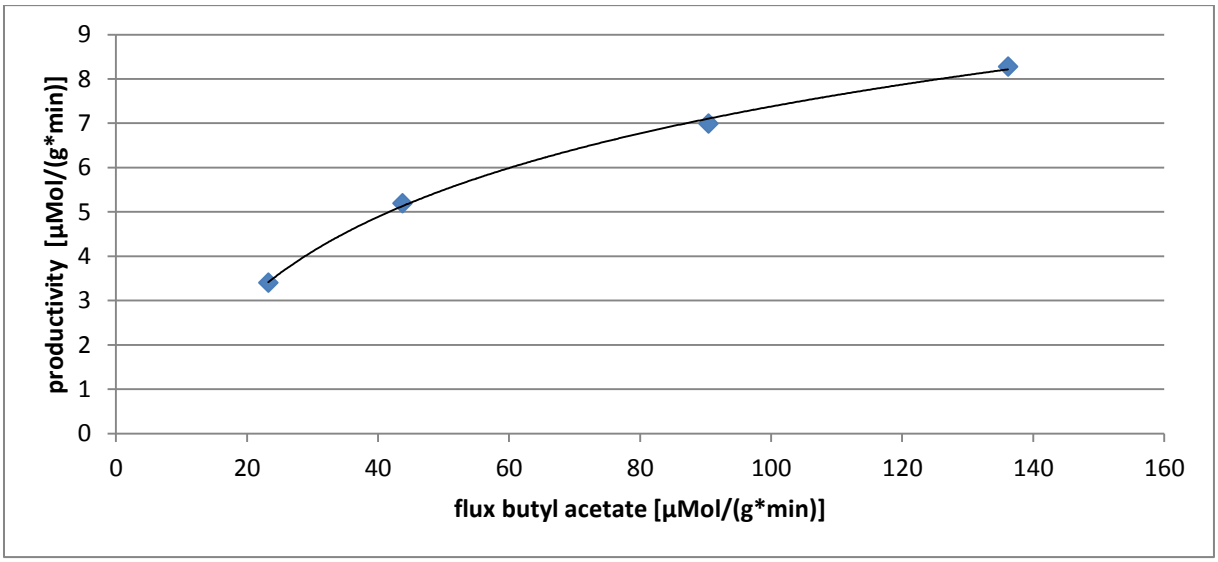

Figure 13: Productivity of hydrolysis of butyl acetate-in aqueous medium with packed bed reactor as a function of time - 3h in buffer; 37°C; 2,17g beads

From the graph in Figure 13 one can see that the specific productivity [µMol/(g*min)] is a function of substrate flux. The productivity increases at higher specific substrate fluxes. The explanation for this phenomenon is that experiments were apparently carried out in a range where substrate limitation takes place. As the activity can only be measured without substrate limitation the experiment was carried out again at higher substrate fluxes. To

study the effect of enzyme stability the next day the same beads where used for the experiment and measurements were repeated also at low fluxes (1, 2, 5, 8, 10 mL/min; 23, 44, 114, 181,229 µMol/(g*min)). The beads were rinsed and stored in phosphate buffer overnight.

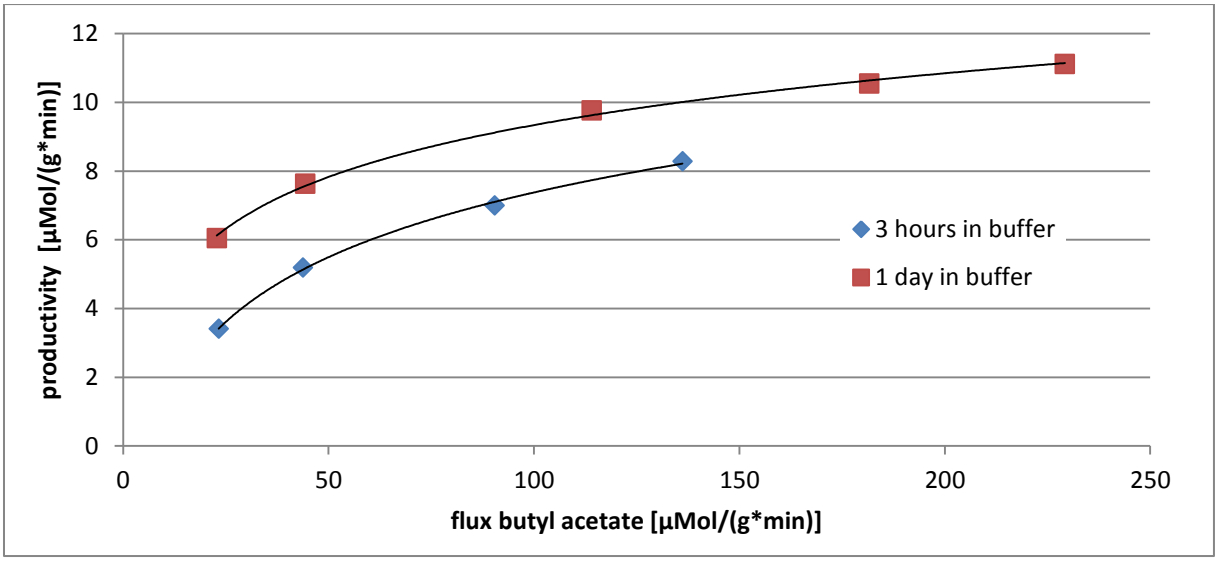

Figure 14: Productivity of hydrolysis of butyl acetate-in aqueous medium with packed bed reactor as a function of time - 3h in buffer + 1day in buffer; 40°C; 2,17g beads

Towards higher fluxes the productivity increases more slowly but there is still substrate limitation (see Figure 14). It is very interesting that an increased productivity of the beads after leaving them in phosphate buffer overnight can be observed. That shows, that rehydrating the enzymes takes a long time. In addition it could be proven, that no desorption and no loss of activity takes place even after several hours of rinsing and using the beads for hydrolysis.

In order to increase the flux of feed solution a new pump (gear pump) had to be installed as the flux of the HPLC was limited to a maximum of 10 mL/min. For the next experiment the same beads as in the prior two experiments were used and they were stored in phosphate buffer for two days. With the gear pump the flux was increased up to 19 mL/min; 441 µMol/(g*min). The results of all three experiments are summarised in Figure 15.

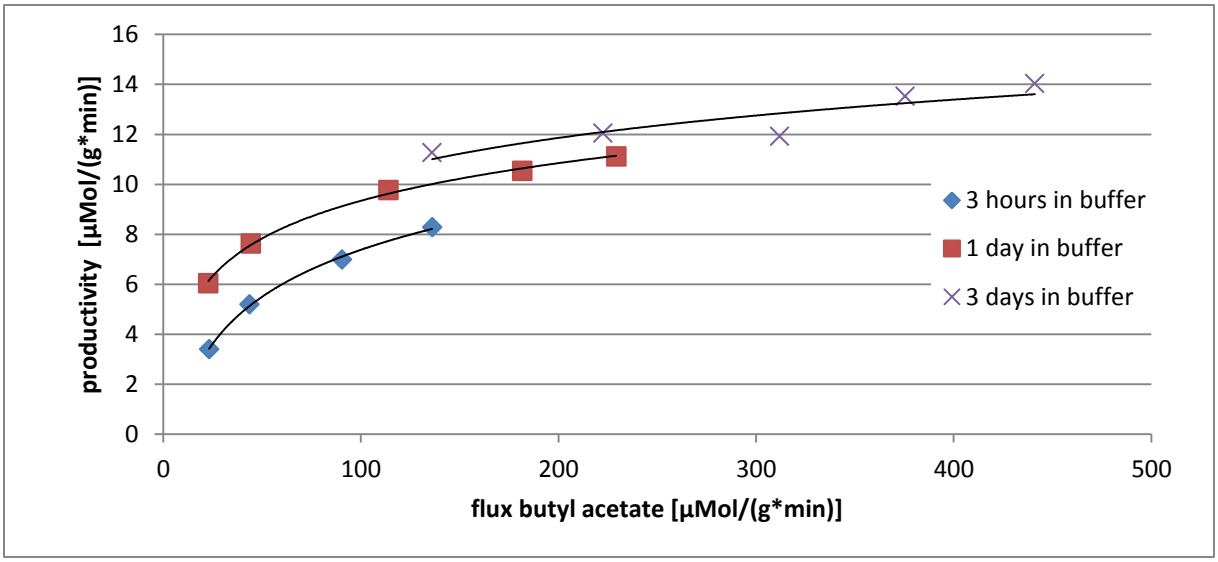

Figure 15: Productivity of hydrolysis of butyl acetate-in aqueous medium with packed bed reactor as a function of time - summary; 40°C; 2,17g beads

Once again a slight increase of productivity can be observed when leaving the beads in phosphate buffer for another two days but the increase is much smaller compared to the difference between the first and the second day.

The productivity graph levels off at higher substrate fluxes and the activity can be estimated from Figure 15.

C.4.1.Conclusion from packed bed hydrolysis

After drying the beads with immobilised enzymes it takes a long time (up to several days) until they are rehydrated and gain their actual activity.

No desorption of the immobilised enzymes takes place in aqueous medium, neither in batch nor in continuous mode operation.

Even after several hours of synthesis and storing the beads in phosphate buffer overnight no loss of activity was observed.

An activity above 14 µMol/(g*min) was measured for the beads immobilised as stated in chapter B.3 and analysed as stated in chapter B.4.3.

C.5.Packed bed synthesis of butyl acetate in organic solvent

C.5.1.Bibliographic review about conditions for packed bed synthesis of butyl acetate

Experiments were also carried out in organic solvent which are conventionally used for ester synthesis. As the experimental setup is much simpler when working under liquid conditions compared to supercritical conditions an organic solvent was chosen for preliminary experiments. To evaluate certain parameters for the synthesis of butyl acetate under liquid conditions such as solvent, temperature, concentrations, etc. literature was reviewed. In Table 7 the data from literature is summarised.

Table 7: Summary of literature review of conditions for packed bed synthesis of butyl acetate in liquid conditions

	(Martins, Graebin et al. 2011)		**(Lozano, Perez-Marin et al. 2002)**
Enzyme	CALB immobilised on macro porous acrylic resin		CALB immobilised on α-alumina ceramic support
Product	butyl acetate		butyl butyrate
Substrate	n-butanol + acetic acid		n-butanol + vinyl butyrate
		Optimum	
Temperature	31,25-43,75°C	40°C	40°C
Substrate concentration	0,1-1 M (acid)	0,4 M (acid)	0,5M (butyrate)
Substrate ratio	1:1-5:1 (alc:acid)	3:1 (alc:acid)	(1:5) (alc:butyrate)
Solvent	n-hexane		n-hexane, acetone, acetonitrile
Enzyme concentration	1-10% of substrate wt.	7,5% of substrate wt.	100 mg of active membrane
Added water	0-0,75 % of substrate wt.	0,25 % of substrate wt.	< 3% v/v of solvent

As both reactions stated in Table 7 are catalysed by CALB and the products and substrates are comparable to those used in this work similar conditions as in the work of Lozano et al. and Martins et al. (Lozano, Perez-Marin et al. 2002, Martins, Graebin et al. 2011) were used. A reaction temperature of 40°C, hexane as solvent and a substrate concentration of 0,5 M were chosen.

As the substrate ratio can play a role in reaction performance a ratio of 1:1 was used for the first experiment. Further experiments with different substrates ratios were carried out to

determine whether an excess of one of the substrates leads to higher productivity (see chapter C.5.2).

The information about the enzyme concentration was not very precise in those two publications and the activity of CALB regarding synthesis of butyl acetate was not known. Thus an appropriate enzyme concentration had to be found.

No water was added to the system as in both publications the best results were obtained at very little water contents or when no water was added at all.

C.5.2.Preliminary experiment with CALB L

Before starting experiments in the packed bed reactor with the immobilised enzymes experiments were carried out in batch mode with free enzymes.

Determination of sufficient amount of CALB L

In a first step the amount of enzymatic solution to obtain a reasonable yield (> 10%) in a reasonable time (about 2-4 hours) had to be found. For this experiment the concentration for both substrates (n-butanol and vinyl acetate) was kept at 0,5M.

The conditions for the first experiment were the following:

The reaction was carried out in flask immerged in a water bath at 40°C equipped with a magnetic stirrer. To 50mL of reactant solution (0,5M n-butanol; 0,5M vinyl acetate; solvent: hexane) 40µL of enzymatic solution CALB (=1,6mL$_{CALB\ L}$/Mol$_{Substrate}$) was added and samples were analysed every 20-30 minutes with GC.

Traces of product (butyl acetate) could be detected by GC but as the yield was very low its exact value could not be quantified.

In a second experiment the reaction conditions were the same (100mL hexane; 0,5M n-butanol; 0,5M vinyl acetate, 40°C), but the amount of CALB L was increased (0,4mL CALB L = 8mL$_{CALB\ L}$/Mol$_{Substrate}$). Figure 16 shows the yield of the reaction as a function of time.

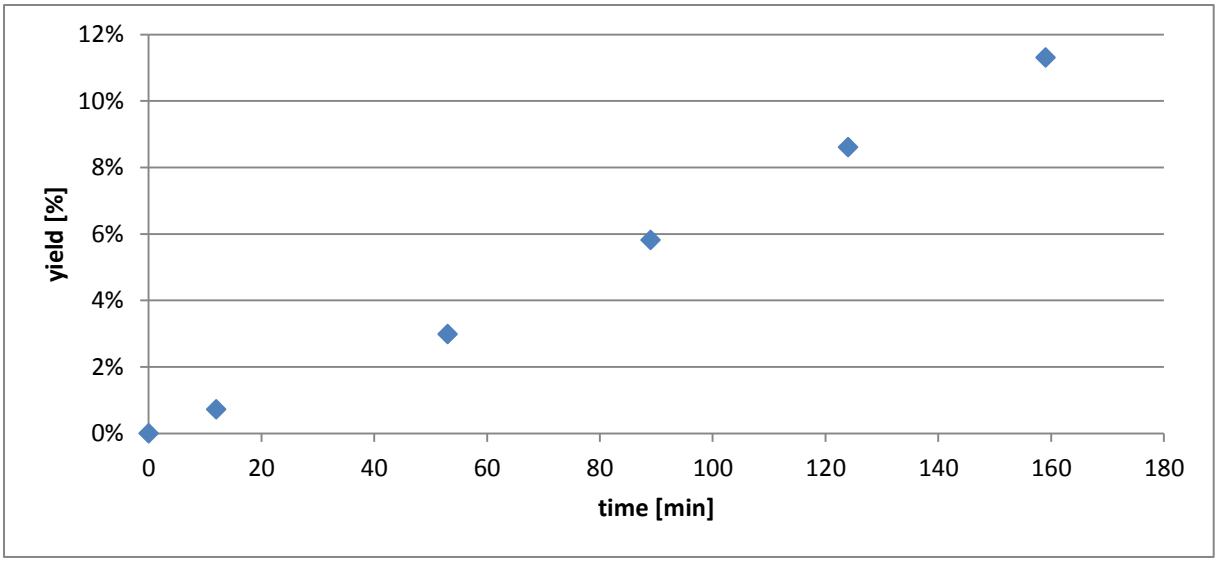

Figure 16: Yield of butyl acetate in liquid phase experiment as a function of time (batch, 40°C; 0,5M n-butanol; 0,5M vinyl acetate; solvent: hexane; 8mL$_{CALB\ L}$/Mol$_{Substrate}$).

The selected amount of enzymatic solution/Mol substrate turns out to be suitable as conversions of about 10% were achieved after 2-3 hours.

At the same time a blank test without enzymes was carried out and resulted in a yield of <0,1% after three hours.

Evaluation of optimum ratio between n-butanol and vinyl acetate

In a next step it was to evaluate whether an excess of alcohol or acetate would trigger higher yields. Three experiments were carried out at the same time under the same conditions with the exception of the ratio between vinyl acetate and n-butanol.

Three solutions of 100mL hexane with different amounts of reactants were stirred and heated to 40°C. Then 0,5mL of CALB L (= 10mL$_{CALB\ L}$/Mol$_{Substrate}$) was added and samples were taken every 30 minutes.

The experiments were carried out at the following ratios (see Table 8)

Table 8: Conditions of experiments for evaluation of optimum ratio between vinyl acetate and n-butanol

Experiment	***vinyl acetate : n-butanol***	
	ratio	**molarity**
No. 1	1 : 1	0,5M : 0,5M
No. 2	3 : 1	1,5M : 0,5M
No. 3	1 : 3	0,5M : 1,5M

Figure 17 shows the yield of the three reactions with different substrate ratios as a function of time.

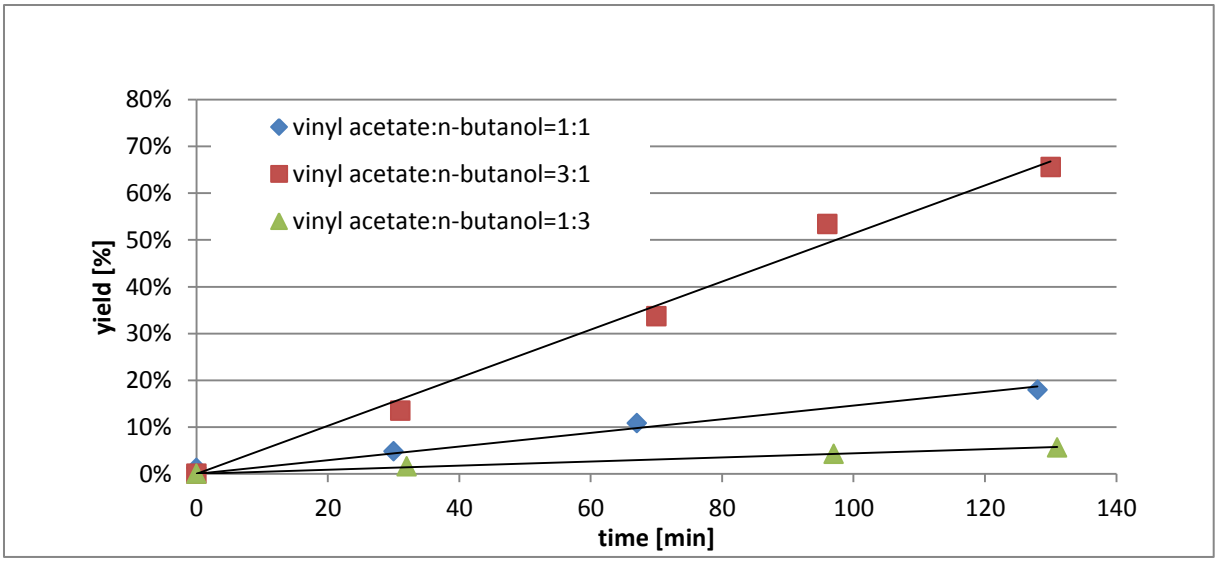

Figure 17: Yield of synthesis of butyl acetate with free enzymes in hexane batch as a function of time - different ratios of substrate (batch; 40°C; solvent: hexane; $10mL_{CALB\ L}/Mol_{Substrate}$)

The results show, that an excess of vinyl acetate results in a yield which is more than three times as high as at a ratio of 1:1. It also shows, that an excess of n-butanol does not improve the yield, but has an undesirable effect on the enzymatic activity resulting in lower yields compared to the experiment with a ratio of 1:1.

Thus the following experiments were carried out at a ratio vinyl acetate:n-butanol 3:1.

C.5.3.Preliminary experiment with immobilised beads

Before the beads were characterised in the packed bed reactor in continuous mode batch experiments were carried out.

Synthesis of butyl acetate - batch

At the beginning a small amount of beads (2,17g; same amount that fits in small packed bed reactor) was used for a batch experiment that was carried out under the same conditions as when working with free enzymes (hexane; 0,5M butanol; 0,5M vinyl-acetate; 40°C, agitated) but the working volume was increased to 200mL so that bigger samples could be taken and analysis is more accurate. A blank test was made under the same conditions but beads without immobilised enzymes were added to the solution. Figure 18 shows the yield of the reaction with and without enzymes as a function of time.

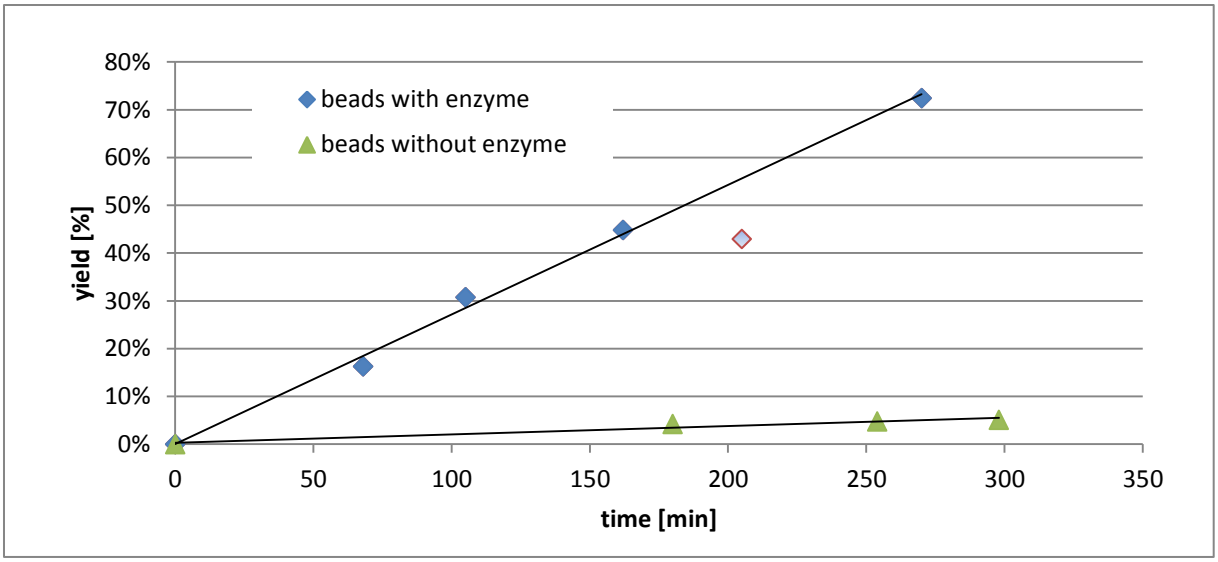

Figure 18: Yield of synthesis of butyl acetate with immobilised enzymes in hexane batch + blank test as a function of time (40°C; 2,17g beads; molar ratio vinyl acetate: n-butanol - 3:1; 0,5M n-butanol; 1,5M vinyl acetate; solvent: hexane)

As can be seen in Figure 18 after six hours a yield of about 75% could be achieved. This experiment shows that immobilised enzymes work very well in organic media for the synthesis of butyl acetate. A blank test with ceramic beads without immobilised enzymes showed a yield of about 6% after 6 hours.

The activity of hydrolysis in aqueous media cannot be used to make assumptions about how much beads are needed for synthesis of butyl acetate when the amount of CALB L is known. This can be explained with the fact that to different reactions with different substrate affinities, different reaction rates and different solvent were tried to be compared. Far less beads were needed than expected showing once again the importance of immobilising the enzymes for this process.

A blank test was carried out with gamma-alumina beads and shows that the beads themselves have little catalytic activity. Three different experiments were carried out under the same conditions: without beads, with ceramic beads and with immobilised enzymes on ceramic beads. The experiment without beads showed yields <0,1% while a yield of 3% was achieved with ceramic beads. An activity almost 10 times higher was observed when using the immobilised enzymes showing the importance of the catalytical activity of the enzymes.

C.5.4.Synthesis of butyl acetate – continuous mode

As under aqueous conditions the beads where characterised regarding their activity of synthesis of butyl acetate in continuous mode.

As in chapter B.5.1 stated the experiment was carried out in a packed bed reactor with 12 g beads at a temperature of 40°C and a feed flux between 0,5-10mL/min i.e. 20-420 µMol/min.

The experiment was done twice and two different loads of beads were used. The yield and the activity of the two reactions are shown in Figure 19 and Figure 20 in two different colours.

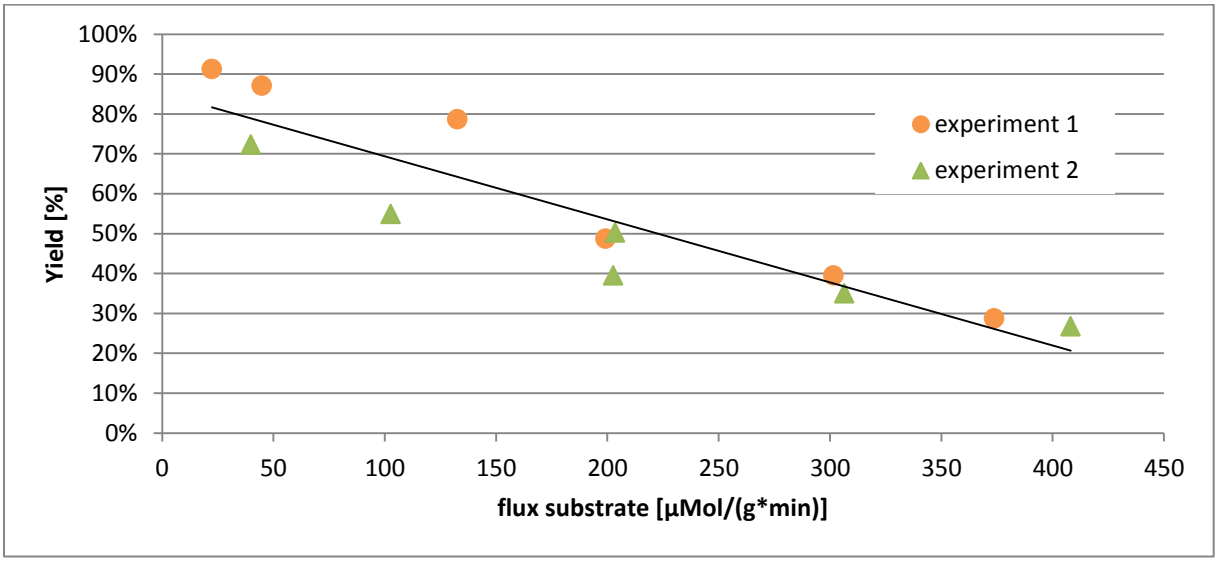

Figure 19: Yield of synthesis of butyl acetate as a function of specific substrate flux (40°C; 12g beads; packed bed; solvent: hexane 0,5M n-butanol; 1,5M vinyl acetate)

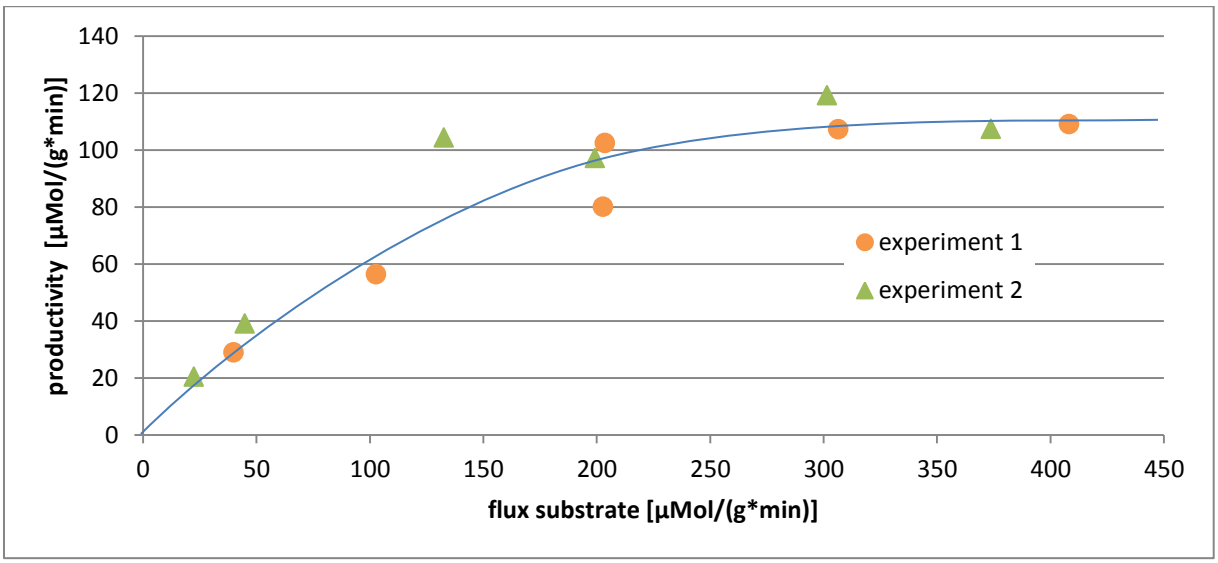

Figure 20: Productivity of synthesis of butyl acetate as a function of specific substrate flux (40°C; 12g beads; packed bed; solvent: hexane 0,5M n-butanol; 1,5M vinyl acetate)

As expected the yield decreases with higher substrate flux (see Figure 19) and on the other hand the activity increases and levels off at a value of about 110 µMol/(g*min) (see Figure 20). To prevent systematic errors the experiments were not carried out in ascending or descending order regarding the substrate flux but in random order.

The reason for the shape of the curve of the productivity is that at low fluxes there is substrate limitation.

The measured activity for synthesis of butyl acetate in hexane is 110 µMol/(g*min).

C.6. Synthesis of butyl acetate under supercritical conditions

For ecological and practical reasons as stated in chapter A.4 sc-CO_2 was used in recent studies as solvent a solvent instead of conventional organic solvents. As CALB is known to catalyse the synthesis of esters the aim of this work was to produce esters that can be used as food additives in flavour industry.

Recently the synthesis of a specific ester catalysed by CALB under supercritical conditions was studied by Ben Ameur (Ben Ameur 2012). Experiments were carried out with CALB immobilised on a ceramic membrane resulting in low yields (about 50%). An approach was made to use CALB immobilised on a macroporous resin (Novozym 435) in a packed bed reactor. Yields of 100% were obtained but due to swelling of the beads with immobilised enzymes the pressure drop of the reactor increased immensely and the experiment had to be stopped. Furthermore Ben Ameur observed solubility problems of the substrates and products for the synthesis of anisyl acetate.

For several reasons stated in chapter C.1 butyl acetate was chosen as product synthesised by n-butanol and vinyl acetate. Enzymes were immobilised by adsorption on ceramic beads (see chapter C.3) and were used to catalyse the reaction under supercritical conditions.

C.6.1.Choice of reaction conditions

In order to establish the reaction conditions literature was reviewed and preliminary experiments were taken in account.

Reaction temperature and pressure

When choosing the reaction temperature and pressure different aspects were taken in account.

a) Minimum temperature and pressure for critical conditions of carbon dioxide
b) Maximum pressure of the pilot plant
c) Conditions from literature with high yields and similar substrates/products
d) Conditions of best solubility for both substrates and products
e) Denaturation temperature of enzymes

As the critical temperature of CO_2 is 31,1°C and the critical pressure is 73,8 bar reaction temperature and pressure had to be chosen above those values. In order to achieve stable conditions it is recommendable not working too close to the critical point as little changes in temperature and pressure change the properties of the sc-CO_2 a lot.

The maximum pressure was limited by the maximum working pressure of the pilot plant which was 200 bar.

CALB in its free form is known to have its optimum performance at temperatures between 40°C and 60°C. When working with immobilised enzymes above 60°C denaturation can occur and can lead to a loss of activity after several hours (Habulin, Sabeder et al. 2007). Depending on the substrate where enzymes are immobilised thermostability has been reported up to 80°C. The thermostability of CALB immobilised on gamma alumina could not be found in literature and was not known.

Oliveira et al. (Oliveira, Rebocho et al. 2009) investigated the synthesis of decyl acetate from vinyl acetate and decanol catalysed by Novozym 435. Different temperatures (35°C-45°C) at a pressure of 100 bar were tested and a positive effect was observed when increasing the temperature. Due to experimental limitation the temperature in their work couldn't be set above 45°C.

Habulin et al. (Habulin, Sabeder et al. 2007) studied the synthesis of citronellol laurate from citronellol and lauric acid and used Novozym 435 as catalyst. They tested temperatures between 50°C and 70°C at a pressure of 100bar and observed a higher activity at 60°C compared to 50°C but a decrease in activity when work at 70°C. Normally most enzymes denature at 60°C but when immobilised on macroporous resin CALB is thermostable until 70°C according to the supplier Habulin et al. credit the decrease of production also to

changes in density of the solvent or other changes in the solvent mixture by increasing temperature.

Oliveira et al. (Oliveira, Rebocho et al. 2009) also tested different pressures (102-190bar, 35°C). Experiments were carried out in batch mode and the conversion after one minute decreased when increasing the pressure. Oliveira et al. related this effect to changes in density-dependent properties of sc-CO_2 that indirectly regulate the activity, specificity and stability of enzymes.

Dijkstra et al. (Dijkstra, Merchant et al. 2007) also observed a negative effect on activity when increasing the pressure from 110 bar to 150/180 bar (40°C, CLEA-CALB, isoamyl alcohol + acetic acid → isoamyl acetate).

Lozano et al. (Lozano, Villora et al. 2004) depict that the activity of CALB (Novozym 435) is strongly related to the density of sc-CO_2. Best results were achieved at high temperatures (40-60°C were tested) and low pressures (80, 95, 120, and 150 bar were tested). This effect can be described as low densities of sc-CO_2 result in a decrease of internal diffusion limitations into the enzyme particle. Similar results regarding the change of activity related to fluid density were observed by Kamat et al. (Kamat, Critchley et al. 1995).

According to literature the optimum reaction temperature for this reaction appears to be between 50°C and 60°C with an optimum pressure between 80 bar and 100 bar.

Solubility data for vinyl acetate, n-butanol and butyl acetate was found, transformed into [mMol/kg] and plotted against pressure (see Figure 21, Figure 22 and Figure 23). Unfortunately there is not a lot of data about the solubility in CO_2 available in literature.

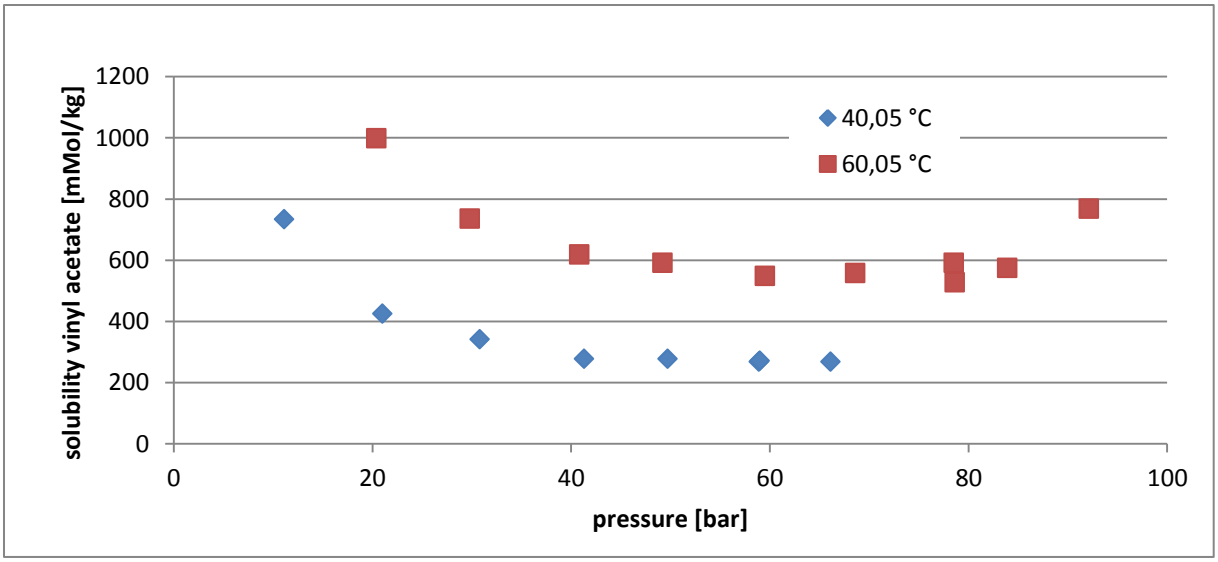

Figure 21: Solubility of vinyl acetate [mMol/kg_{CO2}] plotted against pressure [bar] at 40,05°C and 60,05°C (Stevens, Shen et al. 1997)

The solubility of vinyl acetate was known from literature at 40,05°C and 60,05°C. As shown in Figure 21 by increasing the pressure the solubility of vinyl acetate decreases for both temperatures. After reaching the critical pressure of 73,8 bar (no data for 40°C available) the solubility increases with higher pressures.

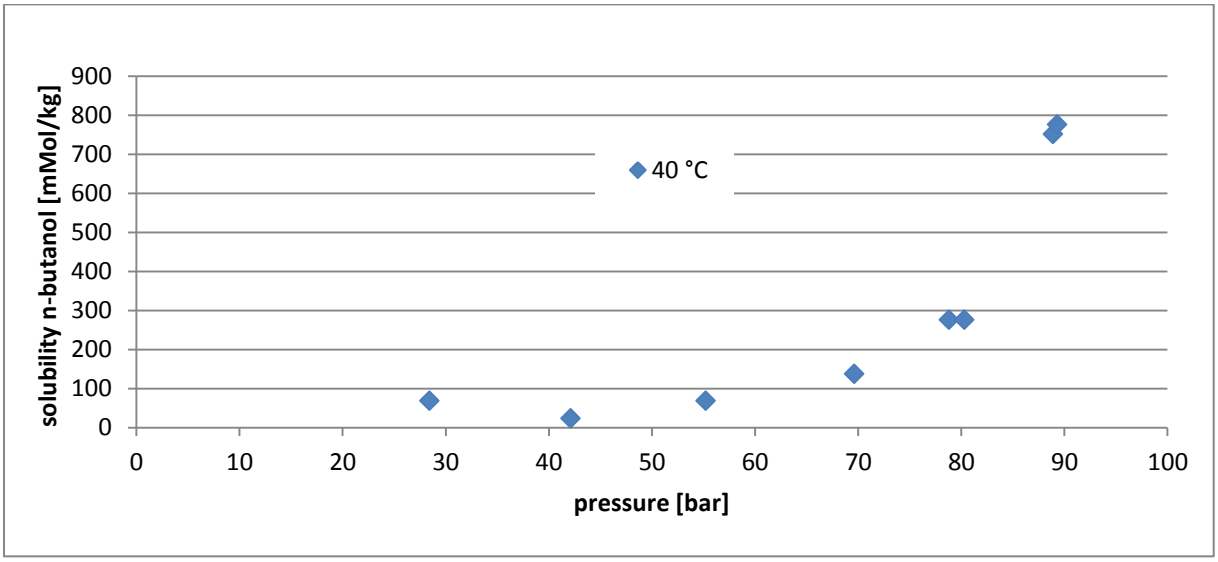

Figure 22: Solubility of n-butanol [mMol/kg_{CO2}] plotted against pressure [bar] at 40°C (Eissier and Friedrich 1988)

For n-butanol there was only solubility data at 40°C available. As can be seen in Figure 22 the solubility of n-butanol increases as a function of pressure. Especially between 70 and 90 bar a fast ascent can be registered.

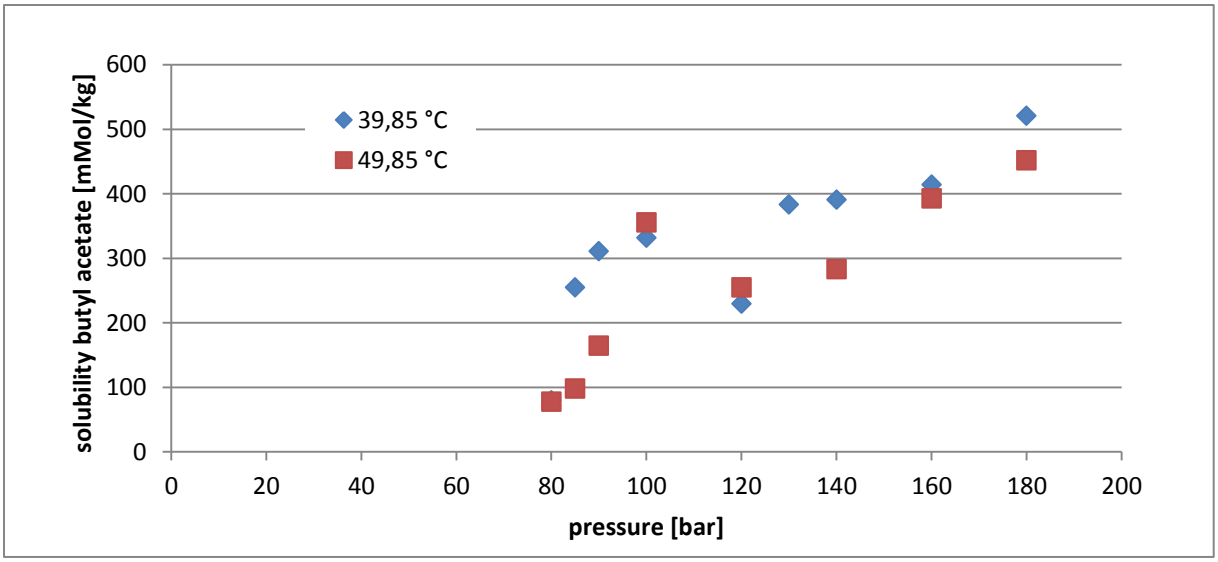

Figure 23: Solubility of n-butanol [mMol/kg_{CO2}] plotted against pressure [bar] at 39,85°C and 49,85°C (Ghaziaskar and Nikravesh 2003)

The solubility of butyl acetate in CO_2 was found in literature for 39,85°C and 49,85°C. Figure 23 shows an increase of solubility until 100 bar. After a drop of solubility at 120 bar the solubility appears to increase at pressures above 130 bar.

No solubility data were found about acetaldehyde which is also formed in this process. According to literature the highest solubilities for the substrates and products used in the chosen reaction appear to be at high pressure (90-100 bar) and elevated temperatures (50-70°C).

Summary

The optimal reaction conditions from literature research appear to be at a pressure between 80 bar and 100 bar and at temperatures between (50-60°C).

To assure stable conditions and as solubility of most components appears to be better at a higher pressure the reaction pressure was set to 100 bar.

In order to prevent denaturation of the enzyme and as the thermostability of CALB immobilised on gamma alumina beads was not known the maximum reaction temperature was chosen to be 50°C.

As these conditions are within the range of possible temperature and pressure of the pilot plant all further experiments of this work were carried out 50°C and 100 bar.

In further studies an optimisation of those conditions is planned. The stability of CALB is to be tested at higher temperatures as in most publications better yields were achieved at higher temperatures (50-70°C)(See 0)).

Choice of ratio of substrates

To evaluate optimum conditions concerning the ratio of substrates literature was reviewed and the results of preliminary experiments were taken in account.

Preliminary experiments were carried out in hexane at different substrate ratios to evaluate optimum conditions (see chapter C.5.2). Those experiments showed that an excess of vinyl acetate leads to higher yields compared to an equimolar ratio. An excess of n-butanol has even a negative effect on the activity of CALB compared to an equimolar ratio. When changing the solvent conditions from liquid to supercritical the optimum substrate ratio can differ. Though similar results proofing that an excess of acyl donor has a positive effect on the activity were found in literature for organic as well as for supercritical media.
Oliveira et al. (Oliveira, Rebocho et al. 2009) explained the decrease in activity of CALB at high alcohol concentrations as competitive inhibition of the alcohol leads to deactivation of the enzyme. As the alcohol can bind to the active site of the enzyme in its native state it forms a dead-end enzyme-alcohol complex and not the product.

As a result of the preliminary experiments and the literature research a substrate ratio of 3:1 (vinyl acetate: n-butanol) was chosen for all experiments to avoid enzyme inhibition caused by an excess of alcohol.
Experiments to evaluate the optimum substrate ratio and to study the effect of substrate inhibition are planned for the future (see 0).

Choice of concentration of substrates

To ensure that all components remain soluble in the reactor throughout the whole reaction the concentration of the substrates and products was chosen by using the solubility data from literature (see Figure 21 - Figure 23).
As the solubility data in literature was not measured under the same conditions (temperature and pressure) as required for the reaction in this study the solubility had to be estimated by the assumption that the evolution of solubility keeps the same trend.

The crucial component was vinyl acetate as it was used in excess (ratio 3:1 vinyl acetate:n-butanol) and it had to be assured that all of it is soluble. From Figure 21 the solubility was estimated to be about 600 mMol vinyl acetate per kg CO_2 at 100 bar and 50°C. To be on the safe side the maximum working concentration was chosen at half of the value obtained from estimation (300mMol/kg). The resulting maximum concentrations for butyl acetate and n-butanol (ratio 3:1) were both 100mMol/kg CO_2. Those concentrations should not pose a problem as the solubility limit for n-butanol (estimated >800mMol/kg CO_2) and butyl acetate (~350mMol/kg CO_2) are a lot higher as the used concentrations. For acetaldehyde no solubility data was available in literature so no statement can be given about its solubility.

To prove that the chosen concentrations do not exceed solubility limit preliminary experiments were carried out. The mass balance of the pilot plant was verified by measuring the feed flux at the entry of the reactor and at its exit under standard operation conditions with beads without immobilised enzymes to prove that all compounds that enter the installation can be recovered.

C.6.2. Preliminary experiments

Mass balance

For most substances there is not a lot of data about their solubility available in literature. As a result the solubility for the compounds of the used reaction (n-butanol + vinyl acetate → butyl acetate + acetaldehyde) had to be estimated from the data found in literature.

To assure that all components remain soluble in the reactor throughout the whole reaction all substances where injected at their maximum possible concentration under standard operation conditions and tried to be recovered at the exit of the reactor. Acetaldehyde has a boiling point of 20,2°C and the experimental setup did not allow collecting this substance without major loss due to evaporation. As a result this experiment was only carried out for vinyl acetate, n-butanol and butyl acetate but not acetaldehyde.

The experiment was carried out at 50°C, 100 bar and a CO_2 flux of 1,8 kg/h. The small packed bed reactor filled with 2,17g beads without immobilised enzymes was used for this experiment and vinyl acetate (300 mMol/kg CO_2), n-butanol (100 mMol/kg CO_2) and butyl acetate (100 mMol/kg CO_2) were constantly injected via the HPLC pump. The fluxes and concentrations of the different components are summarised in Table 9.

Table 9: Concentrations of substrates and products for mass balance experiment at 1,8kg CO_2/h

		Vinyl acetate	n-Butanol	Butyl acetate	
Ratio		3	1	1	Sum
concentration	mMol/kg	300	100	100	500
concentration	g/kg	25,83	7,41	11,62	44,86
flow	µMol/min	9000	3000	3000	12000
flow	g/h	46,5	13,3	20,9	80,74
flow	mL/h	49,77	16,47	23,76	90

Every 30 minutes samples were withdrawn from the product separators S51 and S52 (see Figure 2). The samples where weighed and their composition was analysed by gas chromatography. The total flux at the exit of the reactor was calculated and plotted as a function of time (see Figure 24). The value for the total flux at the entry of the reactor was given by the flux of the HPLC pump and is also indicated in Figure 24.

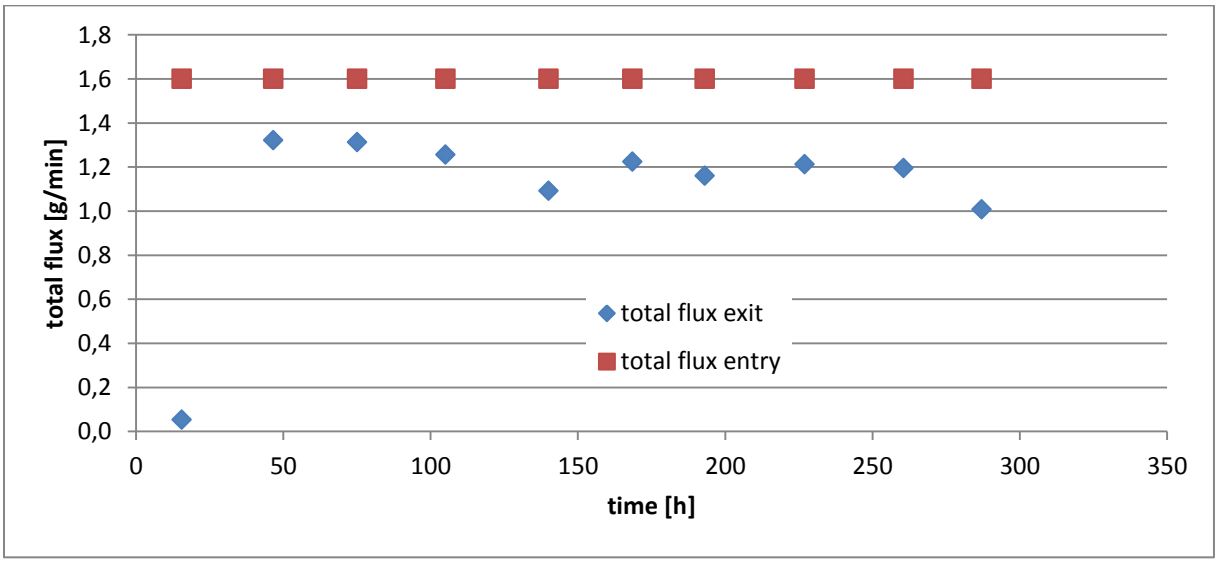

Figure 24: Evolution of total flux at entry and exit of the reactor as a function of time (50°C; 100 bar; 1,8kg CO_2/h; 20g beads; 9000 $\mu Mol_{vinyl\ acetate}$/min, 3000 $\mu Mol_{butanol}$/min, 3000 $\mu Mol_{butyl\ acetate}$/min)

The graph shows that after about 30-60 minutes a constant flux was obtained at the exit of the reactor. Only about 75% of the flux entering the system was collected at its exit. At the beginning it takes about 15-20 minutes until the first product arrives at the exit. This is due to the dead volume of the system. The reason why only 75% of the feed that was sent into the system can be recovered at the exit is probably not due to a loss of product inside the reactor. It is probably caused by a loss of product during sample taking. Inside the separators there is still a pressure of about 50bar (pressure of the CO_2-bottle). When opening the sample valve of the separator a vapour exits at high pressure. Even when withdrawing the

samples in a big bottle a certain loss of product could not be avoided due to evaporation and the small droplets that could not be collected but got carried away by the CO_2 stream.

Samples were analysed regarding their composition by gas chromatography. The measured values for n-butanol and butyl acetate were plotted as a function of time (see Figure 25). The initial concentrations of the feed solution are indicated in Figure 25 at t=0 in purple colour.

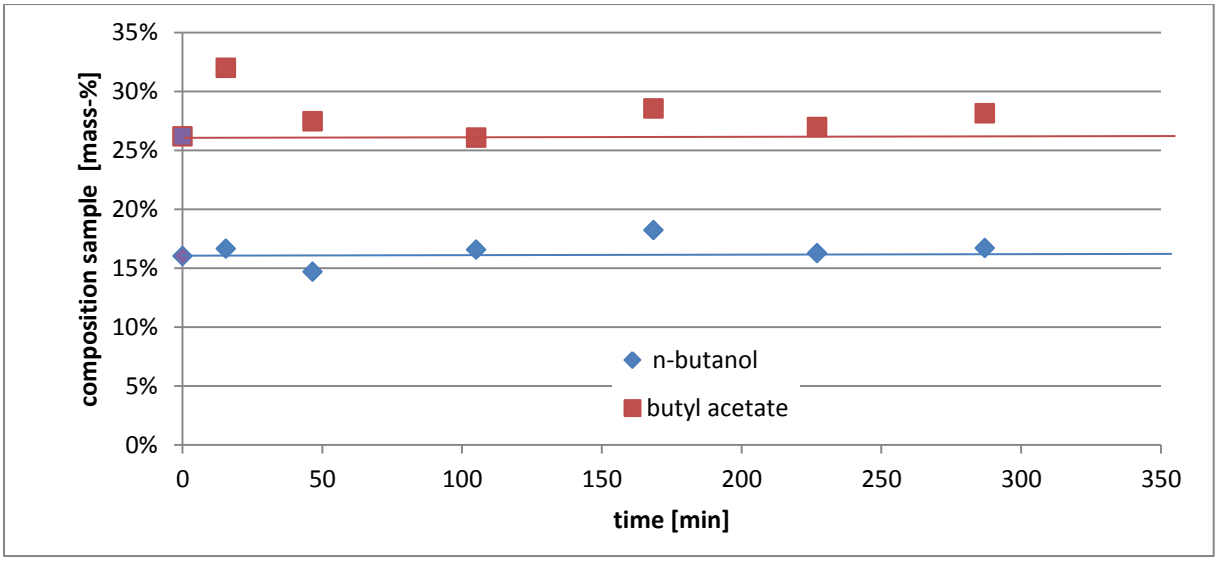

Figure 25: Evolution of composition of different components as a function of time (50°C; 100 bar; 1,8kg CO_2/h; 20g beads; 9000 µMol$_{vinyl\ acetate}$/min, 3000 µMol$_{butanol}$/min, 3000 µMol$_{butyl\ acetate}$/min)

Figure 25 shows that the measured concentration of n-butanol and butyl acetate do not vary in course of time and are equivalent to the concentration of the initial feed solution. As a result the concentration of the third component vinyl acetate which could not be quantified by the used gas chromatography method has to stay constant as well. This indicates equal solubilities for all three components.

With the total flux of the components at the exit and the composition of the sample the specific flux for each component was calculated and plotted in course of time (see Figure 31). The specific substrate flux that enters the system is indicated at t=0 in purple colour.

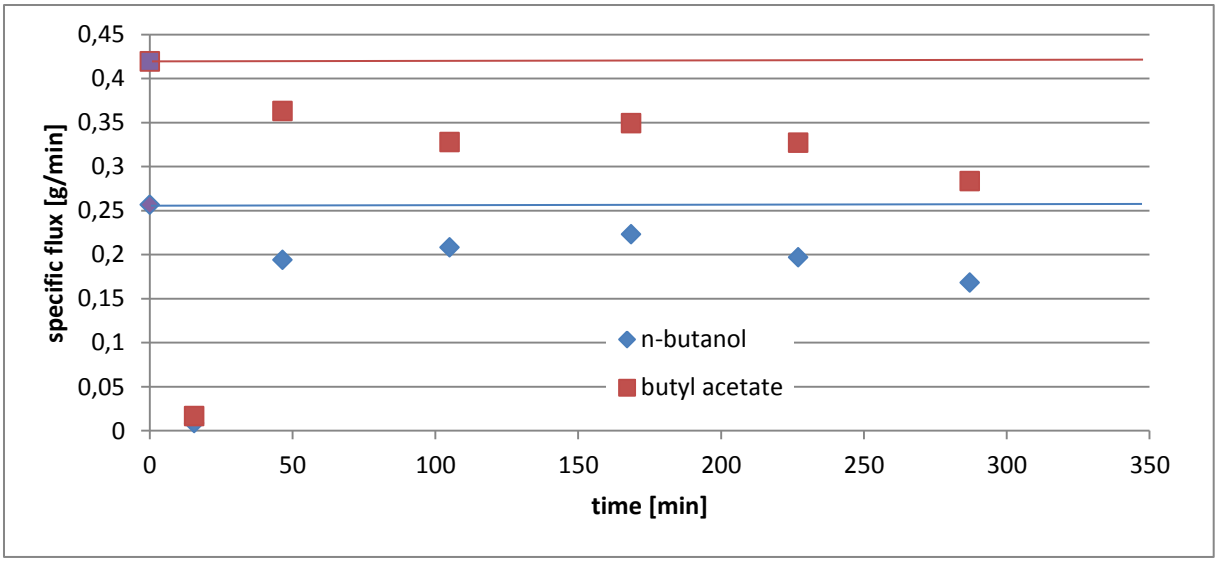

Figure 26: Evolution of mass flux of different components as a function of time (50°C; 100 bar; 1,8kg CO_2/h; 20g beads; 9000 $\mu Mol_{vinyl\ acetate}$/min, 3000 $\mu Mol_{butanol}$/min, 3000 $\mu Mol_{butyl\ acetate}$/min)

Mass balance experiment - flux of different components

Figure 26 shows that after a period of 30-60 minutes the substrate flux is constant.

In Figure 27 the loss of mass flux in per cent is plotted against time for different components making it easier to compare the different compounds.

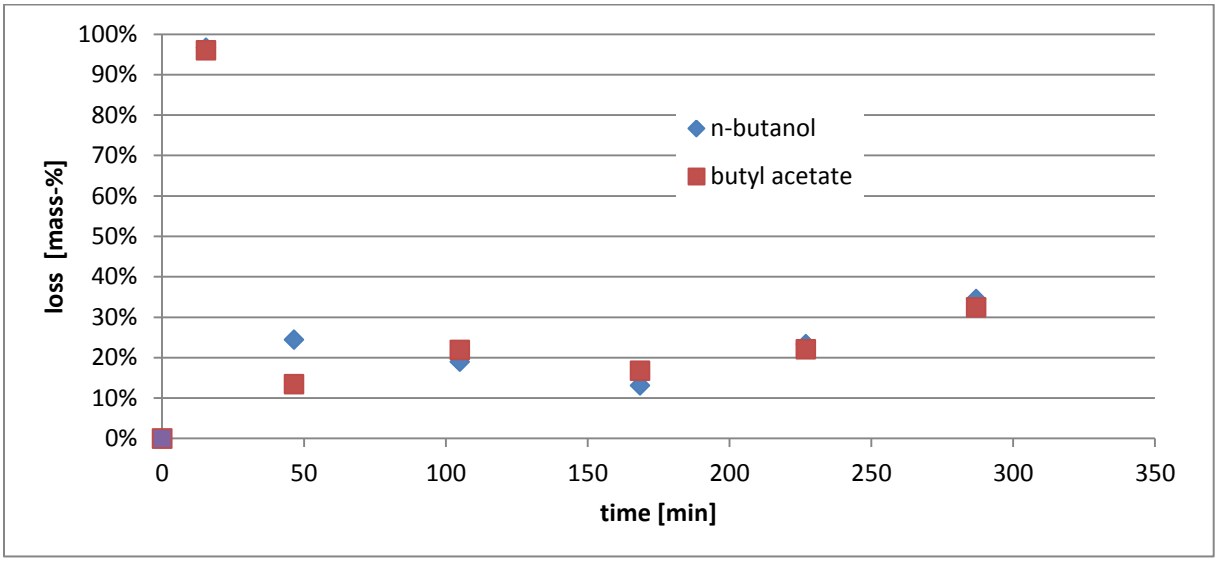

Figure 27: Evolution of the loss of mass flux of different components as a function of time (50°C; 100 bar; 1,8kg CO_2/h; 20g beads; 9000 $\mu Mol_{vinyl\ acetate}$/min, 3000 $\mu Mol_{butanol}$/min, 3000 $\mu Mol_{butyl\ acetate}$/min)

As one can see the composition of the samples does not change significantly in course of time. At the beginning (until 30-60 min) not all of the feed injected into the system can be collected at the exit of the reactor. This is due to the dead volume of the reactor. When comparing the two compounds there is also hardly any difference between the loss of n-butanol and butyl acetate.

As already mentioned the loss of reaction solution at the end of the reactor is probably not due to solubility problems but a result of sample taking. As the compounds exit the separators at a pressure of 50 bar a certain amount of reaction solution gets lost due to evaporation and small droplets that get carried away be the CO_2 stream. In order to minimise the loss of sample and to prove that there is no loss of product inside the reactor a second separator could be installed in series after separator S51 and S51 (see 0). Similar experiments with higher concentrations of all compounds of this reaction can be carried out in future to be able to increase the substrate flux during synthesis without risking solubility problems.

Evaluation of Pressure drop of packed bed reactor

In previous studies at the IEM Ben Ameur used CALB immobilised on a macroporous resin (Novozym 435) as catalyst in sc-CO_2. Due to swelling of the beads the pressure drop increased immensely and the experiment had to be stopped.

In this work CALB was immobilised on a ceramic support (gamma alumina beads) which are known to be inert and no increase of pressure drop was expected due to swelling. Experiments were carried out for eight hours to evaluate the initial pressure drop of the two reactors and to verify that no increase of pressure takes place.

The experiments were carried out at standard reaction conditions (50°C, 100 bar, 1,8kg CO_2/h) and a feed flux of 280 µMol/(g*min) (small reactor with 2,17 g beads) respectively 337µMol/(g*min) (big reactor with 10g beads).

During the whole experiment the pressure drop stayed constant for both reactors. For the small reactor a pressure drop of 1 bar was measured and for the big reactor a pressure drop of 2 bar. No modification of the ceramic beads was visible after using them under supercritical conditions.

C.6.3. Feasibility study

In this part of the work experiments were carried out to verify that CALB immobilised on ceramic beads catalyses the production of butyl acetate from n-butanol and vinyl acetate under supercritical conditions. A blank test with ceramic beads without immobilised enzymes was carried out to prove that no or only very little product can be obtained in the absence of enzymes under similar conditions.

Calculation of productivity and yield

To screen the reactions samples were taken and analysed by gas chromatography and the yield [%] and the productivity [µMol$_{butyl\ acetate}$/(g$_{beads}$*min) were monitored. There are two different ways of calculating the yield (see Equation 1 and Equation 2) and the productivity (see Equation 3 and Equation 4) of a reaction.

$$yield\ 1\ [\%] = \frac{butyl\ acetate\ (exit)[\frac{\mu Mol}{min}]}{butanol\ (entry)[\frac{\mu Mol}{min}]}$$

Equation 1: Yield 1

$$yield\ 2\ [\%] = \frac{butanol\ (entry)\left[\frac{\mu Mol}{min}\right] - butanol(exit)[\frac{\mu Mol}{min}]}{butanol\ (entry)[\frac{\mu Mol}{min}]}$$

Equation 2: Yield 2

$$productivity\ 1\left[\frac{\mu mol_{butyl\ acetate}}{g_{beads} * min}\right] = \frac{butyl\ acetate\ (exit)[\frac{\mu Mol}{min}]}{amount\ beads\ [g_{beads}]}$$

Equation 3: productivity 1

$$productivity\ 2\left[\frac{\mu mol_{butyl\ acetate}}{g_{beads} * min}\right] = \frac{butanol\ (entry)\left[\frac{\mu Mol}{min}\right] - butanol(exit)[\frac{\mu Mol}{min}]}{amount\ beads\ [g_{beads}]}$$

Equation 4: productivity 2

For all reactions the yield and productivity was calculated in both ways.

Synthesis without enzymes (blank test)

The blank test without enzymes to show that there is only very little product obtain without using enzymes was carried out under standard reaction conditions (50°C, 100 bar, 1,8 kg CO_2/h). The small packed bed reactor was filled with 2,17 g gamma alumina beads without immobilised enzymes and a specific substrate flux of 415µMol/(g*min). The experiment was carried out for four hours and samples were taken, weighed and analysed every 30 minutes to evaluate the yield and the productivity of the reaction.

Only traces of butyl acetate could be detected by gas chromatography showing hardly any conversion of substrates (yield < 0,5%; productivity < 2µMol/(g*min)).

This shows that the product obtained by the spontaneous reaction is negligible and that enzymes are necessary as catalysts to obtain amounts of product that are interesting for an industrial process.

Synthesis with enzymes

An experiment was carried out to prove that synthesis of butyl acetate catalysed by immobilised CALB under supercritical conditions is possible.

For this experiment standard reaction conditions were applied (50°C, 100 bar, 1,8 kg CO_2/h) and the big packed bed reactor (filled with 20g beads) was used at a specific substrate flux of 75 $\mu Mol_{n\text{-}butanol}/(g*min)$ respectively 225 $\mu Mol_{vinyl\ acetate}/(g*min)$.

Figure 28 and Figure 29 present the yield and the activity of this experiment as a function of time. The yield and the activity were calculated by the two different ways mentioned at the beginning of this chapter. The blue diamonds indicate the yield/productivity calculated by the measured product (butyl acetate) at the exit of the packed bed reactor whereas the red squares indicate the yield/productivity calculated by the used substrate (n-butanol) at the exit of the packed bed reactor.

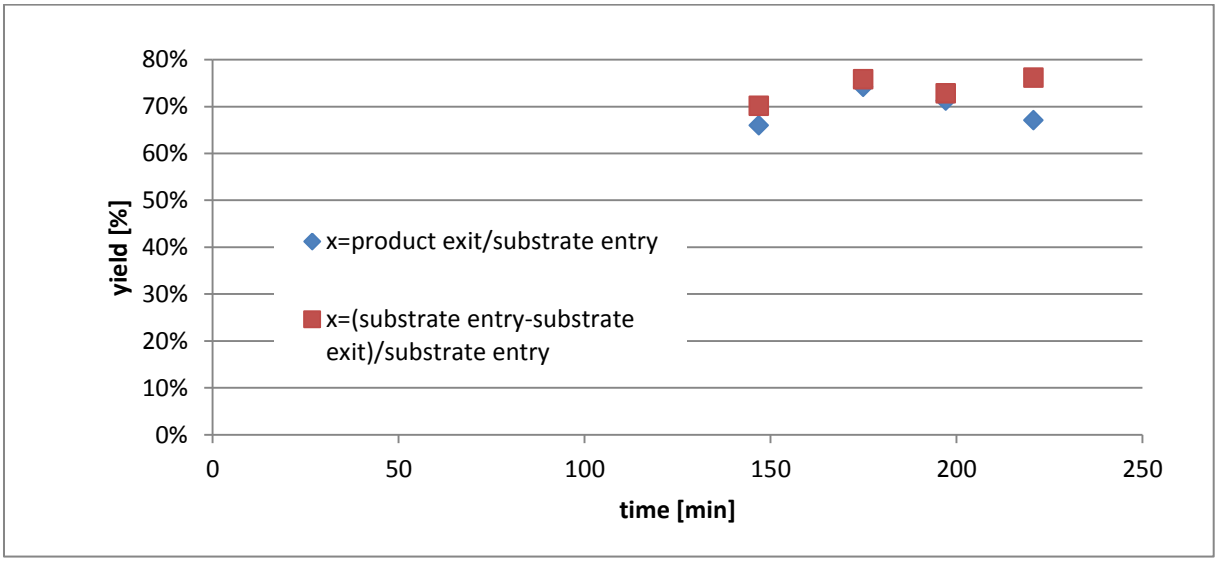

Figure 28: Evolution of the yield of synthesis of butyl acetate calculated by two different ways as a function of time (50°C; 100 bar; 1,8 kg CO_2/h; 20g beads; molar ratio vinyl acetate: n-butanol - 3:1; 75 $\mu Mol_{n\text{-}butanol}/(g*min)$; 225$\mu Mol_{vinyl\ acetate}/(g*min)$)

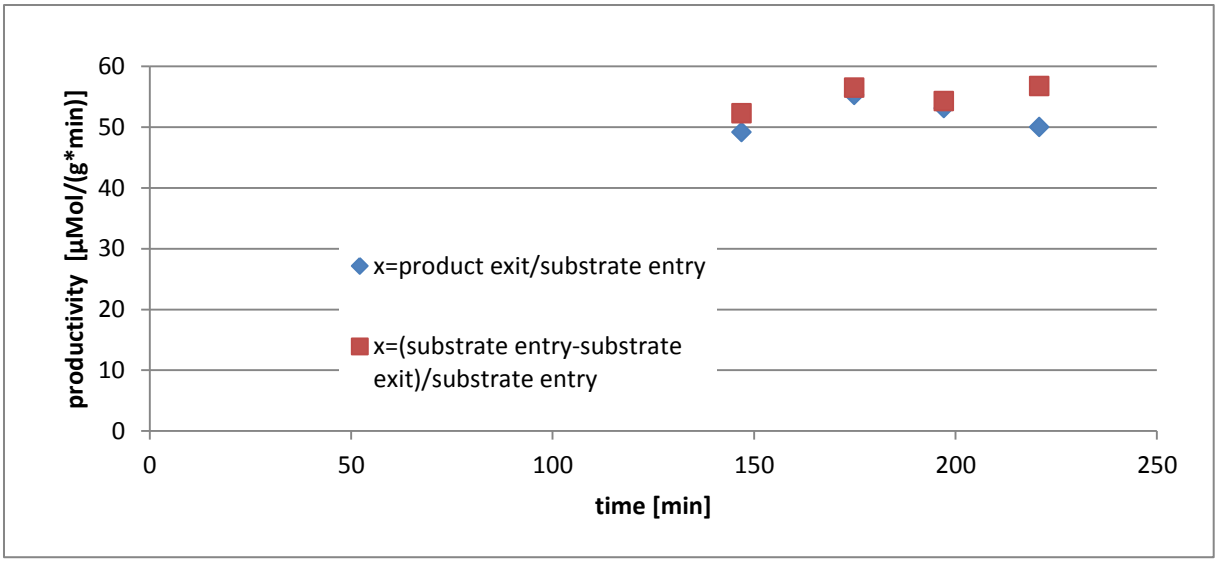

Figure 29: Evolution of the productivity of synthesis of butyl acetate calculated by two different ways as a function of time (50°C; 100 bar; 1,8 kg CO_2/h; 20g beads; molar ratio vinyl acetate: n-butanol - 3:1; 75 $\mu Mol_{n\text{-}butanol}$/(g*min); 225$\mu Mol_{vinyl\ acetate}$/(g*min))

Figure 28 and Figure 29 show that under the stated conditions a yield of about 72% and a productivity of about 54 µMol/(g*min) can be observed. The values calculated by the two ways do not differ a lot and yield and productivity appears to stay constant.

The data obtained through those two experiments shows that CALB catalyses the synthesis of butyl acetate under supercritical conditions. While negligible amounts of product were detected in the absence of enzymes a high yield (72%) and a productivity of about 54 µMol/(g*min) was measured when using ceramic beads with immobilised enzymes.

C.6.4.Study of the effect of the substrate specific flow

Experiments were carried out to evaluate the activity of the enzymes immobilised on ceramic beads. In case of substrate limitation only the productivity but not the activity can be measured. At high yields (as in the experiment of chapter C.6.3 “Synthesis with enzymes” where yields of 72% were achieved) the effect of substrate limitation can play a major role. To minimise the effect of substrate limitation higher specific substrate flux rates were applied. This can either be achieved by increasing the feed flux or decreasing the amount of enzyme respectively the amount of beads with immobilised enzymes. To assure staying in a concentration range where all substrates and products are soluble the feed flux was not increased (as the CO_2 flux was already almost at the pilot plant’s maximum and an increase of feed flux would have resulted in a higher substrate concentration) but a smaller amount

of beads (2,2 g) was used for the following experiments. The experiments were carried out under standard reaction conditions (50°C, 100 bar, 1,8 kg CO_2/h) with 2,17g beads and a specific substrate flux between 180 and 1300 $\mu Mol_{butanol}/(g_{beads}*min)$ respectively 540 and 3900$\mu Mol_{vinyl\ acetate}/(g_{beads}*min)$.

Each experiment was carried out for at least three hours to reach the steady state and the beads were not reused but changed after every experiment. As soon as the yield/productivity in each experiment was constant (after about 30-60 minutes) several samples were taken over a period of about 2-3 hours and the average yield and productivity were calculated. Then those average values of yield and productivity were plotted over the specific substrate flow rate to determine the activity as well as to study the effect of substrate limitation (see Figure 30 and Figure 31).

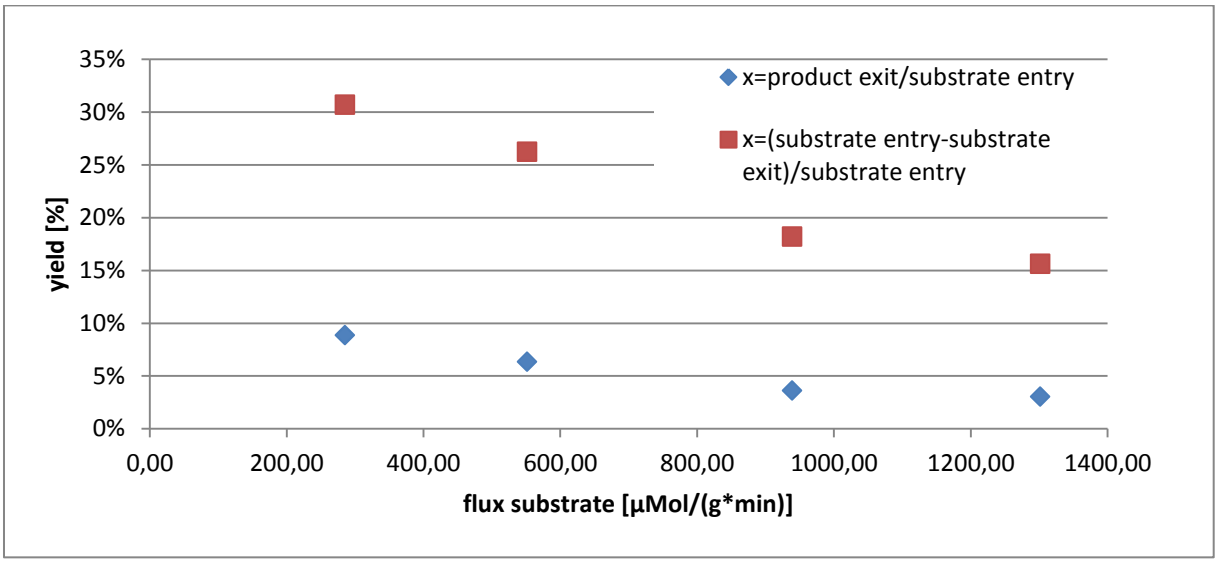

Figure 30: Evolution of yield as a function of different specific substrate flux of n-butanol (50°C; 100 bar; 1,8 kg CO_2/h; 2,17g beads; molar ratio vinyl acetate: n-butanol - 3:1; 285-1300 $\mu Mol_{n\text{-}butanol}/(g*min)$; 855-3900 $\mu Mol_{vinyl\ acetate}/(g*min)$)

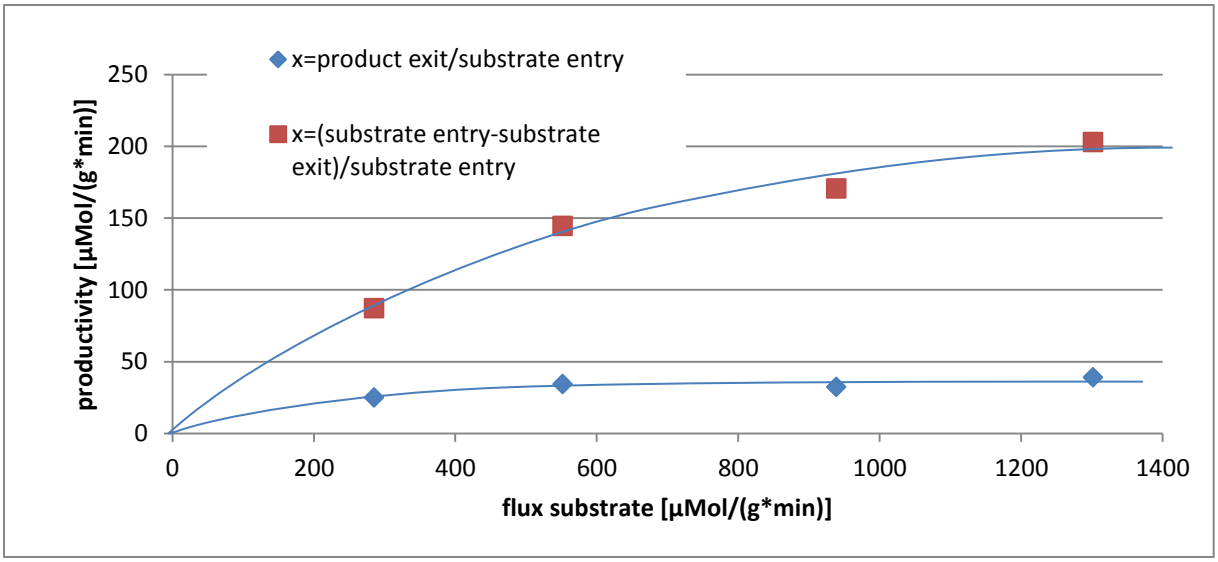

Figure 31: Evolution of productivity as a function of different specific substrate flux of n-butanol (50°C; 100 bar; 1,8 kg CO_2/h; 2,17g beads; molar ratio vinyl acetate: n-butanol - 3:1; 285-1300 $\mu Mol_{n\text{-}butanol}/(g*min)$; 855-3900 $\mu Mol_{vinyl\ acetate}/(g*min)$)

The two figures (Figure 30 and Figure 31) show a great difference between the values obtained by the two different ways of calculation.

There are hypothesis for the difference between the values obtained by different ways of calculation:

1. The loss of reaction solution at the exit of the reactor during sample withdrawing can either result in an over- or underestimation of the yield/productivity depending on the method of calculation.
2. Different sample preparation for analysis of n-butanol and butyl acetate thus obtaining more or less precise values for yield/productivity depending on the method of calculation.

On the one hand the loss of reaction solution during sample withdrawing at the exit of the reactor can lead to an underestimation or an overestimation of yield/productivity.

Effect of underestimation: As a result of the loss of sample at the exit of the separators the measured amount of butyl acetate is smaller than the actual value. The value for n-butanol at the entry of the reactor is fairly precise as it is calculated by the feed concentration and the flux of the feed pump. That leads probably to an underestimation of the yield/productivity.

Effect of overestimation: Calculating the yield/productivity from the difference between substrate at the entry and at the exit of the reactor also brings an error. The measured amount of n-butanol at the exit of the reactor is lower than the actual amount due to loss of

sample whereas the n-butanol at the entry of the reactor is calculated by the flux of the feed pump giving a precise value. Thus when calculating the yield/productivity by the used substrate they are probably overestimated.

On the other hand the difference in preparation of samples for GC to analyse n-butanol and butyl acetate can result in different values for yield/activity due to different errors in sample preparation. For analysing the amount of n-butanol the ratio of the internal standard octanol: reaction solution was about 1:3 -1:6. When quantifying butyl acetate the amount of internal standard was very little (ratio (1:20-1:60).

To find the best way to calculate the yield/productivity the results obtained for a lower specific substrate flux (75µMol/(g*min)) where the values for yield and productivity are equal for both ways of calculation can be taken in account.

For a specific feed flux of 75µMol/(g*min) (same value for both ways of calculating the yield and productivity see Figure 32) the amount of lost reaction solution is in the same order in magnitude as for the other experiments where big differences between the values obtained by the two different methods were noticed (10%-25% see Table 10).

Table 10: Comparison of different yields/productivities and loss of reaction solution for experiments carried out under supercritical conditions.

Feed flux [µMol/(g*min)]	Productivity [µMol/(g*min)]	Yield [%]	Productivity [µMol/(g*min)]	Yield [%]	Loss [mass %]
	(by formed butyl acetate)		(by used n-butanol)		
1302	39	3,0%	203	15,6%	13%
552	34	6,3%	145	26,2%	25%
939	32	3,6%	171	18,2%	13%
285	25	8,8%	87	30,7%	28%
75	53	70%	54	73%	26%

This shows that hypothesis one i.e. the effect of over and underestimation by loss of product plays only a minor role and that there is another reason for the difference between the values obtained by the two different methods of calculation.

As stated in chapter C.6.3 “Synthesis with enzymes” the experiment at a specific substrate flux of 75µMol/(g*min) was carried out with a higher amount to beads (20 g) to obtain a low specific substrate flux and high yields/productivities.

The difference between the two ways of calculation only occurred when low amounts of product (butyl acetate) were obtained. When high yields (between 40 and 70%) were obtained the amount of substrate (n-butanol) and product (butyl acetate) at the exit of the reactor were in the same order in magnitude. Thus the preparation and analysis of one sample was sufficient to determine the yield/productivity whereas at low yields the difference between the amount of butyl acetate and n-butanol in the reaction solution was too high to quantify both compounds with one analysis. As already stated when analysing the amount of butyl acetate the ratio of internal standard to reaction solution was very little (1:20-1:60) compared to the analysis of butyl acetate (1:3-1:6). Thus hypothesis two i.e. difference of yield/productivity due to sample preparation appears to be more plausible.

As a result of weighing and analysis errors the measured value for the amount of butyl acetate is probably less accurate than the value of n-butanol. This makes the values for yield/productivity calculated by the difference of n-butanol at the entry and the exit of the reactor more plausible.

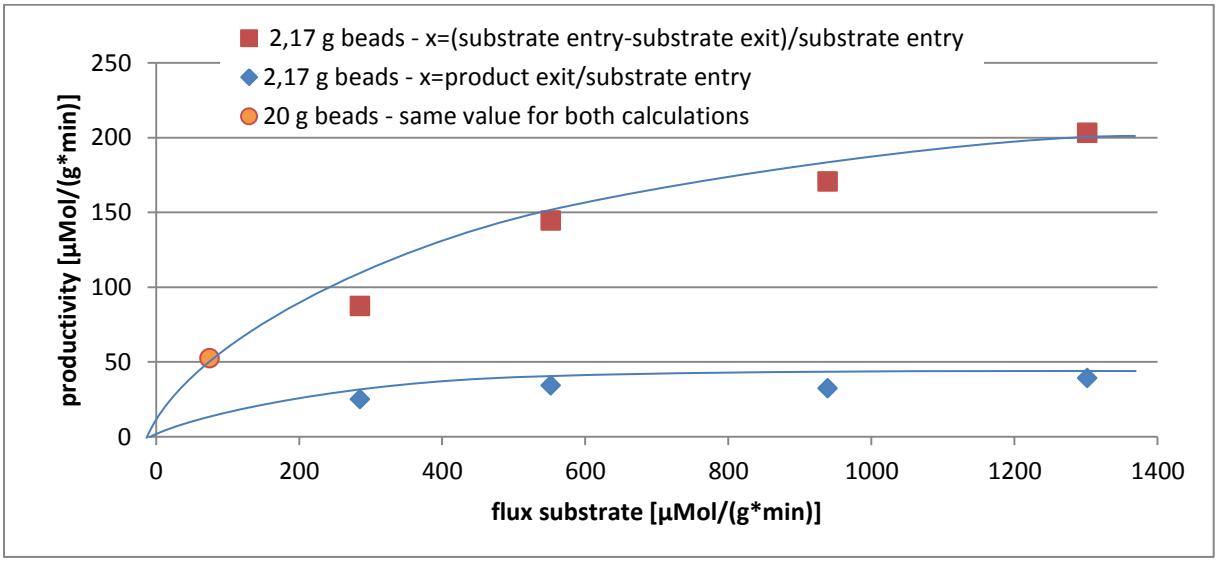

Figure 32: Comparison of values of productivity obtained by two different calculation methods as a function of different specific substrate flux of n-butanol (50°C; 100 bar; 1,8 kg CO_2/h; molar ratio vinyl acetate: n-butanol - 3:1)

In addition Figure 32 shows that the data point at a specific substrate flux of 75 µMol/(g*min) which is reliable fits better on the curve of productivity calculated by the used

substrate at the end of the reactor and not on the curve of productivity calculated by measured product (butyl acetate) at the exit of the reactor.

This proves that the values for productivity obtained by measuring the amount of used substrate at the exit of the reactor is more plausible. In the following experiments all stated yields and activities were calculated by the amount of used substrate at the exit of the reactor.

When looking at the evolution of productivity as a function of specific substrate flux in Figure 32 (red squares) it appears that the productivity is increasing as a function of the specific substrate flux. That proves that until a specific substrate flux of 1300 $\mu Mol_{n\text{-}butanol}/(g*min)$ there is substrate limitation. However, a trend of levelling off can be seen for the experiments carried out at higher fluxes.

Thus an activity higher than 200$\mu Mol_{butyl\ acetate}/(g_{beads}$*min) can be estimated for the beads with immobilised enzymes under supercritical conditions regarding their activity to synthesise butyl acetate.

C.6.5.Long term experiments

Evolution of productivity over eight hours

As the effect of temperature and pressure on CALB immobilised enzymes on ceramic beads is not known the evolution of productivity of in course of time was tested in experiment lasting 8h.

The experiment was carried out under standard conditions (50°C, 100 bar, 1,8kg CO_2/h) in the small packed bed reactor with 2,17 g beads and a substrate flow of 280$\mu Mol_{n\text{-}butanol}/(g*min)$.

Figure 33 and Figure 34 show the measured yield and activity in course of time.

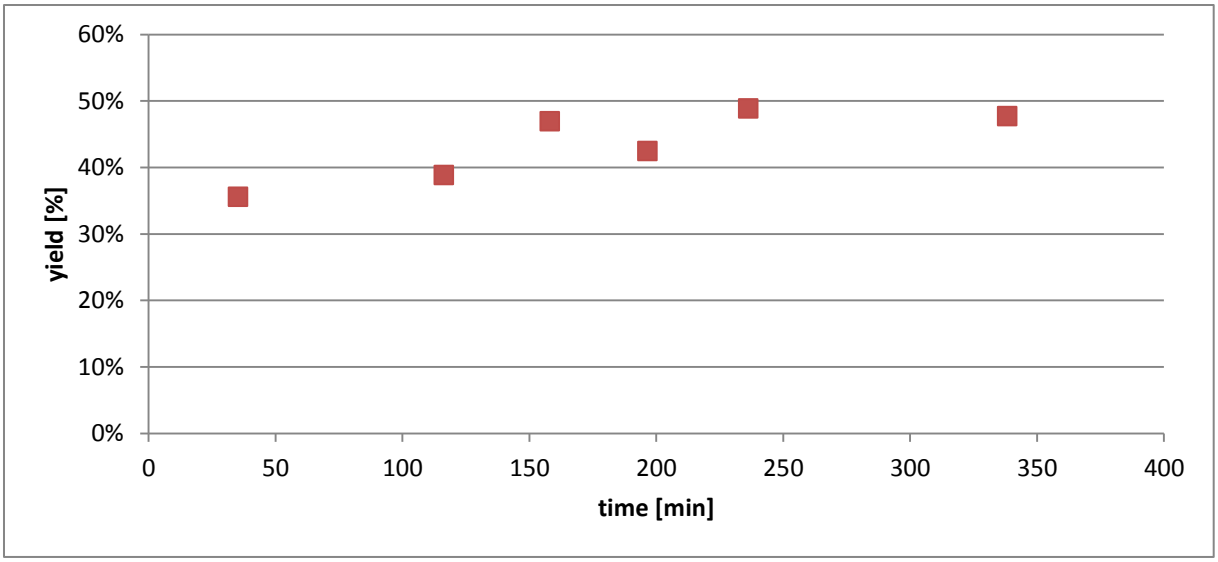

Figure 33: Evolution of yield as a function of time (50°C; 100 bar; 1,8 kg CO_2/h; 2,17g beads; 280 $\mu Mol_{n\text{-}butanol}/(g_{beads}*min$; 840$\mu Mol_{vinyl\ acetate}/(g*min)$)) – 8 hours

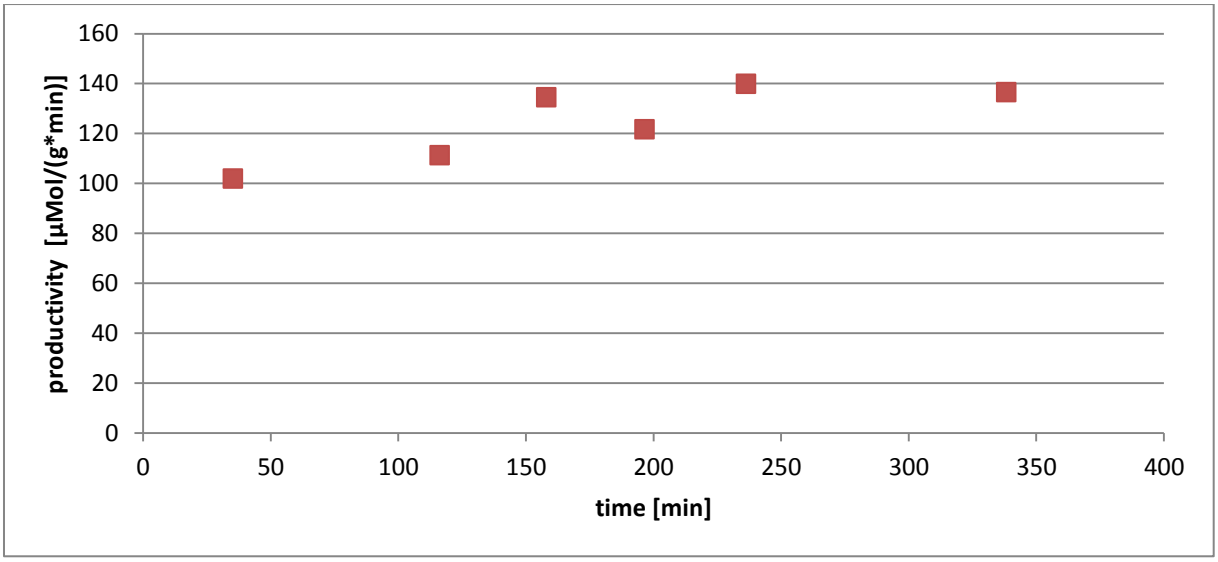

Figure 34: Evolution of productivity as a function of time (50°C; 100 bar; 1,8 kg CO_2/h; 2,17g beads; 280 $\mu Mol_{n\text{-}butanol}/(g_{beads}*min$; 840$\mu Mol_{vinyl\ acetate}/(g*min)$)) - 8 hours

As can be seen in Figure 33 and Figure 34 it takes about 1-2 hours until the fluxes are stable and the productivity stays constant. After two hours the productivity does not decrease when using the immobilised enzymes under supercritical conditions for almost eight hours. Reactions could not be carried out during the night limiting this experiment to eight hours. But similar results were obtained by Ben Ameur (Ben Ameur 2012) who observed that there is no loss of activity when using CALB immobilised on a ceramic support for several days and CALB is known in literature to be a very stable enzyme that can be used thousands of hours without losing its activity (Heldthansen, Ishii et al. 1989). This experiment also shows that no

desorption of the enzymes take place in sc-CO_2 when immobilising enzymes on gamma alumina through adsorption.

Effect of depressurisation on productivity of enzymes

The effect of depressurisation on the productivity of CALB immobilised on gamma alumina is not known. In the following experiment the beads were used under standard conditions, depressurised and stored at reaction temperature overnight. The next day the productivity of those beads was reanalysed to evaluate whether the change of pressure affects the productivity of the immobilised enzymes.

After the experiment described in "Evolution of productivity over eight hours" the beads were stored in the packed bed reactor under atmospheric pressure over night at 50°C. The next day the system was re-pressurised and synthesis of butyl acetate was carried out under the same conditions as the day before (50°C; 100 bar; 1,8 kg CO_2/h; 2,17g beads; 280 $\mu Mol_{n\text{-}butanol}/(g_{beads}*min$; 840$\mu Mol_{vinyl\ acetate}/(g*min)$). The obtained values for the yield and the activity were plotted as a function of time (see Figure 35 and Figure).

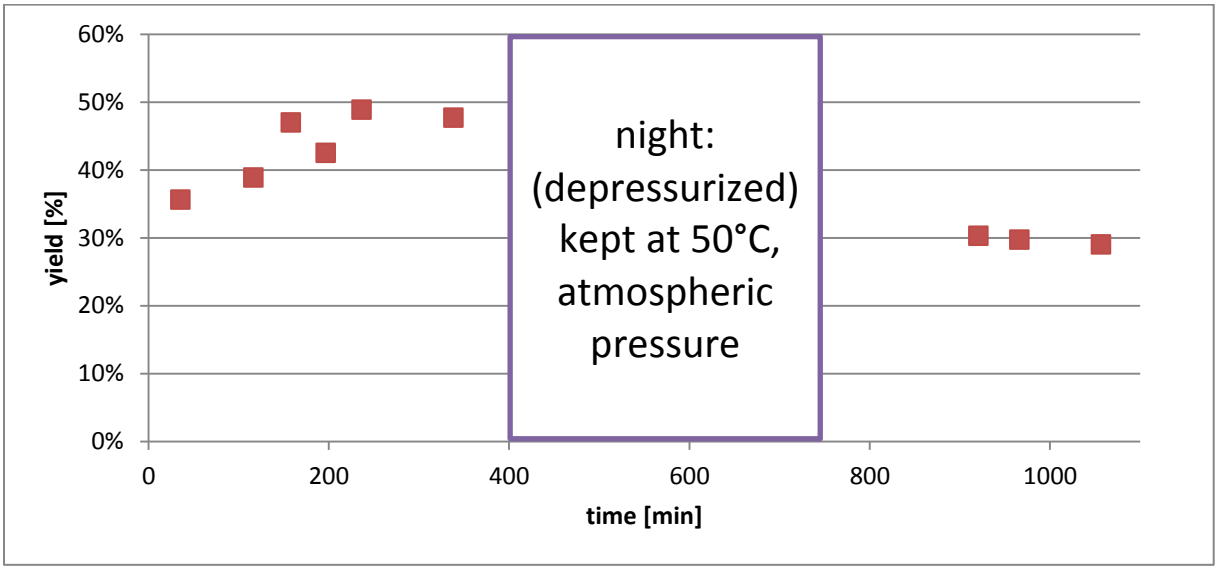

Figure 35: Evolution of yield as a function of time (50°C; 100 bar; 1,8 kg CO_2/h; 2,17g beads; 280 $\mu Mol_{n\text{-}butanol}/(g_{beads}*min$; 840$\mu Mol_{vinyl\ acetate}/(g*min)$)) – 2 days with depressurisation

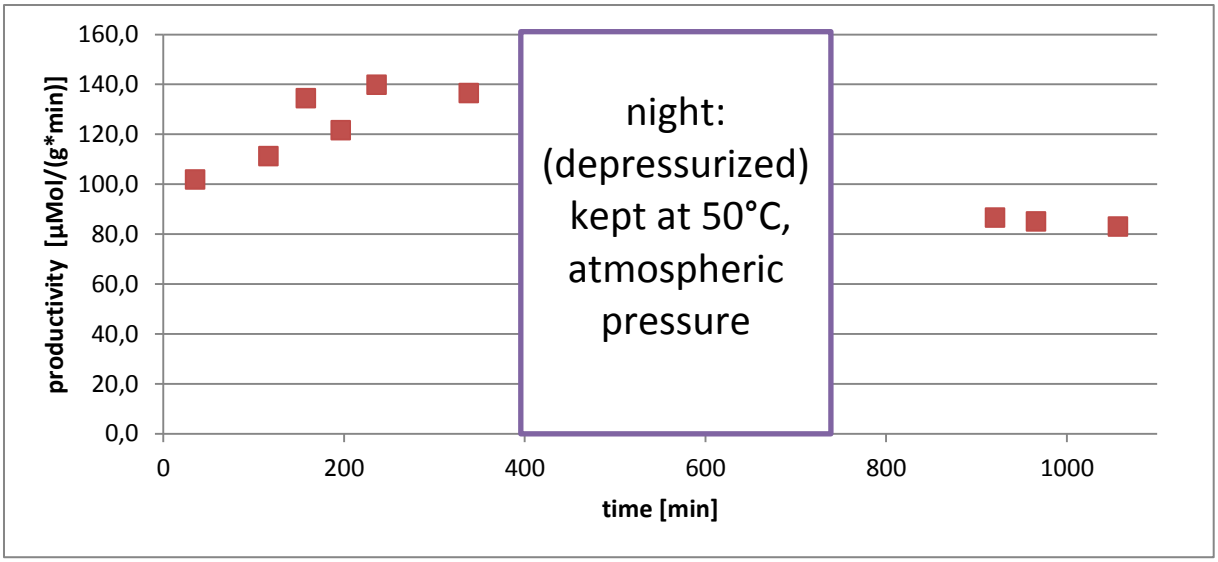

Figure 36: Evolution of productivity as a function of time (50°C; 100 bar; 1,8 kg CO_2/h; 2,17g beads; 280 $\mu Mol_{n\text{-}butanol}/(g_{beads}*min$; 840$\mu Mol_{vinyl\ acetate}/(g*min)$)) – 2 days with depressurisation

Figure 35 and Figure 36 show that the measured yield and productivity was lower at the second day compared to the first day (45% loss of productivity). That indicates that enzymes immobilised on gamma alumina are sensitive to pressure changes and that their productivity decreases after depressurisation.

When working with immobilised enzymes immobilised on acrylic resin (Novozym 435) enzymes are reported not to lose their activity after six cycles of depressurisation and storage at room conditions over night.

Dijkstra et al. (Dijkstra, Merchant et al. 2007) also reported no significant activity loss after depressurising four times when working with cross linked enzyme aggregates of *Candida Antarctica* lipase B.

From previous work at the IEM it is known that enzymes immobilised on a ceramic membrane lose their activity after depressurisation. Apparently enzymes are more sensitive to pressure changes when adsorbed on a ceramic support.

As the experimental setup did not allow keeping the beads under supercritical conditions during the night and as a sufficient amount of beads with immobilised enzymes was available new beads were used for every experiment.

Discussion storage time and water content of beads

To investigate the effect of storing the beads for a different time in a desiccator after immobilisation serveral experiments were carried out.

After immobilising the enzymes on beads they were dried and stored in a desiccator. When storing the beads after immobilisation for different time intervals in the desiccator before using them, yield and productivity were measured. Figure 37 and Figure 38 show the yield and the activity of the immobilised enzymes plotted against the specific substrate flux when storing the beads for two and four weeks

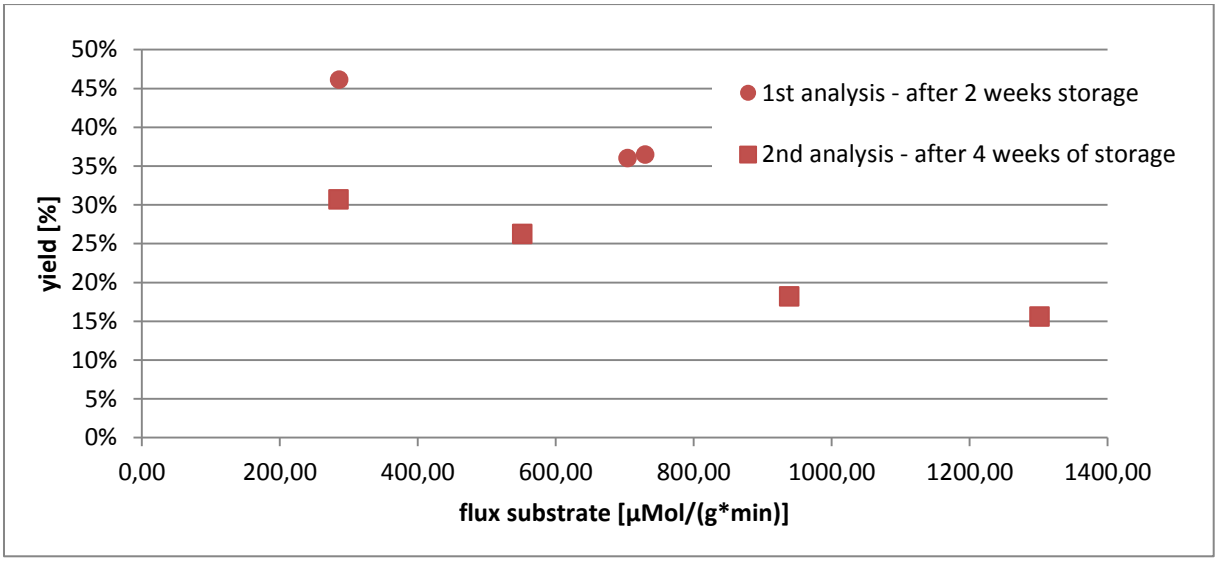

Figure 37: Evolution of yield after storing beads for 2 respectively 4 weeks in a desiccator at different substrate fluxes as a function of time (50°C; 100 bar; 1,8 kg CO_2/h; 2,17g beads)

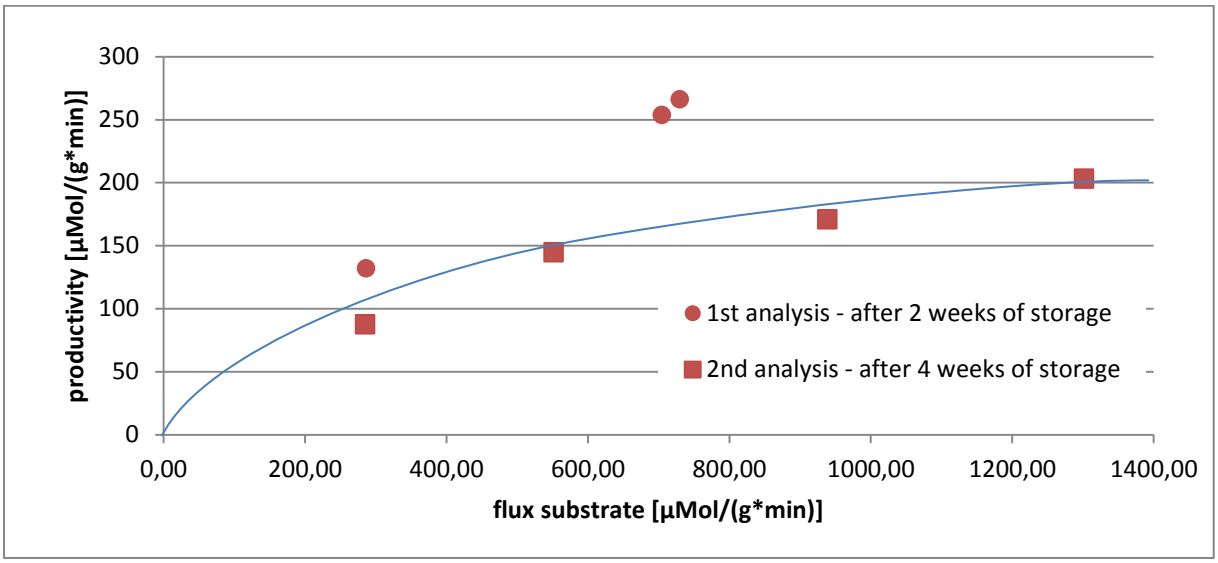

Figure 38: Evolution of yield after storing beads for 2 respectively 4 weeks in a desiccator at different substrate fluxes as a function of time (50°C; 100 bar; 1,8 kg CO_2/h; 2,17g beads)

Figure 37 and Figure 38 show that yield and activity for the beads stored for two weeks are higher than those obtained when the beads were stored for four weeks.

For the interpretation of these results it is important to know that after two weeks of storing the beds in a desiccator they were not yet completely dry (weight did not stay constant while storing in desiccator) but due to time limitation they were analysed.
There are two possible explanations for the change in productivity.

- The beads lose their activity after being stored for a certain time
- The enzymes were dehydrated and lost their activity as a result of insufficient water content.

It is rather improbable that the enzymes lose their activity after being stored for four weeks. Ben Ameur studied the effect of storing enzymes immobilised in a ceramic membrane. She tested the membrane before and after storing it at room conditions for 10 days and no negative effect on the activity was observed.

A more plausible explanation is the effect of dehydration of the enzymes that leads to a decreased activity. As reported in literature the enzyme acquires higher structural flexibility leading to increased catalytical activity when the hydration level is increased (Pirozzi and Greco 2004). Chowdary and Prapulla (Chowdary and Prapulla 2002) also stated that the hydration level of the enzyme has a large impact on its activity and that only small variations in water activity can significantly change the catalytic activity and selectivity of an enzyme.

This shows that the water activity of the enzymes is a crucial parameter for their productivity and that there is a lot of optimisation potential. The water activity can be influenced by the extent of drying but also by the water content of substrates that are added.
There are several options of controlling the water activity in a non-aqueous media. Salt hydrates and molecular sieves can be used to keep the amount of free water constant but water concentration can also be controlled offline by drying the substrates or adding water to the reaction medium. (Dijkstra, Merchant et al. 2007)
Further experiments have to be carried out to study the effect of water activity and find optimum conditions.

C.6.6.Comparison of different packed bed reactors

Experiments were carried out in two different reactors in order to be able to vary the amount of beads: a small one (13cm length, 8 mm diameter) and a big one (120cm length, 2cm diameter). The small reactor was always filled with about 2,2 g beads. The big reactor had a maximum capacity of about 150 g beads. As described in chapter B.6.1 the big reactor

was not always completely filled with ceramic beads with immobilised enzymes. The needed amount of beads with immobilised enzymes was filled into the reactor and the remaining volume was filled up with glass beads without enzymes. The results of the experiments with different reactor types and different amounts of beads are summarised in this section.

Figure 39 and Figure 40 show the yield and the productivity of the experiments carried out in different packed bed reactors with different amounts of beads plotted against the specific substrate flux.

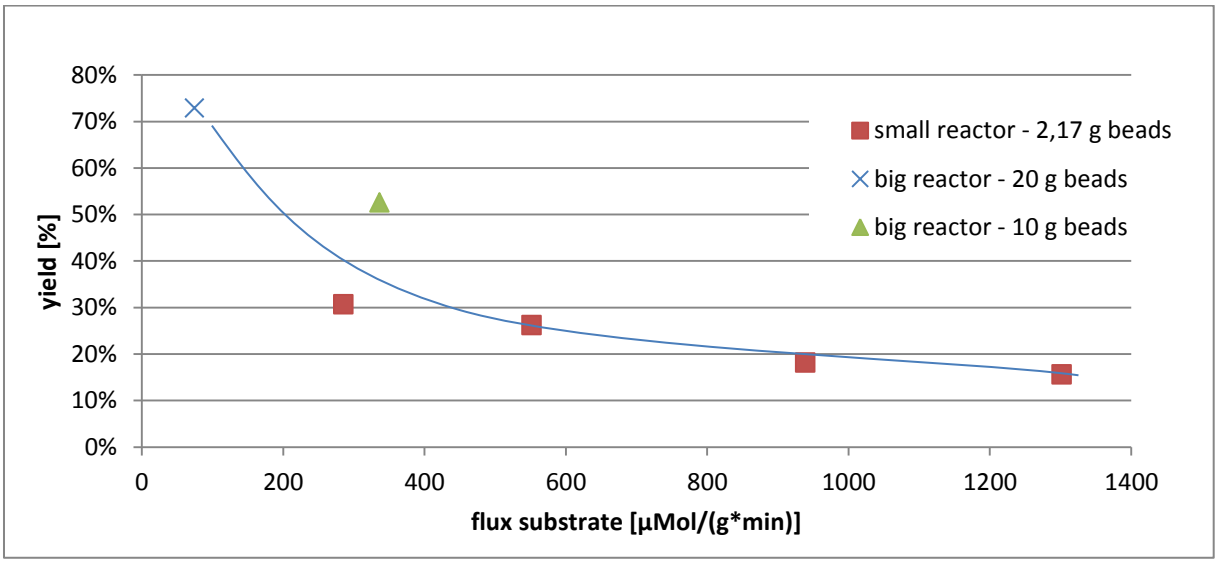

Figure 39: Comparison of yields as a function of specific substrate flux in different packed bed reactors (50°C, 100 bar, 1,8kg CO2)

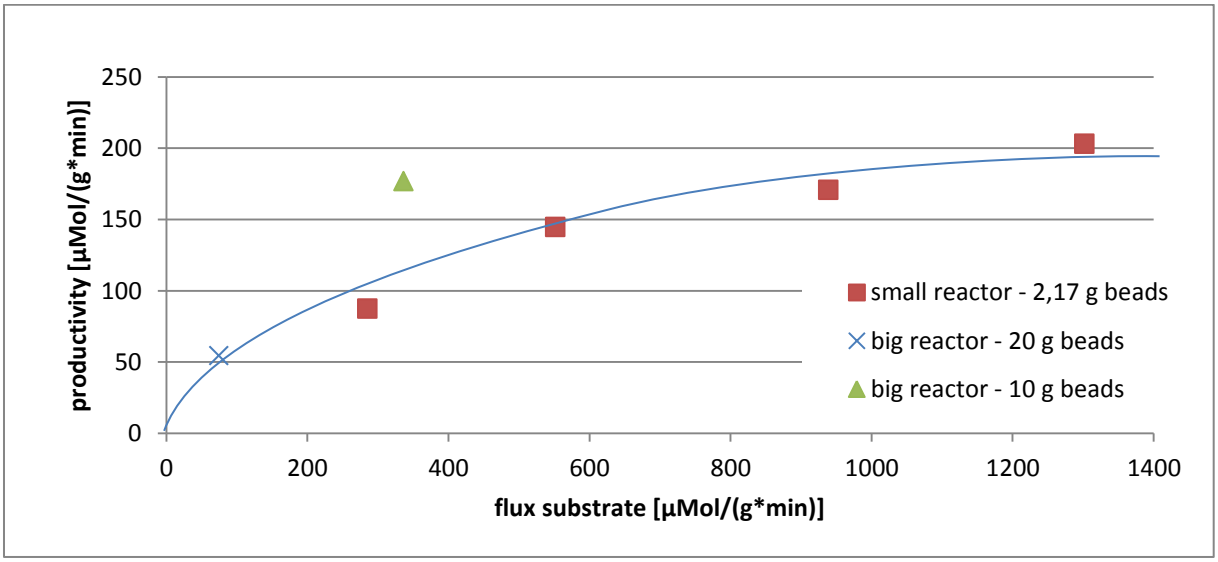

Figure 40: Comparison of productivity as a function of specific substrate flux in different packed bed reactors (50°C, 100 bar, 1,8kg CO_2)

Figure 39 and Figure 40 show that the yields and productivities obtained for the two different reactors do not vary a lot. For the experiment carried out with 10 g beads in the big packed bed reactor slightly higher yields and activities were measured. This can be a result of the following aspects.

The reactor volume per gram beads in the big reactor (2,66mL/g_{beads}) is slightly larger than in the small packed bed reactor (2,31mL/g_{beads}). Thus the beads in the big reactor are not as compacted as in the small reactor and enzymes might be better accessible for substrates. In addition residence time in the big reactor is longer and can be a reason for the slightly higher yield and productivity.

When using the big packed bed reactor the substrates have to pass the bed of glass beads before entering the enzymatic packed bed. This assures perfect homogenisation and can also contribute to better results of the big packed bed reactor.

C.7.Comparison of synthesis in hexane vs. sc-CO_2

In this work the synthesis of butyl acetate catalysed by immobilised CALB was studied in hexane and in sc-CO_2. Preliminary experiments were carried out in hexane as it is easier to handle compared to experiments in sc-CO_2 which had to be carried out in the pilot plant of industrial scale at a pressure of 100bar. The results gained by these preliminary experiments were used for choosing reaction conditions under supercritical conditions. The activity of synthesis of butyl acetate was evaluated for the immobilised enzymes in both solvents. Figure 41 shows the measured productivity in hexane and in sc-CO_2 under the following conditions:

Hexane: 40°C; 12 g beads; 20-420 $\mu Mol_{n\text{-}butanol}/(g_{beads}*min)$; 60-1260 $\mu Mol_{vinyl\ acetate}/(g_{beads}*min)$

Sc-CO_2: 50°C; 100 bar; 2,17 g beads; 75-1300$\mu Mol_{n\text{-}butanol}/(g*min)$; 225-3900 $\mu Mol_{vinyl\ acetate}/(g_{beads}*min)$

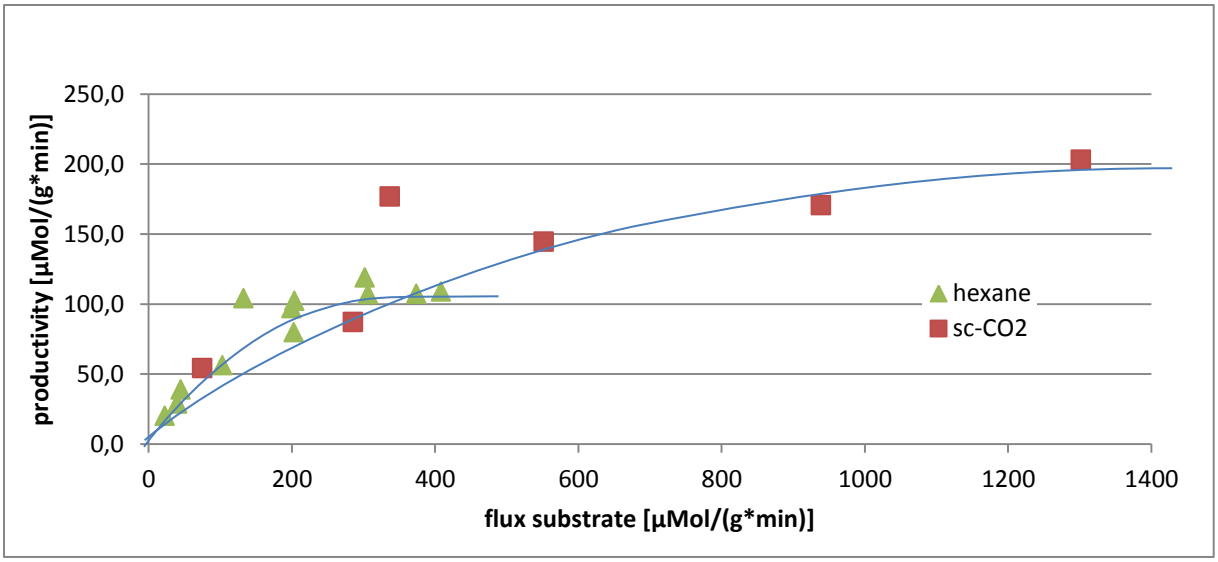

Figure 41: Comparison of enzyme activity under liquid and supercritical conditions as a function of specific substrate flux

Figure 41 shows that the activity of the same beads under critical conditions (about 200µMol/(g*min)) is almost twice as high compared to the activity in hexane (110 µMol/(g*min)). Substrate limitation can be observed in hexane until a specific substrate flux of about 250 $\mu Mol_{n\text{-}butanol}/(g*min)$ whereas in supercritical CO_2 it can be seen until a specific substrate flux of about 1300 $\mu Mol_{n\text{-}butanol}/(g*min)$.

This shows that CALB works more effectively under supercritical conditions compared to the conventional organic solvent hexane. Apart from the increased activity working with sc-CO_2 this solvent brings numerous advantages as already stated in chapter A.4.

D. Conclusion and perspective

The goal of this master thesis was to develop a process to synthesise a flavour that can be used for food industry with an enzymatic packed bed reactor under supercritical conditions.

In the first part of this work a flavour had to be chosen and a way to immobilise the enzymes (*Candida antarctica* lipase B) on an inorganic support had to be found. Butyl acetate was chosen as it is permitted as food additive by the US Food and Drug Administration, has a pleasant flavour (pineapple, tropical), can be analysed easily by gas chromatography and has a suitable solubility in sc-CO_2.

Adsorption was chosen as immobilising technique being simple and effective and the enzymes were immobilised on gamma alumina beads. After preliminary experiments where

adsorption followed by precipitation was tested the immobilisation conditions were optimized.

In the second part of this study the beads were characterised in three different media.

At the beginning hydrolysis of butyl acetate in aqueous conditions was chosen as model reaction to screen the immobilisation efficiency when developing a protocol of immobilisation. The productivity of hydrolysis of butyl acetate was monitored by keeping the pH constant and measuring the needed amount of NaOH to neutralise the formed acetic acid. Simple adsorption was found to be as affective but easier to handle as adsorption followed by precipitation and it was also verified that no desorption of immobilised enzymes takes place in aqueous media using this immobilisation technique. After immobilisation of the enzymes the beads were dried in a desiccator and it was discovered that it takes up to several days until the beads are rehydrated again which is necessary to reach their optimum performance in aqueous conditions.

Then the beads were characterised in packed bed reactors in aqueous medium, in an organic solvent and in supercritical carbon dioxide (sc-CO_2).

In aqueous conditions the beads with immobilised enzymes were characterised regarding their activity to hydrolyse butyl acetate. Substrate limitation could be observed until a substrate flow of about 500 $\mu Mol_{butyl\ acetate}/(g_{beads}*min)$ and an activity of 14-15 µMol/(g*min) was measured when rehydrating the beads for three days in phosphate buffer.

When using hexane and sc-CO_2 as solvent the beads were characterised regarding their activity of synthesis of butyl acetate from vinyl acetate and n-butanol. In order to monitor the productivity of the beads a gas chromatography method had to be developed. Different solvents were tested with an optimum using dichloromethane. By optimising the temperature programme better resolution and nicer peak shapes were obtained. Furthermore an analysis could be carried out in less than 20 minutes.

The activity of CALB immobilised on ceramic beads in organic solvent was about 110 µMol/(g*min) regarding synthesis of butyl acetate while in sc-CO_2 an activity higher than 200 µMol/(g*min) was measured.

When working with CALB immobilised on macroporous resin (Novozym 435) swelling of the beads leading to a high pressure drop was observed in previous works. As in this study the

enzymes were immobilised on ceramic beads the pressure drop of the packed bed was small (1-2 bar) and stayed constant.

The evolution of activity of the immobilised enzymes under supercritical conditions was studied over a period of 8h and the effect of depressurisation of the enzymes was studied. No loss in activity was observed during an experiment of eight hours but after depressurisation the activity decreased by a percentage of 45%.

The effect of drying of the beads was also studied and it was found out that a certain water activity is necessary for the enzymes to obtain their three dimensional structure and to stay active.

Two packed bed reactors with different dimensions were tested and a slight increase of activity was observed when using the big reactor. This can be attributed to the different reactor geometry, different residence time and better homogenisation of substrates.

A lot of results were obtained in this work. Due to the complexity of this study there are still a lot of aspects that may need further investigation.

The effect of water activity has to be studied as it affects the activity of the enzymes in supercritical conditions a lot. Beads with different water content can be tested and different amounts of water can be added by the feed pump. The stability of the beads for several days can also be verified. Finally the reaction conditions such as substrate ratio, maximum substrate concentration without precipitation, temperature and pressure can be optimised.

The developed process works very well under supercritical conditions and shows higher yield and productivities compared to organic solvents. Furthermore working with enzymes in sc-CO_2 as solvent is a very sustainable and innovative process being environmentally friendly and reducing amounts of waste.

Still it is questionable whether butyl acetate will be produced by this method in the future as the chosen substrates are rather expensive compared to the value of the product. Though with this work many effects of enzymatic ester synthesis under supercritical conditions could be studied and the chosen reaction can be seen as a model reaction.

Thus products of great value can be synthesised by changing the substrates. Similar reaction conditions as found in this study can be used to produce those substances in great demand,

especially long chained esters that are used as flavours. Furthermore CALB is also known to catalyse the synthesis of regio- and enantioselective products thus allowing production of compounds that are not accessible by classical chemical synthesis or usually require multi-stage synthesis.

E. Table of illustrations

References

- Almeida, M. C., R. Ruivo, C. Maia, L. Freire, T. C. de Sampaio and S. Barreiros (1998). "Novozym 435 activity in compressed gases. Water activity and temperature effects." Enzyme and Microbial Technology **22**(6): 494-499.
- Ben Ameur, S. (2012). "Conception et étude d'un réacteur enzymatique á membrane fonctionnant en milieu supercritique." PhD thesis at the "Ecole Nationale Supérieure de Chimie de Montpelier".
- Berger, R. G. (2009). "Biotechnology of flavours-the next generation." Biotechnology Letters **31**(11): 1651-1659.
- Cao, L. (2005). "Carrier-bound immobilized enzymes: principles, applications and design." Weinheim: Wiley-VCH, 1st ed., 53-168.
- Cheung, H., R. Tanke and G. P. Torrence (2011). "Acetic Acid." Ullmann's Encyclopedia of Industrial Chemistry, Weinheim: Wiley-VCH.
- Chowdary, G. V. and S. G. Prapulla (2002). "The influence of water activity on the lipase catalyzed synthesis of butyl butyrate by transesterification." Process Biochemistry **38**(3): 393-397.
- Christensen, M. W., L. Andersen, T. L. Husum and O. Kirk (2003). "Industrial lipase immobilization." European Journal of Lipid Science and Technology **105**(6): 318-321.
- de Castro, H. F., P. C. de Oliveira, C. M. F. Soares and G. M. Zanin (1999). "Immobilization of porcine pancreatic lipase on celite for application in the synthesis of butyl butyrate in a nonaqueous system." Journal of the American Oil Chemists Society **76**(1): 147-152.
- Dijkstra, Z. J., R. Merchant and J. T. F. Keurentjes (2007). "Stability and activity of enzyme aggregates of Calb in supercritical CO2." Journal of Supercritical Fluids **41**(1): 102-108.
- Eissier, R. and J. P. Friedrich (1988). "Estimation of supercritical fluid-liquid solubility parameter differences for vegetable oils and other liquids from data taken with a stirred autoclave" Journal of the American Oil Chemists Society **65(5)**: 764-767.
- Fahlbusch, K., F. Hammerschmidt, J. Panten, W. Pickenhagen, D. Schatkowski, K. Bauer, D. Garbe and H. Surburg (2003). "Flavors and Fragrances." Ullmann's Encyclopedia of Industrial Chemistry, Weinheim: Wiley-VCH.
- Gaffar, R., S. Kermasha and B. Bisakowski (1999). "Biocatalysis of immobilized chlorophyllase in a ternary micellar system." Journal of Biotechnology **75**: 45-55.
- Gandhi, N. N. (1997). "Applications of lipase." Journal of the American Oil Chemists Society **74**(6): 621-634.
- Garcia, S., N. M. T. Lourenco, D. Lousa, A. F. Sequeira, P. Mimoso, J. M. S. Cabral, C. A. M. Afonso and S. Barreiros (2004). "A comparative study of biocatalysis in non-conventional solvents: Ionic liquids, supercritical fluids and organic media." Green Chemistry **6**(9): 466-470.

- Ghaziaskar, H. S. and M. Nikravesh (2003). "Solubility of hexanoic acid and butyl acetate in supercritical carbon dioxide." Fluid Phase Equilibria **206**(1-2): 215-221.
- Habulin, M., S. Sabeder, M. Paljevac, M. Primozic and Z. Knez (2007). "Lipase-catalyzed esterification of citronellol with lauric acid in supercritical carbon dioxide/co-solvent media." Journal of Supercritical Fluids **43**: 199-203.
- Heldthansen, H. P., M. Ishii, S. A. Patkar, T. T. Hansen and P. Eigtved (1989). "A New Immobilized Positional Nonspecific Lipase for Fat Modification and Ester Synthesis." ACS Symposium Series **389**: 158-172.
- The Good Scent Company, "Database about properties of flavours and fragances", date of access 2013/01/03, http://www.thegoodscentscompany.com.
- Kamat, S., G. Critchley, E. J. Beckman and A. J. Russell (1995). "Biocatalytic Synthesis of Acrylates in Organic-Solvents and Supercritical Fluids: III. Does Carbon-Dioxide Covalently Modify Enzymes?" Biotechnology and Bioengineering **46**(6): 610-620.
- Kirk, O. and M. W. Christensen (2002). "Lipases from Candida antarctica: Unique Biocatalysts from a Unique Origin." Organic Process Research & Development **6**: 446-451.
- Knez, Z., S. Kavcic, L. Gubicza, K. Belafi-Bako, G. Nemeth, M. Primozic and M. Habulin (2012). "Lipase-catalyzed esterification of lactic acid in supercritical carbon dioxide." Journal of Supercritical Fluids **66**: 192-197.
- Liaw, E. T. and K. J. Liu (2010). "Synthesis of terpinyl acetate by lipase-catalyzed esterification in supercritical carbon dioxide." Bioresource Technology **101**(10): 3320-3324.
- Liu, K. J. and Y. R. Huang (2010). "Lipase-catalyzed production of a bioactive terpene ester in supercritical carbon dioxide." Journal of Biotechnology **146**(4): 215-220.
- Lozano, P., A. B. Perez-Marin, T. De Diego, D. Gomez, D. Paolucci-Jeanjean, M. P. Belleville, G. M. Rios and J. L. Iborra (2002). "Active membranes coated with immobilized Candida antarctica lipase B: preparation and application for continuous butyl butyrate synthesis in organic media." Journal of Membrane Science **201**(1-2): 55-64.
- Lozano, P., G. Villora, D. Gomez, A. B. Gayo, J. A. Sanchez-Conesa, M. Rubio and J. L. Iborra (2004). "Membrane reactor with immobilized Candida antarctica lipase B for ester synthesis in supercritical carbon dioxide." Journal of Supercritical Fluids **29**(1-2): 121-128.
- Martins, A. B., N. G. Graebin, A. S. G. Lorenzoni, R. Fernandez-Lafuente, M. A. Z. Ayub and R. C. Rodrigues (2011). "Rapid and high yields of synthesis of butyl acetate catalyzed by Novozym 435: Reaction optimization by response surface methodology." Process Biochemistry **46**(12): 2311-2316.
- Minovska, V., E. Winkelhausen and S. Kuzmanova (2005). "Lipase immobilized by different techniques on various support materials applied in oil hydrolysis." Journal of the Serbian Chemical Society **70**(4): 609-624.

- Mori, M., R. G. Garcia, M. P. Belleville, D. Paolucci-Jeanjean, J. Sanchez, P. Lozano, M. Vaultier and G. Rios (2005). "A new way to conduct enzymatic synthesis in an active membrane using ionic liquids as catalyst support." Catalysis Today **104**(2-4): 313-317.
- Oliveira, M. V., S. F. Rebocho, A. S. Ribeiro, E. A. Macedo and J. M. Loureiro (2009). "Kinetic modelling of decyl acetate synthesis by immobilized lipase-catalysed transesterification of vinyl acetate with decanol in supercritical carbon dioxide." Journal of Supercritical Fluids **50**(2): 138-145.
- Olsen, T., F. Kerton, R. Marriott and G. Grogan (2006). "Biocatalytic esterification of lavandulol in supercritical carbon dioxide using acetic acid as the acyl donor." Enzyme and Microbial Technology **39**(4): 621-625.
- Pirozzi, D. and G. Greco (2004). "Activity and stability of lipases in the synthesis of butyl lactate." Enzyme and Microbial Technology **34**(2): 94-100.
- Radzi, S. M., W. A. F. Mustafa, S. S. Othman and H. M. Noor (2011). "Green Synthesis of Butyl Acetate, A Pineapple Flavour via Lipase-Catalyzed Reaction." World Academy of Science, Engineering and Technology **59**: 677-680.
- Randolph, T. W., H. W. Blanch, J. M. Prausnitz and C. R. Wilke (1985). "Enzymatic Catalysis in a Supercritical Fluid." Biotechnology Letters **7**(5): 325-328.
- Romero, M. D., L. Calvo, C. Alba, M. Habulin, M. Primozic and Z. Knez (2005). "Enzymatic synthesis of isoamyl acetate with immobilized Candida antarctica lipase in supercritical carbon dioxide." Journal of Supercritical Fluids **33**(1): 77-84.
- Serra, S., C. Fuganti and E. Brenna (2005). "Biocatalytic preparation of natural flavours and fragrances." Trends in Biotechnology **23**(4): 193-198.
- Sheldon, R. A. (2007). "Enzyme immobilization: The quest for optimum performance." Advanced Synthesis & Catalysis **349**(8-9): 1289-1307.
- Varma, M. N. and G. Madras (2007). "Synthesis of biodiesel from castor oil and linseed oil in supercritical fluids." Industrial & Engineering Chemistry Research **46**(1): 1-6.

F. Appendix

F.1.Calibration data for gas chromatography

F.1.1.n-Butanol

Table 11: Data of n-butanol (But) calibration with octanol (Oct) as internal standard

sample	m(But)	m(oct)	$\frac{m(Oct)}{m(n-But)}$	A(But)	A(oct)	$\frac{A(Oct)}{A(n-But)}$
DJW040	0,5271	0,5016	0,952	771,1	749,6	0,972
DJW042	0,5271	0,5016	0,952	367,5	361,9	0,985
DJW043	0,4935	0,2948	0,597	729,4	442,8	0,607
DJW044	0,3076	0,2064	0,671	471,1	310,6	0,659
DJW068	0,3501	0,5039	1,439	646,8	934,0	1,444
DJW069	0,2545	0,4994	1,962	487,7	954,5	1,957

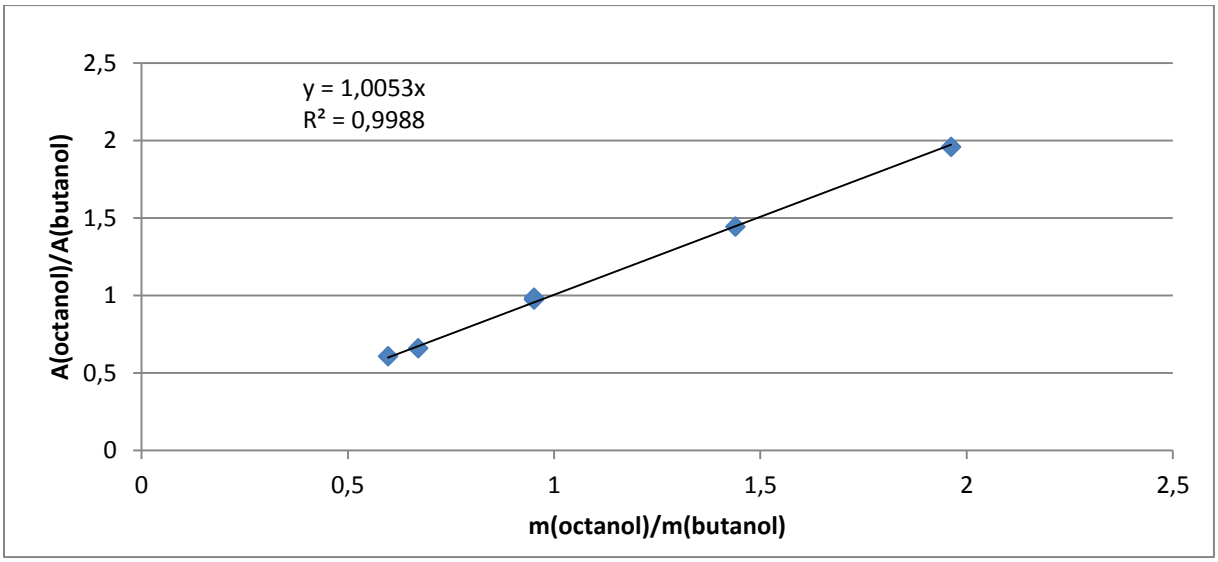

Figure 42: Calibration n-butanol

The slope of the calibration was determined to be **1,0053** with R^2=0,9988

F.1.2.Butyl acetate

Table 12: Data of butyl acetate (But-Ac) calibration with octanol (Oct) as internal standard

sample	m(But-Ac)	m(Oct)	$\frac{m(Oct)}{m(But-Ac)}$	A(But-Ac)	A(Oct)	$\frac{A(Oct)}{A(But-Ac)}$

DJW029	0,5021	0,5062	1,008	614,6	856,7	1,394
DJW030	0,504	0,2822	0,560	720,8	527,7	0,732
DJW031	0,504	0,2822	0,560	616,9	426,7	0,692
DJW032	0,2501	0,5428	2,170	323,8	935,5	2,889
DJW033	0,5144	0,456	0,886	559,6	668,9	1,195
DJW034	0,5166	0,2501	0,484	663,1	446,8	0,674
DJW068	0,3497	0,5039	1,441	487,5	934,0	1,916
DJW069	0,2809	0,4994	1,778	406,2	954,5	2,350

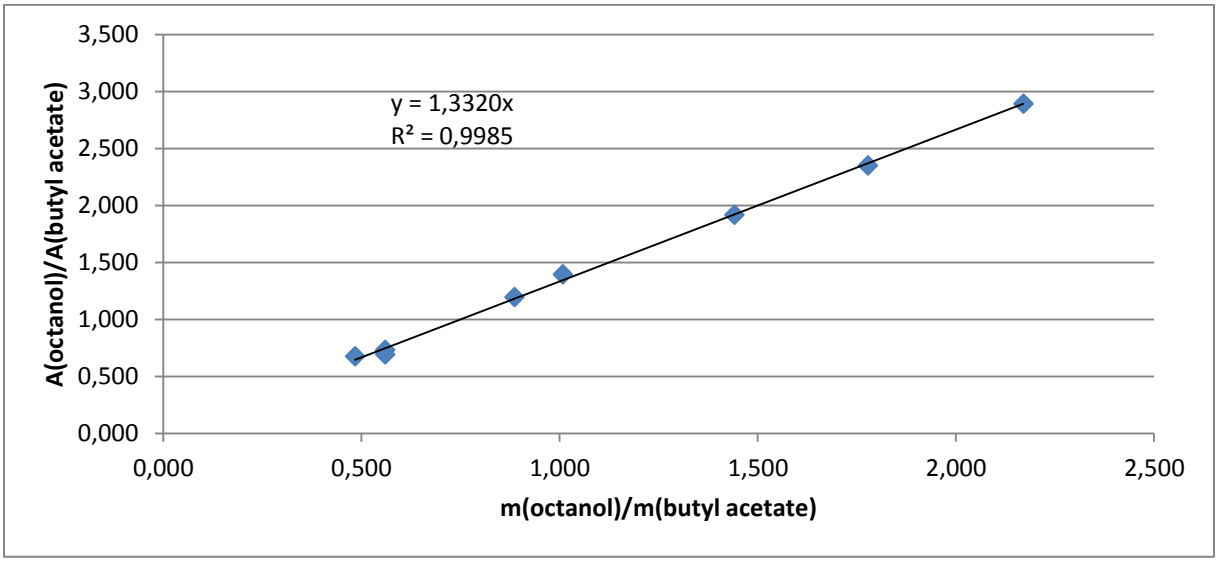

Figure 43: Calibration butyl acetate

The slope of the calibration was determined to be **1,3320** with R^2=0,9985.

F.2.Preparation of solutions for analysis with pH-Stat

In order to measure the enzymatic activity with a pH-Stat solutions were prepared as follows (Ben Ameur 2012):

a) **30mM solution of NaOH** which is added to the solution to be analysed by ph-Stat to maintain a constant pH

b) **20mM solution of oxalic acid** to determine the exact concentration of the 30mM solution of NaOH

c) **10mM phosphate buffer** at pH 7,8 to prepare the substrate solution and to prepare the diluted solutions of enzymes to be added

Preparation of 100mM buffer:

- 42,5 mL of Solution A at 0,2M: 31,2g/L $NaH_2PO_4.2H_2O$
- 475,5 mL of Solution B at 0,2M: 35,6g/L $Na_2HPO_4.2H_2O$
- Fill up to 1L with deionised water

Preparation of 10mM buffer:

- Dilute 100mM buffer 10 times with deionised water
- Verify pH (7,8) after dilution

d) 50mM butyl acetate substrate solution

- Dilute 0,66 mL of butyl acetate with 5 mL acetone and fill up to 100mL with phosphate buffer
- Cover bottle with aluminium foil (to prevent auto hydrolysis caused by light) and leave it at 37°C in an ultrasonic bath until homogenisation

e) Diluted solutions of enzymes (1-5mL/L)

- Solutions of 1,2,3,4 and 5µL CALB solution pure/mL buffer have to be prepared