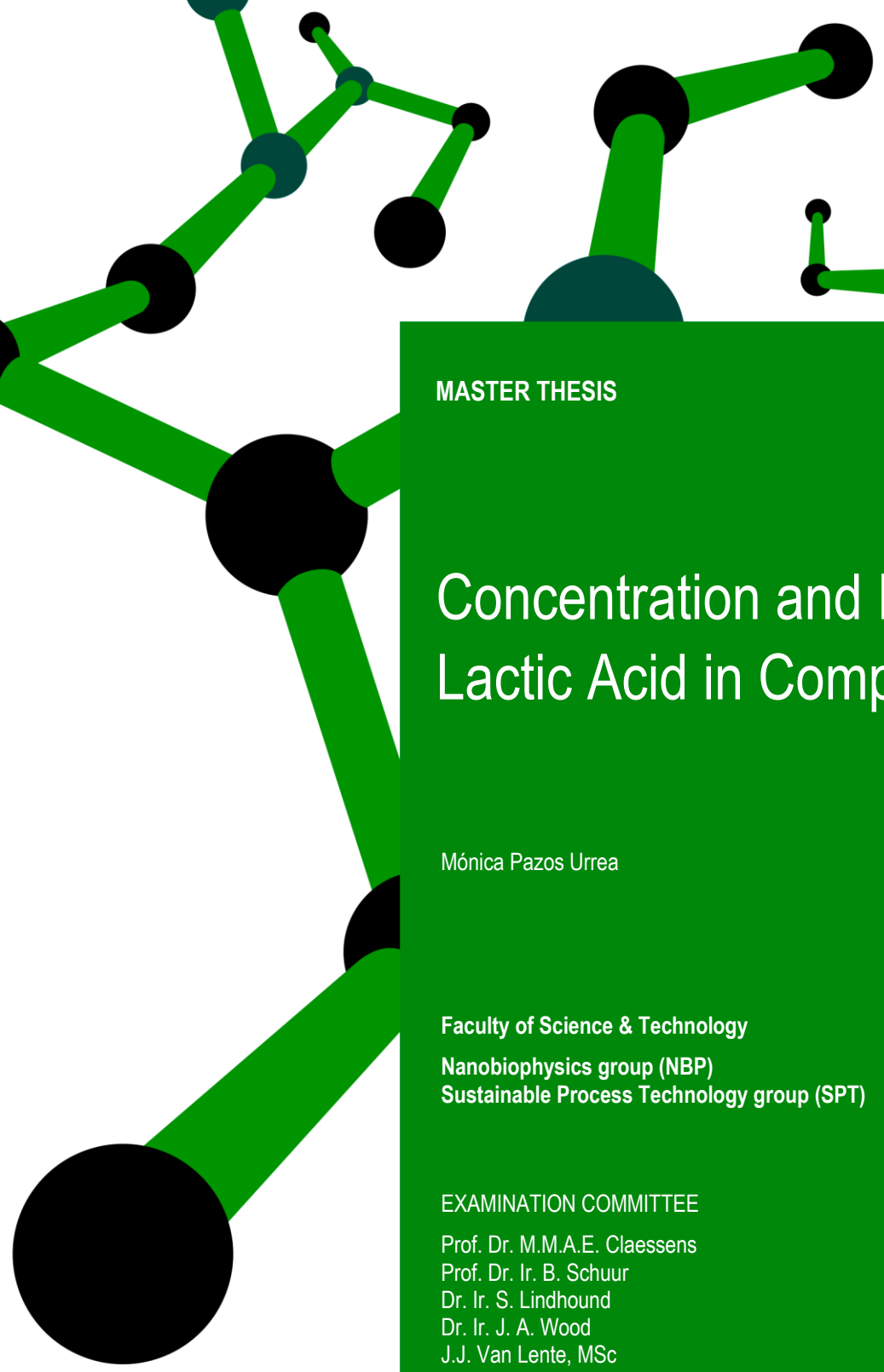

MASTER THESIS

Concentration and Esterification of Lactic Acid in Complex Coacervates

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August 27, 2019

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Abstract

Efficient recovery of lactic acid from the fermentation broth is an area of interest for the industrial production of this organic acid and further applications including the production of biodegradable polymers and green solvents. The viability of complex coacervates to concentrate the acid and further convert it into a valuable compound is evaluated. In the first step, the concentration of lactic acid, butanol, lipases and the ester butyl lactate in complex coacervates is studied. The effect of various parameters such as complex composition, total polyelectrolyte concentration, ionic strength, and temperature on the distribution of the molecules between the dilute phase and complex coacervate phase were discussed. The components presented distribution coefficients greater than one, indicating that the molecules are being incorporated in the complex coacervates. The factor with the greatest influence on the enzymes and lactic acid incorporation was the ionic strength while for butanol distribution the temperature presented the more substantial effect.

In the second step, the esterification of lactic acid with butanol was performed in the presence of an enzyme-filled complex coacervate. The results indicate that lactic acid can be converted to butyl lactate at a low yield (7-9%) at the conditions evaluated (50°C, 170 hours). These experiments represent the first approach to assess this possible application of complex coacervates. Several factors can be explored to improve the ester production, such as an increase in enzyme concentration, increase in temperature or higher concentration of the reagents.

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Contents

List of figures

Abbreviations

LA	**L**actic **A**cid
PLA	**P**oly-**L**actic **A**cid
PBT	**P**oly-**B**utylene **T**erephthalate
PPL	**P**orcine **P**ancreas **L**ipase
CALA	**A**-**l**ipase from ***Ca****ndida antarctica*
CALB	**B**-**l**ipase from ***Ca****ndida antarctica*
PAA	**P**oly(**A**crylic **A**cid)
PEI	**P**oly(**E**thylen**i**mine)
PAH	**P**oly(**A**llylamine **H**ydrochloride)
K_D	**D**istribution coefficient
pI	**I**soelectric **P**oint
IC	**I**on **C**hromatography
TGA	**T**hermogravimetric **A**nalysis

Chapter 1. Introduction

Nowadays, most of the polymer materials used are derived from petrochemical sources. The quest for renewable materials has become a research horizon in the chemistry field to shift a fossil-based society towards a sustainable and carbon-neutral society. In line with this ideal, polylactic acid (PLA), a biodegradable polyester derived from renewable resources, is presented as a promising substitute to replace the standard petroleum-based polymeric materials[1].

The general synthesis of PLA is a combination of fermentative production of lactic acid (LA) from biomass with chemical polymerization, based on two main routes; direct LA condensation or ring opening polymerization from esters of the acid[2]. Both methods require high purity monomers, high temperatures and vacuum for the reaction conditions, besides, while the first one produces a low molecular weight polymer due to equilibrium limitations, the second one usually, contaminates the polymer produced due to the usage of toxic heavy metals based catalyst[3].

Thus, not only the lactic acid recovery from the fermentation broth becomes a variable of concern for the PLA production, but so does searching for an efficient process to obtain high purity LA at low production cost to be competitive in the polymer market. But also, the introduction of alternative processes to decrease the environmental impact in the production of PLA.

The possibility of producing biodegradable polyesters through polycondensation catalyzed by enzymes, including lipases and cutinases, have been studied for the last thirty years[4]. Enzymatic polymerization is presented as an environmentally friendly process that involves high selectivity, low boiling solvents and milder reactions conditions such as low temperatures, neutral pH and atmospheric pressures[5].

An extensive application of enzymes as bio-catalyst for polymer synthesis is hindered by the low stability in chemical reactors, limiting its usage in industrial applications[6]. Nonetheless, immobilization of enzymes with water-insoluble particles, gels, or hollow fibers enhances the enzyme stability, recyclability, and reuse; offers a different path for enzymes utilization[4].

Recently, it has been demonstrated that complex coacervates, liquid phases formed by oppositely charged macromolecules, can incorporate proteins up to high concentrations[7] while retaining their catalytic activity[8] and can easily release them by changing the surrounding conditions, such as increasing the ionic strength[9]. Moreover, small organic acids like acetic acid or butyric acid can be concentrated inside the complex. McCall et al., 2018 reported the spontaneous partitioning and polymerization of the cytoskeletal protein actin inside complex coacervates, demonstrating the possible application of coacervates as bioreactors for studying biomolecular reactions[10].

With these findings, it is interesting to evaluate the viability of using the enzyme-filled complex coacervate as a reaction media to concentrate the lactic acid from a fermentation broth, and further enzymatically polymerize it to PLA. Assuming that by the disintegration of the complex coacervate the polyelectrolytes and the protein remain in the solution whereas PLA precipitates, being easily recovered (Figure 1.1). This approach will significantly simplify the production of PLA, combining the recovery and transformation of LA, introducing a greener process.

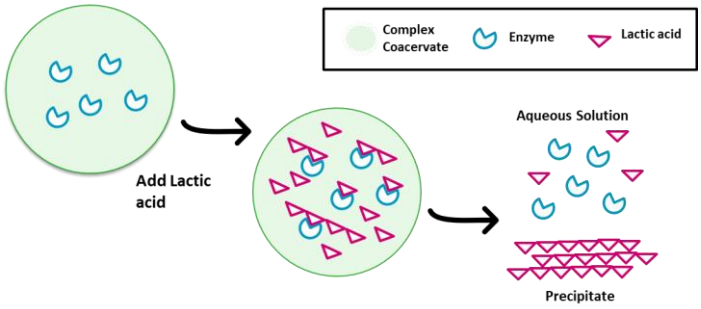

Figure 1.1. Proposed method to produce PLA from extracted LA

The formation of PLA is based on an esterification reaction. Therefore, this project will evaluate a simplified version of the previously stated overview: the formation of an ester inside the enzyme-filled complex coacervate. For this purpose, this research will assess the extent at which LA can be concentrated inside the complex coacervates, as one of the primary aims — followed by the evaluation of an esterification reaction between LA and butanol to obtain butyl lactate in a complex coacervate.

This model reaction was selected knowing that esters of lactic acid produced with alcohols of low molecular weight, such as butanol, are considered green solvents: non-toxic and biodegradable. They present excellent solvent properties including high boiling points, low vapor pressures and low surface tensions that could potentially replace the toxic alternatives for a wide range of industrial applications [11]. Hence, this study will provide new insights into the possible extraction of lactic acid with complex coacervates and the formation of a biodegradable ester; that will set an initial background to further development towards PLA production inside complex coacervates.

1.1. Outlook of the Thesis

The prospective study was designed to investigate the possible usage of complex coacervates as extraction media for lactic acid. Taking advantages of the coacervates capabilities to not only extract the acid but transform it into valuable chemicals. The ultimate purpose for long term research would be to reach suitable conditions to produce PLA inside the complex coacervate. Thus, as an initial approach, it is essential to investigate whether the enzymes can catalyse an esterification reaction inside the complex coacervates. The aim of this project was limited to evaluate the possible formation of an ester of lactic acid with butanol inside the complex coacervate.

Initially, the evaluation of the partitioning of the enzymes that will be used as a catalyst for the esterification reaction is evaluated. Therefore, enzyme incorporation is explored to determine suitable conditions to obtain high concentrations inside the complex coacervate.

Subsequently, the LA incorporation in complex coacervates is studied. The effect of different variables such as temperature, compositions, and concentrations are explored to assess at what extent LA can be concentrated inside the complex coacervate. Moreover, butanol and butyl lactate partitioning are evaluated to verify whether these components allocate inside the complex coacervate.

Once the concentrations of the reactants and catalyst are stablished, the evaluation of the esterification reaction takes place. At first, the reaction is studied at conventional conditions in organic

solvents to verify the activity of the enzymes to form esters of the acid. Then, the possible formation of the ester inside the complex coacervate is assessed.

The report has been divided into six chapters. In Chapter 2, a brief overview of the conventional production of LA is presented, giving examples of some alternatives that have been tested to enhance the LA production. Then, the concept of enzymatic polymerization is explained, describing the most relevant enzymes used for esterification reactions. Lastly, an introduction to complex coacervates and their potential applications is described. In Chapter 3, the methodologies followed for the different experimental setups are described, including the materials and equipment for analysis. In Chapter 4, the experiments completed during the period of the thesis concerning the partitioning of the molecules of interest in the complex coacervate are presented, divided into four sections. The first section corresponds to the partitioning of the proteins. The second section offers the partitioning of LA, and the last two sections of this chapter discuss butanol and butyl lactate distribution. Chapter 5 covers the esterification reaction, initially studying this reaction in the presence of organic solvents, proceeding with its evaluation inside complex coacervates. Finally, in Chapter 6, the conclusions of the thesis are exposed, including recommendations for future research.

Chapter 2. Background

In this chapter, the theoretical background of the project is introduced. Initially, an overview of the production of lactic acid is presented. Subsequently, the lactic acid enzymatic polymerization is described, including the most common enzymes used and the reaction pathway. Finally, the formation of the complex coacervates is explained, together with essential findings regarding its possible applications.

2.1. Lactic acid

Lactic acid is an organic acid containing a dual functional group: hydroxyl and a carboxylic acid (Figure 2.1) a characteristic that makes it attractive for the production of many valuable chemicals[12]. It is widely used in food, cosmetic and pharmaceutical industry taking advantages of its chiral structure [13]. Furthermore, it can be used as a feedstock for the production of green solvents and as the building block for the production of a biodegradable polyester: PLA, a promising polymer for the substitution of petroleum-based thermoplastics. PLA presents some comparable properties to conventional polymers, such as gloss, clarity, and processability similar to polystyrene and barrier properties analogous to polyethylene terephthalate. Additionally, its viscosity, purity, and tensile strength make it suitable for applications in the medical field, fibers, and packaging materials[12].

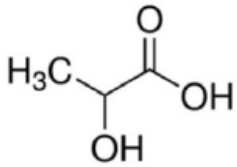

Figure 2.1. Lactic acid structure

LA can be produced via chemical synthesis or microbial fermentation. Acetaldehyde is the starting material for the chemical route (Figure 2.2. Overview of the chemical synthesis of LAFigure 2.2) providing a racemic mixture of LA, where hydrogen cyanide is added in the presence of a base to produce lactonitrile at high atmospheric pressures, followed by distillation to recover the lactonitrile crude, and hydrolysis with concentrated H_2SO_4, to obtain lactic acid and ammonium salt. Furthermore, to achieve a higher purity, the lactic acid reacts with methanol to produce methyl lactate, passing through a distillation step to be further hydrolyzed by water, obtaining lactic acid and methanol[14].

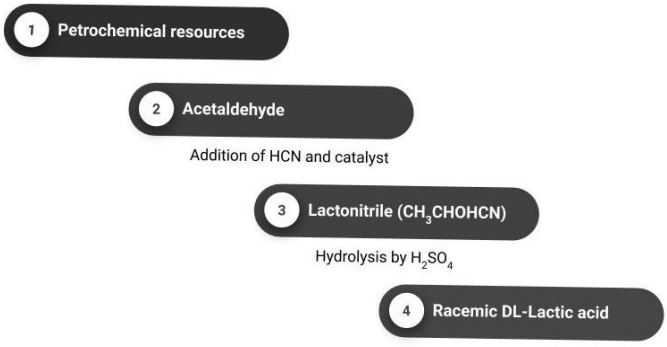

Figure 2.2. Overview of the chemical synthesis of LA

On the other hand, fermentation of renewable feedstock is the preferred path for the production of LA, representing about 90% of the total LA worldwide produced[15]. Beet sugar, molasses, whey, and barley malt are commonly used for its commercial production; nonetheless, different starting materials can be implemented, from dairy wastes containing lactose and sugars to beverage industry wastes containing sucrose, where price and availability play an essential role in its selection[16].

Depending on the feedstock, the process conditions are adjusted, but generally, the fermentation takes place at 35 to 45°C, and the pH is maintained at a constant value between 5 to 6.5 adding a suitable base such as hydroxides, carbonates or ammonia to ensure the performance of the microbial strains[14]. The rate of the fermentation process depends on the pH, temperature, and initial substrate concentration and the reported yield after the fermentation stage is around 90 – 95% based on initial carbohydrates concentration[17].

The fermentation broth contains salts of lactic acid, other organic acids, unreacted materials, nutrients, microorganism, among others. Thus, separation and recovery steps are necessary to achieve the required purity. The conventional process to obtain LA (Figure 2.3) involves the precipitation of calcium lactate, which is reacidified with a strong acid to obtain crude lactic acid[18]. Subsequently, the calcium sulfate is removed by filtration; the filtrate is purified using carbon columns and evaporated to produce technical grade lactic acid.

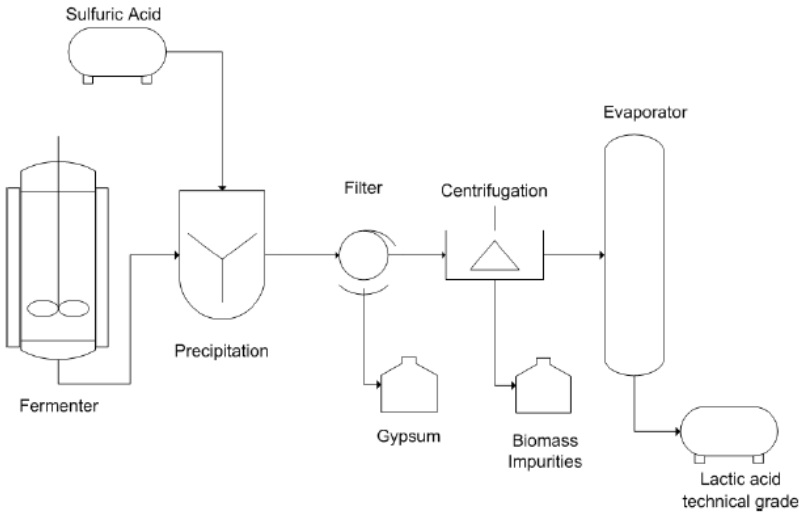

Figure 2.3. Layout of the conventional process for lactic acid recovery by precipitation[19]

Nonetheless, by this conventional recovery process, a large amount of calcium sulfate cake is produced with some organic impurities making it harder to dispose of: approximately one ton of calcium sulfate is produced for every ton of lactic acid produced, generating an environmental concern[12]. This route have made large-scale production economically and environmentally unattractive, creating an opportunity to overcome the separation barriers.

2.2. Downstream process of Lactic acid

It has been a challenge to design an efficient and cost-effective downstream process for LA purification. Not only due to the strong affinity of LA towards water, its low volatility, and its probable decomposition when it is exposed to elevated temperatures, but also due to the presence of different

organic acids in the fermentation broth that present similar properties to LA [20]. Making conventional separation approaches such as distillation or solvent extraction with standard organic solvents unprofitable[21].

To date, several techniques have been tested for the recovery of LA from the fermentation broth looking to reduce production cost, amount of effluents and thus, decreasing the negative impact towards the enviroment[18]. Precipitation, adsorption, solvent extraction, reactive distillation, and membrane separation process are some of the technologies that have been used as an alternative for the conventional process[15].

The introduction of these separation technologies presented benefits, developing a more efficient production of LA; however, still, some drawbacks are reported. In solvent extraction, high distribution coefficients (*Kd*) are required to establish an economically attractive extraction, referring to the ratio of the concentration of LA in the solvent phase to the concentration of LA in the aqueous phase [12].

Preliminary work on the distribution coefficient of LA in aqueous solution and organic solvents has observed a strong dependence on pH and initial concentration of LA, obtaining the highest distribution coefficients at a pH between 5,5 and 5,8 related to the complete deprotonation of LA[21].

Regarding the dissociation behavior of LA in an aqueous solution (2.1), it is completely dissociated at a pH >> pKa = 3,85, meaning only lactate ions are present in the solution[21]. Figure 2.4 illustrates the equilibrium of a lactic acid aqueous solution at a concentration of 100mM, where the concentration of lactic acid [HA] and lactate ions [A-] are calculated as a function of the hydrogen ions, as can be seen in the following equations:

$$HA_{(aq)} + H_2O \Leftrightarrow {H_3O^+}_{(aq)} + {A^-}_{(aq)} \qquad 2.1$$

$$[A^-] = \frac{[HA]_{tot}[H_3O^+]}{[H_3O^+] + K_a} \qquad 2.2$$

$$[HA] = \frac{[HA]_{tot}\, K_a}{[H_3O^+] + K_a} \qquad 2.3$$

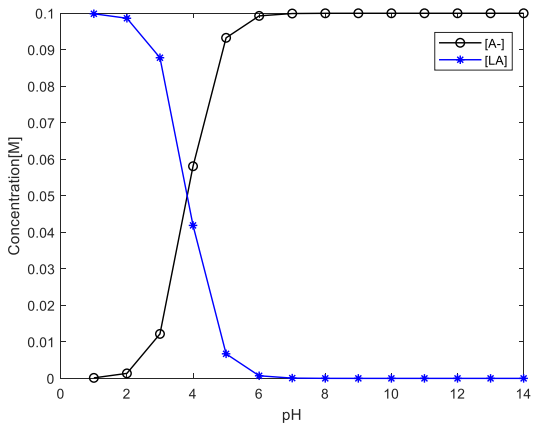

Figure 2.4. Acid-base equilibrium of lactic acid at a concentration of 0,1 M. Concentration of lactate ion [A-]. The concentration of lactic acid [LA] (*adapted from*[21])

Various studies have assessed the efficacy of solvents for LA extraction, with a significant focus in amines, known as effective extractants for carboxylic acids in aqueous solutions[12]. A summary of these findings is presented in Table 2.1, including silica beads with nitrogen-based functionalities[22].

Table 2.1. Solvents tested for LA extraction. (adapted from [12]*)*

Extractant	Diluent	LA initial concentration	Distribution coefficient	Reference
Tri-n-octylamine-Tripropylamine (2:8w/w, 1 mol/kg)	1-octanol/n-heptane (3:7 w/w)	2,3 M (20%w)	0,8	[23]
Tri-n-butyl phosphate	-	2,3 M (20%w)	0,95	[24]
Tri-n- octylamine (1M)	Chlorobutane	2,3 M (20%w)	1,4	[25]
Alamine 336 (0,3 M)	MIBK	1,3 M (11%w)	8,13	[26]
Alamine 336 (50%)	Oleyl alcohol	0,11 M (1%w)	13	[27]
Alamine 336 (40%)	MIBK	1,6 M (14%w)	4,24	[28]
Alamine 336 (30%)	Octanol	0,38 M (3,4%)	25,95	[28]
Alamine 336 (40%)	Decanol	0,76 M (6,8%)	23,37	[29]
Silica beads functionalized with N-N-didodecylpyridin-4-amine	n-Octanol	0,013 M (0,12%)	27	[22]

As can be seen in Table 2.1, there is a potential application for non-conventional solvents with favourable distribution ratios for LA. However, the requirement of expensive equipment to be able to provide a high exchange area for efficient separation and the in-situ application limited by the toxicity of the extractants towards the microorganism, reduce the utilization of liquid-liquid extraction in the industrial process[19].

As far as membrane filtration is concerned, electrodialysis and nanofiltration present promising results, including low energy requirement, low chemical consumption and low effluent generation [13]. It can be implemented in situ to remove the lactic acid continuously from the fermentation broth, maintaining the operational pH. A double electrodialysis process has been developed successfully to produce concentrated lactic acid using an initial electrodialysis unit to remove the multivalent ions followed by a water-splitting unit with bipolar membranes achieving a high recovery yield (> 95%)[30]. Nonetheless, polarization and fouling problems limits the application of electrodialysis on large scale, besides additional process steps should be implemented to achieve lactic acid with high purity for polymerization applications[19].

In terms of obtaining high purity lactic acid, reactive distillation becomes an attractive alternative, where the lactic acid reacts with alcohol, followed by a distillation of the ester and hydrolysis to obtain the free lactic acid and alcohol[12]. Esterification is the only downstream process that allows the separation of lactic acid from other organic acids, due to the differences in boiling points of their ester compounds[20]. Generally, these reactions present thermodynamics limitations, therefore, a large excess of alcohol and rapid removal of water are common practices to obtain high yields (between 60% to 100% - depending on the water removal method implemented). However, the presence of impurities in the feed stream such as residual sugars and proteins affect the performance of the catalyst, cation exchange resins, complicating steady state operation[12].

Thus far, this section has elucidated some of the possible downstream processes that can be used to separate and purify lactic acid from the fermentation broth in industrial processes. Some improvements to the conventional method have been found over the years, but still, there is room to

further investigations to develop a more efficient and economically attractive process to potentialize the production of LA.

2.3. Enzymatic polymerization of lactic acid

In the last two decades, enzymatic polymerization has become a significant synthetic method in polymer chemistry[31]. Not only are enzymes able to catalyse polymerization reactions under mild conditions, such as low temperatures and low boiling solvents, but they promote the formation of good quality polymers due to its high enantio-, chemo- and region-selectivity[4].

These bio-catalysts are derived from renewable resources, being eco-friendly, recyclable and non-toxic materials[3,4,32]. Thus, enzymatic processes are presented as environmentally benign, that can synthesize various polymers with well-defined structures.

Several studies have been reported exploring the applications of the enzymes in polymerization reactions. Lipases are one of the most promising catalysts due to their extensive range of applications[32]. Lipases are a very versatile class of biocatalyst stable in organic solvents and over a wide pH range, and, are specific towards ester bonds, reducing the possibility of the formation of undesired by-products[15].

Enzymatic polyester synthesis can take place via polycondensation of hydroxyacids, illustrated in Figure 2.5 for the production of PLA directly from lactic acid accompanied by the formation of water as a by-product. The polycondensation reactions are determined by equilibrium, where usually the by-product is removed from the reaction zone to drive the equilibrium in the direction of the products[4].

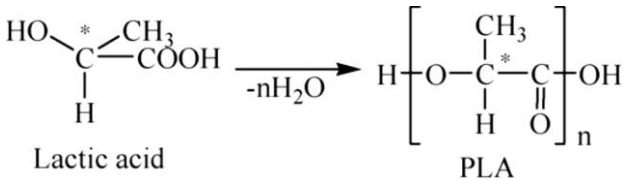

Figure 2.5. PLA formed by polycondensation of LA[33]

Generally, enzymatic production of polyesters takes place in organic solvents such as toluene, heptane, hexane and di-isopropyl ether. The reactions rate for these type of processes are low, taking into account several days to notice a significant conversion of the monomers[34]. A great deal of previous research into polyester synthesis has focused on lipases from mammalian (Porcine Pancreatic lipase) (PPL)) fungal (*Candida antarctica* Lipase B (CALB) or bacterial origin (*Pseudomonas cepacian* PCL)[3].

PPL has a molecular weight of 50-52 kDa; it is a small globular protein composed of 449 amino acids that reach its highest catalytic activity at alkaline pH medium between 7,3 and 9 [35]. Commercially, PPL crude is obtained as a complex mixture of various enzymes, containing other hydrolases: esterases amylases and proteases as impurities in a significant amount[36]. It is one of the most economical sources of lipases when compared to other commercial lipases, thus, it is extensively used for biotransformation reactions. It has been reported that PPL performed satisfactorily in organic environments with low water concentration, presenting thermal stability and high catalytic activity at high temperatures in a range of 20°C to 80°C[35].

Divakar *et al*. (2003) studied the polymerization of lactic acid catalysed by PPL, and two types of immobilized enzymes from *Rhizomucor miehie*, evaluating the influence of the substrate lipase/ratio and the initial concentration of LA on the average molecular weight of the PLA. They observe that PPL was the most suitable catalyst from a molecular weight and conversion point of view, achieving a molecular weight of 1423 Da with an 80,2% conversion at 69°C in hexane: Methyl isobutyl ketone mixture (7:1) [37]. Sonwalkar *et al*. (2003) studied the polycondensation of LA in the organic solvents in the presence of silica gel with PPL as biocatalyst. They noticed that the conversion is higher in the presence of the silica due to the absorption of the water formation by these particles; but more interestingly, they observed that even higher conversion rates could be achieved with only silica gel particles [38]. Lasalle & Ferreira (2008), detected 96% conversion for PPL in the presence of hexane at 60°C for 96 hours. Nonetheless, low molecular weight oligomers were the principal products of the reaction, suggesting PPL is not as effective for the polymerization of LA[3].

Regarding the lipases from *Candida antarctica,* two different lipases are produced CALA (45 kDa) and CALB (33 kDa). CALB is broadly studied in the field of esterification reactions. Both enzymes are stable over a broad pH range, being CALB more stable at alkaline pH while CALA more stable at acidic pH[39].

CALB is a globular protein composed by 317 amino acids. It presents a broad substrate specificity, including regio- and enantioselectivity. Besides, it is extensively used due to its exceptional catalytic activity and versatility for esterification and transesterification reactions, preferred in its immobilized commercial presentation on an hydrophobic carrier for industrial applications, known as Novozym 435 (Novozymes A/S, Copenhagen, Denmark) or Chirazyme L-2 (Roche Molecular Biochemicals, Mannheim, Germany)[40].

Lasalle & Ferreira (2008), observed that CALB was the most effective catalyst for the production of PLA from direct condensation of LA, in comparison with PPL and a lipase from *Pseudomonas cepacia*; achieving 60% of LA conversion and a polymer recovery of 55% in n-hexane at 60 °C for 96h[3]. The majority of the polyesters produced in the presence of CALB present a low molar mass, therefore, are destinated for biomedical applications, mainly for delivery systems[41] Knez *et al.* (2012) investigated the formation of lactate esters by the direct esterification of n-butanol and LA catalyzed by immobilized CALB in supercritical carbon dioxide. They observe the highest LA conversion with n-hexane as co-solvent at 55 °C and 30MPa using a stirred – tank reactor[42]. Moreover, Pirozzi & Greco (2004) studied the esterification of n-butanol and LA comparing different lipase sources. They observe that the optimal temperature for esterification is around 45°C and no esterification activity is detected above 60°C. Furthermore, low concentration of the substrates are preferred to avoid a negative effect of the acid over the enzyme stability. In these experiments octane was used as a solvent and CALB presented the highest esterification activity among the studied enzymes[11].

CALA is stable above 90°C, considered as one of the most thermostable lipases described to date. Another attractive characteristic of this lipase is the specificity towards highly sterically hindered substrates, being able to accept highly branched acyl groups. When it is compared with other lipases, it is the only one able to catalyse very bulky substrates, being recognized as a potential bio-catalyst not fully exploded yet[39].

In Table 2.2, a summary of relevant properties of the enzyme previously described are presented.

Table 2.2. Some properties of lipases from porcine pancreas and candida antarctica[39,43]

	Lipase from porcine pancreas [PPL]	**Lipase B from *candida antarctica* [CALB]**	**Lipase A from *candida antarctica* [CALA]**
Molecular weight [kDa]	50-52	33	42
pH range stability	6,5-9	7-10	6-9
Isoelectric point (pI)	4,9	6	7,5

2.4. Complex coacervates

Polymers are long molecules formed by a large number of reiterating units (monomers) linked by covalent bonds; they are essential units for synthetic materials such as plastic bags or nylons or can be found in nature including rubber, cellulose, DNA, proteins among others. Polymers that contain charged monomers are known as polyelectrolytes, composed of acidic or basic groups or salts that can dissociate in water[44]. They are classified as weak or strong polyelectrolytes, where the pH determines the degree of dissociation for weak polyelectrolytes, while in most of the pH values strong polyelectrolytes are completely dissociated. Figure 2.6 gives the schematic representation of the effect of the pH in the polyelectrolytes charges, where at low pH weak poly-bases are fully charged and at high pH uncharged, presenting the opposite effect for weak poly-acids[45].

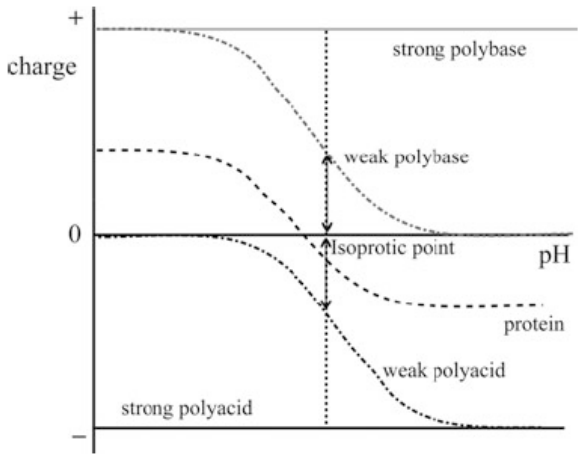

Figure 2.6. Charge of polyelectrolytes as function of the pH [45]

Complex coacervates are dense polyelectrolyte phases that can be formed by mixing two oppositely charged polyelectrolytes in an aqueous solution, leading to liquid-liquid phase separation[9]. The formation of these complexes strongly depends on the molecular architecture, mixing ratio and concentration of the polyelectrolytes; and the environmental conditions such as the ionic strength, temperature and, when weak polyelectrolytes are involved, pH[7,46].

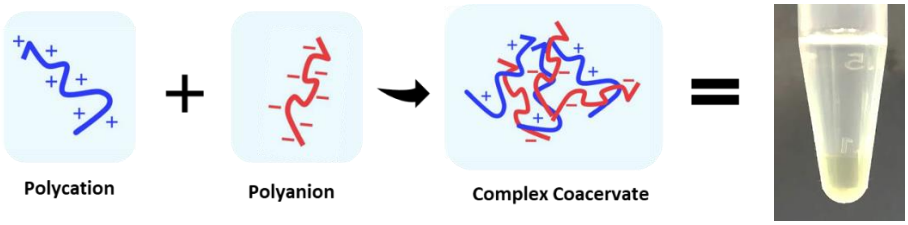

Figure 2.7. Right – Schematic representation of complex coacervate formation in aqueous solutions. Complex coacervate formation by mixing two oppositely charged polyelectrolytes (polyacrylic acid and polyethyleneimine)

The main driving force for polyelectrolyte complexation is believed to be the electrostatic interactions between the oppositely charged polyelectrolytes in water followed by additional molecular interactions such as hydrogen bonding, chirality and hydration in combination with entropic gains associated to the release of counter-ions and restructure of molecules[47,48]. The release of the counter-ions reveal an entropic dependence of the complex formation, expressing a significant effect of overall salt concentration. High salt concentrations destabilize the phase separation by decreasing the entropic driving force for the complex formation[9,48].

The ratio between the positive and negative charges is directly related to the ability of a mixture to form a complex coacervate, seeking to achieve electroneutrality in a given phase. Generally, the formation of a complex coacervate occurs in a range of stoichiometric compositions, obtaining the larger amount of complex around the net neutrality[49]. Therefore, the pH becomes a significant variable when weak polyelectrolytes are taken into account, where the charge present in the polymer is function of the pH.

Furthermore, the temperature has a relatively weak effect on the complex coacervate formation, where only small changes have been detected in the enthalpy and entropy of the complex formation[49,50]. However, it is mentioned that increasing in temperature enhance the hydrophobic interaction and polyelectrolyte dissociation in synthetic polyelectrolytes, thus, it present a larger effect when the total polyelectrolyte concentration is higher[44,51].

2.5. Complex coacervates applications

In previous studies[46,52,53], it has been demonstrated that small molecules can migrate into complex coacervates, mainly due to the low surface tension between the dense polyelectrolyte phase and the diluted phase facilitating the uptake of different component crossing the interphase with a low energetic barrier[46,54]. The ability of complex coacervates to accumulate small molecules expand its possible applications including drug delivery purposes, loading the complex with pharmaceutical compounds[53]; extracting contaminants from aqueous solutions[52] and in the food industry can be used as a protective coating for flavours and oils [55].

Additionally, a great deal of previous research into complex coacervates has focused on the interactions between protein and polyelectrolytes, being proteins weakly charged polyelectrolytes dependent on the pH for its charge density and charge sign[7,10,53,56,57]. Two-component system, the interaction between a protein and an oppositely charged polyelectrolyte or three-component systems where the protein is complexed in a mixture of two oppositely charged polyelectrolytes can

be considered when proteins are taken into account, leading to the formation of a complex coacervate depending on salt concentration and pH[7].

The principal attention on these interactions is that proteins can retain their catalytic activity inside the complex coacervates[9], providing them protection to harsh environmental conditions such as high temperature or proteolysis and enhance stability, when compared to an enzyme in an aqueous solution[58]. Potential applications in the medical field can be explored due to the simplicity, versatile and rapid method to encapsulate proteins and use them as biosensors, therapeutics, and enzyme catalysis[58].

Chapter 3. Experimental procedure

3.1. Materials

Crystalline L-lactic acid was kindly donated by Corbion, the Netherlands. The negatively charged polyelectrolyte poly(acrylic acid) (PAA) (Cat# 06567, MW ~6,000), and the positively charged polyelectrolyte poly(ethylenimine) (PEI) (Cat# 06089, MW ~1,800) were purchased from Polysciences. Sodium Chloride, 1-butanol (analytical reagent grade 99,7%), n-heptane (analytical reagent grade 99%) and n-Butyl lactate (>98% w/w) were purchased from Sigma Aldrich, and ultrapure water dispensed from a Mili-Q water system (PURELAB® flex) at a resistivity of 18,2 MΩ was used in all samples preparations.

Stocks solutions of PAA, PEI, and LA were prepared by dissolving the compounds in ultrapure water. The pH of these solutions was adjusted to 7 with NaOH (Merck) or HCl (Merck) after its total dissolution.

Lipase from porcine pancreas (PPL) was purchased from Sigma Aldrich and used without further purification. A-lipase (CALA) and B-lipase (CALB) from the yeast *Candida antarctica* were donated by Novozymes, Denmark.

Stock solutions of PPL were prepared by dissolving the powder in ultrapure water, the pH of the partly dissolved sample is adjusted to 7 with NaOH or HCl. Subsequently, it is mixed for one hour and then centrifuged for 20 min at 3000rpm (Beckman Coulter BenchTop Centrifuge Allegra), where the precipitate is discarded.

Stock solutions of CALA and CALB were prepared by diluting the sample by a 7-fold dilution for CALA an 14-fold dilution for CALB followed by the addition of NaOH or HCL to adjust the solutions to neutral pH.

3.1.1. Defining complex composition

The complex composition is defined[7,8] according to Equation 3.1, where $[n_+]$ is the concentration of charges on the PEI in solution, and $[n_-]$ is the concentration of chargeable groups of PAA, assuming that both polyelectrolytes are fully charged at pH 7. Thus, the charge of any amount of polyelectrolyte is based on the molecular weight of its composite monomer.

$$F^- = 1 - F^+ = \frac{[n_-]}{[n_-] + [n_+]} \qquad 3.1$$

The molecular structures of the polyelectrolytes used in this project are presented in Figure 3.1. For this case, it is estimated that both polyelectrolytes are fully charged, taking into account eight positive charges for the monomer of PEI with a molecular weight of 288,6 g/mol. While only one negative charge for the monomer of PAA with a molecular weight of 76,74 g/mol.

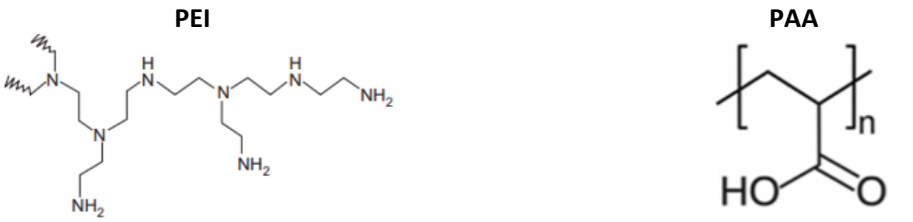

Figure 3.1. Schematic representations of poly(ethylenimine) (PEI) and poly(acrylic acid) (PAA)[59]

Electroneutrality of the mixture is found at 0,5 F-. However, a deviation from this value can be observed experimentally due to the actual number of charges on the weakly charged polyelectrolytes, that is unknown. The charges of these polyelectrolytes are highly dependent on the pH of the system and can be influenced by the presence of another polyelectrolyte in the system, making it challenging to make an accurate estimation[7].

3.2. Methods

3.2.1. Complex coacervates - Partitioning experiments

Complex coacervation leads to the formation of two liquid phases, a dense polymer rich phase (coacervate) and a dilute polymer phase (supernatant) existing in equilibrium. This phase separation can be used to concentrate biomacromolecules such as proteins[7,9,54,60], small organic molecules[46] or, even inorganic nanoparticles[61].

The distribution of lipase, lactic acid, butanol and butyl lactate is studied in this project. Two methods were followed for the calculation of the distribution coefficient of the different molecules, including the evaluation of the effect of specific factors over the partitioning of the components, such as complex composition [F-], total polyelectrolyte concentration, salt concentration, initial component concentration, and temperature.

Method 1

Method 1 is established for the complex coacervate formation and concentration of the small molecules in the complex coacervate phase: lactic acid, 1-butanol, and butyl lactate. It is schematically described in Figure 3.2, using LA as the exemplifying component.

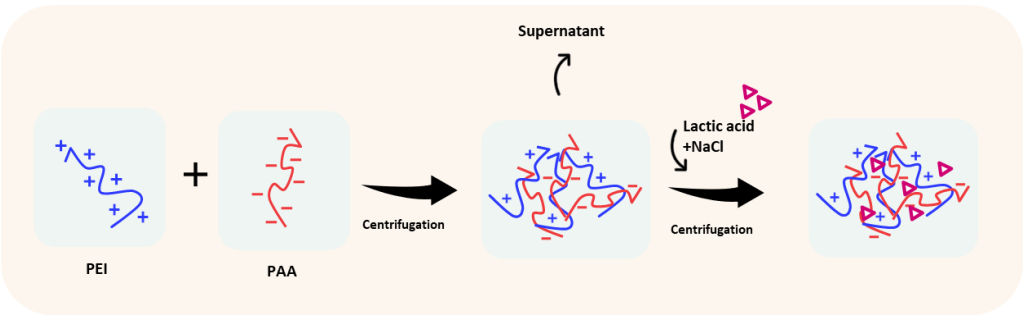

Figure 3.2. Method 1 - Concentration of lactic acid, butanol and butyl lactate in complex coacervates

Initially, the polyelectrolytes solutions are mixed at the desired composition [F-], polyelectrolyte concentration and salt concentration in an aqueous solution reaching a total volume of 500µL. Subsequently, after one night of equilibration, the mixtures are centrifuged using an Eppendorf Centrifuge 5425 for 30 min at 12500 rcf.

Once the complex coacervate is formed, the supernatant is removed and replaced with 500µL of a solution of known concentration containing the molecules of interest i.e. LA, butanol or butyl lactate. Subsequently, the samples are equilibrated overnight and centrifugated one more time for 30 min at 12500 rcf. The concentration of the compounds in the remaining supernatant is measured by ion chromatography or gas chromatography.

The amount of complex coacervate formed in every sample is determined by weighting each sample on an analytical balance (Sartorius BP61S Analytical Balance). To estimate the concentration of the compound of interest in the complex coacervate, the amount of the compound that is incorporated in the complex coacervated is calculated by mass balance, subtracting the total amount of the compound in the solution to the remaining amount present in the supernatant. Additionally, it is assumed that the complex coacervate has the same density as water, due to its water content, that it is roughly between 60 to 80%[50]. The experiments were done in triplicates, and the results are presented as an average with the standard deviation.

Method 2

Method 2 is followed for the incorporation of the bio-macromolecules, i.e., Lipases in the complex coacervate, as can be seen in Figure 3.3.

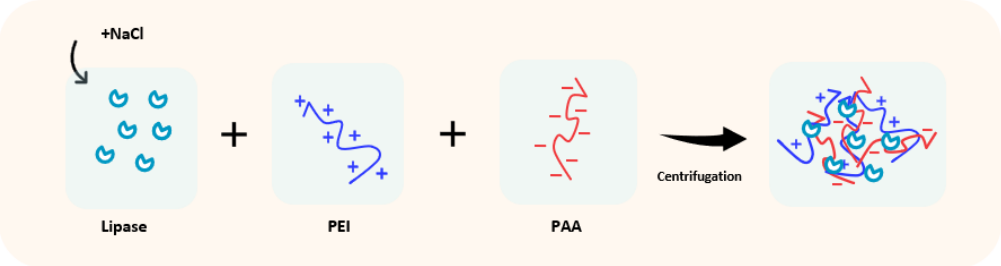

Figure 3.3. Method 2 – Incorporation of lipases inside complex coacervates.

The sample volume for all experiments was fixed at 500µL. The stock solutions were mixed to obtain samples of the desires polyelectrolyte composition, total polyelectrolyte, protein and salt concentrations. Afterwards, the samples were equilibrated at least eight hours, followed by centrifugation for 30 minutes at 12500 rcf (Eppendorf Centrifuge 5425)

The macromolecules were mixed in the following order: ultrapure water, enzyme solution with the desired enzyme and salt concentration, positively charged polyelectrolyte solution and negatively charged polyelectrolyte solution. After the centrifugation, the protein concentration in the remaining supernatant is determined by UV/vis spectrophotometry at 280 nm using the molar extinction coefficient of the lipases. The extinction coefficient of PPL and CALA was calculated according to the

peptide sequence of the enzymes, presented in Appendix B.1; while the extinction coefficient used for CALB is reported by Rabbani *et al*. (2015)[62]. PPL 68715 M^{-1} cm^{-1} (MW = 51,579 kDa)[63], CALA 54570 M^{-1} cm^{-1} (MW=49,262 KDa) and CALB 40690 M^{-1} cm^{-1} (MW = 33 KDa)[62]

Furthermore, the amount of complex coacervate is determined by weighting each sample on a digital scale. The water content of a complex coacervate is roughly between 60 to 80%[50], besides the density of PEI is 1,05 g/L and PAA is 1,15g/L. Therefore, it is assumed that the density of the complex coacervate is the same as water to estimate its volume. The enzyme incorporated inside the complex coacervate is calculated by a mass balance, subtracting the total amount of enzyme present in the supernatant from the total added enzyme. All experiments were done in triplicates, and the results are presented as an average with the standard deviation.

3.2.2. Esterification reaction in an organic solvent

A 2ml sample containing 10mg of the enzyme with 1-butanol and lactic acid in excess of the alcohol is prepared, in a 4:1 ratio. The reagents were diluted in n-heptane at low concentrations (LA 100 mM) to avoid the formation of a two-phase system. The mixtures were incubated for 2 hours in 5-ml glass tubes at 50°C using a magnetic stirrer at 500 rpm, as can be seen in Figure 3.4.

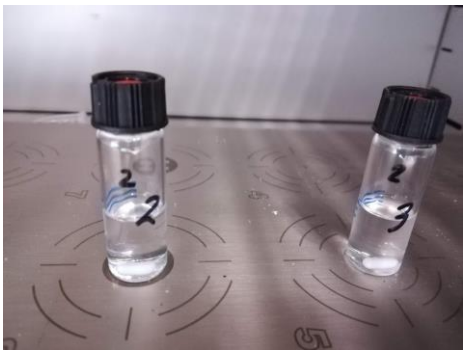

Figure 3.4. Experimental setup for the esterification reaction in the presence of an organic solvent at 50°C and 500rpm.

Butanol and butyl lactate concentrations were measured by a Thermo Scientific Trace 1300 gas chromatograph equipped with a flame ionization detector. One unit of esterification activity of the studied lipases is expressed as one µmol of butanol consumed per mg catalyst per minute [µmolBu/min*mg lipase].

3.2.3. Esterification reaction in the complex coacervate phase

The production of butyl lactate from lactic acid and 1-butanol is studied to determinate if the enzymes are active inside the complex coacervate and are able to catalyse an esterification reaction. To carry out this experiment, the complex coacervate is formed in an aqueous solution containing a known concentration of salt and enzyme by adding the polyelectrolyte solutions at the desired complex composition and polyelectrolyte concentration, as it is described in Method 2 of section 3.2.1

Subsequently, the supernatant is replaced by a solution containing lactic acid, butanol and NaCl at known concentrations. The samples are placed in a Eppendorf ThermoMixer at 50°C and 300rpm for

98 hours, a representation of the procedure is showed in Figure 3.5. The final concentrations of butanol and butyl lactate are determined by Thermo Scientific Trace 1300 gas chromatograph equipped with a flame ionization detector, detecting its presence in each phase: supernatant and complex coacervate. Nonetheless, before the analysis the complex coacervate is dissolved by the addition of 200µL of 2,5M NaCl solution. Every sample analysed by gas chromatography was diluted in a ratio 1:1 with acetone.

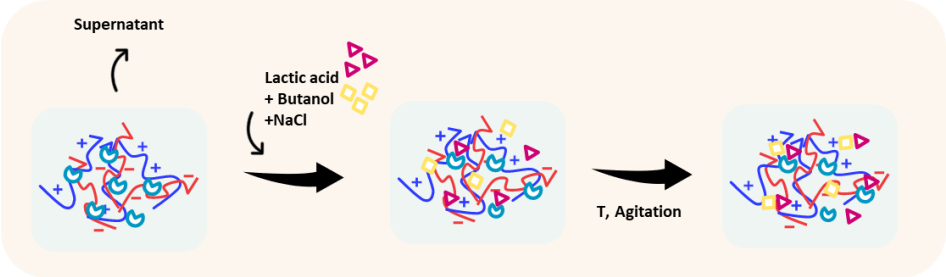

Figure 3.5. Schematic representation of the esterification reaction inside the complex coacervate

The butanol conversion (X) and yield (Y) towards butyl lactate is calculated based on the amount of butanol and butyl lactate that are presented in the sample after the 98 hours of reaction, as can be seen in the following equations:

$$X_{Butanol} = \frac{Initial\ moles_{Butanol} - final\ moles_{Butanol}}{Initial\ moles_{Butanol}} \qquad 3.2$$

$$Y_{Butyl\ lactate} = \frac{Produced\ moles_{\ Butyl\ lactate}}{Initial\ moles_{Butanol}} \qquad 3.3$$

3.2.4. Detection of the components

UV-VIS spectroscopy

The general use of UV-VIS spectroscopy to quantifying the protein concentration in an aqueous solution is seen as a fast and convenient method, where no additional reagents are required. The characteristic ultraviolet absorption of the proteins at 280nm is due to the aromatic amino acids tryptophan, tyrosine, and cysteine contained in their structures. Depending on the relative concentration of these three amino acids, the molar extinction coefficient of the protein is established. Thus, the extinction coefficient is specific of each protein and can be used to quantify its concentration in a solution[64]. Figure 3.6 shows the absorption spectrum of the enzymes studied in this project: PPL, CALB, and CALA at a same concentration illustrating the differences between them.

The absorbance of a component is related to its concentration in the solution and the path length of the solution. This dependence is stablished by the Beer-Lambert law as can be seen in Equation 3.4

$$A = \varepsilon c l \qquad 3.4$$

Where, ε is the molar absorption coefficient, c the molar concentration of the component and l is the path length of the cell containing the solution (generally 1 cm). Thus, once the absorbance of the sample is measured at 280nm, the protein concentration can be calculated using the Beer-Lambert law and the molar extinction coefficient previously determined for the protein[64].

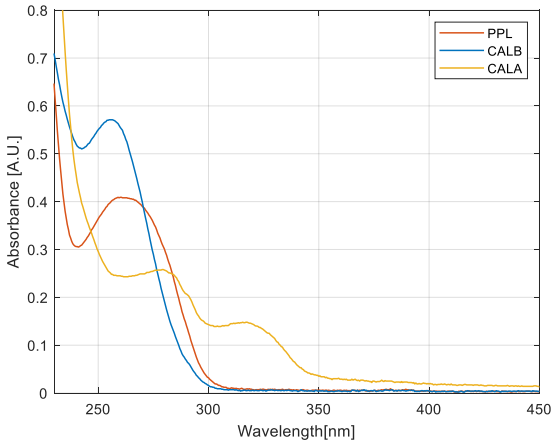

Figure 3.6. Absorption spectrum of PPL, CALB and CALA.

Gas Chromatography

Gas chromatography is an analytical technique generally used for the separation of volatile compounds. It is a widespread technique due to its high efficiency, which allows the separation of the components of a mixture in a reasonable time with accurate quantitation, even for the detection of minimal quantities.

In principle, the sample is injected into the instrument, vaporized and carried by the mobile phase (carrier gas) through the stationary phase contained in a column. The relative vapor pressure and affinities for the stationary phase are the properties responsible for the successful separation of the different components, presenting different retentions time within the analysis. The stronger the interaction is, the more time it takes to migrate through the column (retention time). Then, the detector measures the components at the exit of the column.

This analytical technique was used to measure the butanol and butyl lactate concentration. The calibration curve for each component is presented in Appendix A.

Ion Chromatography

Ion chromatography (IC) is an analytical technique used for the separation of differently charged or ionizable compounds. Electrolytes in its dissociated form are contained in the liquid mobile phase, and functional groups with a fixed charge in an inert organic matrix constitute the stationary phase. The system is electrically neutral due to the presence of the counterions next to the functional groups. The

ionic interactions between the compounds, ions present in the eluent and the ionic functional groups of the chromatographic support are the base for the separation[65].

Two mechanisms play a significant role in the separation in ion chromatography: attraction and repulsion. Ion exchange by attraction occurs due to the competitive ionic binding and repulsion by the ion exclusion between similarly charged ions between the sample and the ions fixed in the stationary phase[65].

This analytical technique is commonly used to determine carboxylic acids of low molecular weight. It offers high detection sensitivity and extensive separation capacity, allowing the separation of a wide range of organic acids[66]. At high pH, the acids are present in their ionized form; thus, they can be separated employing anion exchange chromatography[67].

In anion exchange chromatography, the functional groups of the stationary phase carry a positive charge, while the compounds to be separated, carry a negative charge. When a sample is provided to the column, the ions present in the sample can reversibly replace the counterions bound to the charged functional group on the stationary phase. Thus, its movement throughout the column results at a slower velocity than the mobile phase. The adsorption of the ions to the stationary phase and desorption by the mobile phase ions is repeated within the column, and depending on the individual interactions, the residence time among diverse compounds can differ, allowing the separation[67].

Ion chromatography was used to measure the lactic acid concentration throughout the experimental section of this project. The calibration curve for this analytical technique can be seen in Appendix A.

Chapter 4. Partitioning of molecules inside complex coacervates

In this experimental chapter, the partitioning of the molecules of interest is evaluated. The purpose of this section is to explore the individual interactions between proteins, LA, butanol, and butyl lactate with the complex coacervate, to determine a possible accumulation of these molecules inside the dense-polyelectrolyte phase. The experimental results and discussion are presented in each subs-section, discussing the distribution of the enzymes, LA, butanol and butyl lactate.

4.1. Partitioning of proteins in complex coacervates

Several factors have an effect on the incorporation of proteins within the complex coacervates. In this section, the accumulation of the lipase inside the complex coacervate is studied elucidating significant variables that affect this phenomenon.

Three different lipases, PPL, CALB, and CALA previously studied [3,6,68,69] were selected to evaluate their incorporation into the complex coacervate phase.

First, the purity of the enzymes was evaluated with polyacrylamide gel electrophoresis (SDS-page gel). This technique allows the separation of the proteins according to their molecular weights by applying an electrical field to promote the migration of the proteins through a sieving matrix[70]. The procedure followed for the preparation of the SDS-page gel can be seen in Appendix A.4. A 12% polyacrylamide gel was used according to the molecular weight of the enzymes. Figure 4.1 shows the SDS-page gel for the studied lipases at different concentrations, next to the ladder used to identify the apparent molecular weights (kDa) of the proteins contained in the samples (Thermo Scientific PageRuler Plus Prestained Protein ladder #26619).

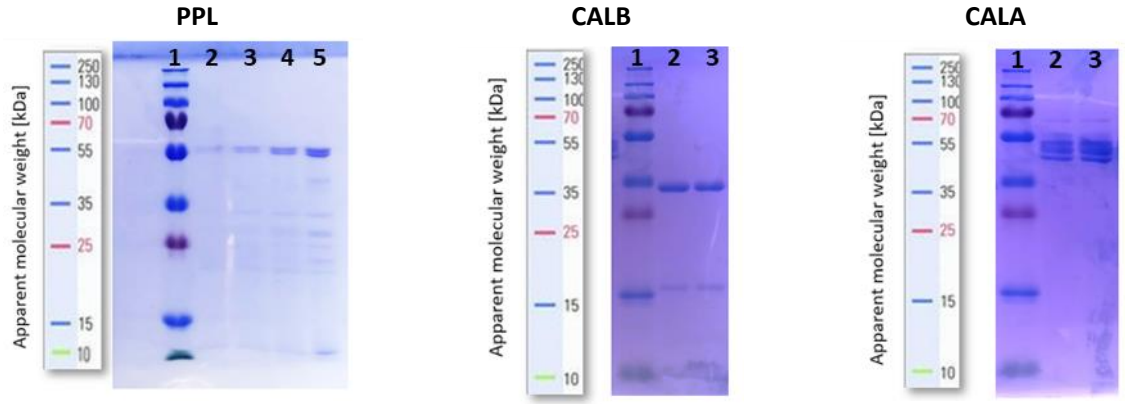

Figure 4.1. 12% SDS-page gel for PPL (1. Ladder, 2. 0,25 g/L, 3. 0,5 g/L, 4. 1 g/L , 5. 2g/L), CALB (1. Ladder, 2. 1 g/L, 3. 2 g/L) and CALA (1. Ladder, 2. 1 g/L, 3. 2 g/L)

As can be seen from Figure 4.1, the lipases selected for this project present diverse molecular weights and different levels of purity. The commercial crude of PPL contains a spread variety of proteins, from 10 kDa up to 55kDa, with PPL detected in a more significant concentration in the mixture around 55 kDa. Next, CALB is observed at 33kDa, with a few impurities at 15 and 10 kDa. And CALA is seen around 45 kDa. In previous studies no purification of the enzymes is reported[3,37,71]; thus, in the following

sections, the proteins would be used without any further purification. Nonetheless, these results will be taking into account in the analysis of the data, seeing PPL as the least pure product.

4.1.1. Protein incorporation as function of F-

Having defined the purity of the enzymes, it is time to discuss the incorporation of these proteins in the complex coacervate. Electrostatic interactions play a major role in the partitioning of different molecules into complex coacervates. For this reason, the complex composition, defined by the concentration of the positive and negative charges in the complex, is selected as one of the variables of evaluation.

For this experiment, method 2 (-section 3.2.1) is implemented at neutral pH, with a total polyelectrolyte concentration of 5 g/L and a low ionic strength of 10mM. Previous experiments were performed to observe the formation of complex coacervates between PEI and PAA; the details of this experiment can be seen in Appendix B.2. In this assessment, it was observed that the creation of the complex coacervate only occurs at a complex composition in the range of 0,09 and 0,66 F-. Thus, the effect of this factor is evaluated in this range for the following experimental section.

Figure 4.2 illustrates the results once the complex coacervate has incorporated the lipases. From this figure the differences between the lipases studied can be seen. CALB and CALA are liquid products presented as a transparent-dark coloured solution. PPL is a cream colour powder that forms a transparent solution when diluted and filtered in water. Thus, while the complex with PPL present a yellowish colour similar to the complex coacervate without any enzyme, the complex formed for CALA and CALB presents the colour of the stock protein solution as well.

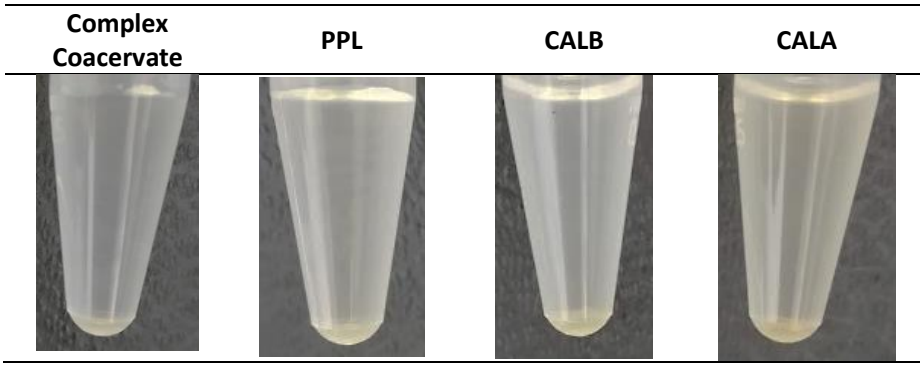

Figure 4.2. Complex coacervates with the incorporated enzymes at 0,26 F- complex composition.

The results obtained from the partitioning of the lipases are presented in Figure 4.3, where the distribution coefficient *[K_D]* is plotted as a function of F-. The distribution coefficient is defined as the ratio of the lipase concentration in the complex coacervate and the lipase concentration in the supernatant, stated in Equation 4.1.

$$K_D = \frac{[lipase]_{coacervate}}{[lipase]_{supernatant}} \qquad 4.1$$

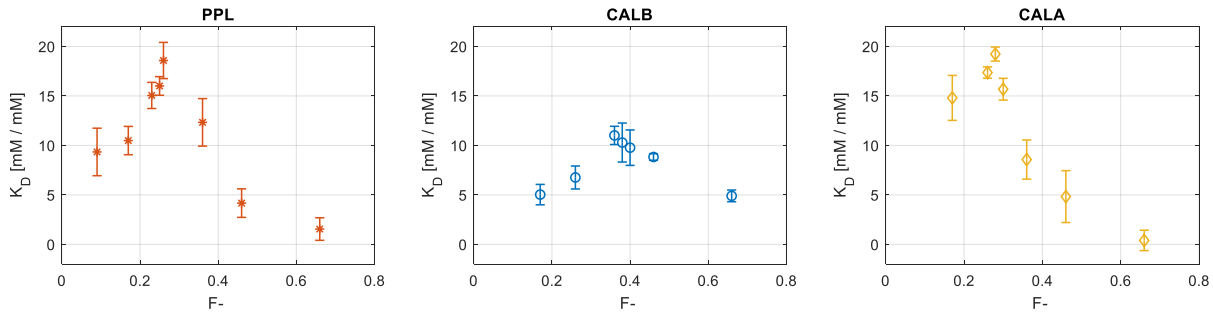

Figure 4.3. Lipases distribution coefficient [K_D] as function of the polyelectrolyte composition F-. PPL (left) initial concentration 0,083 mM. CALB (middle) Initial concentration 0,052 mM. CALA (right) Initial concentration 0,035 mM. The error bars show the standard deviation over three experiments.

From Figure 4.3, it can be seen that there is a significant effect of the complex composition on the accumulation of the enzymes in the complex coacervate. The highest distribution coefficient for PPL occurs at 0,26F- with a value of 18,5 ± 1,8. A similar result is observed for CALA at 0,28F- a distribution coefficient of 19,2 ± 0,7 is noticed. Meanwhile, for CALB, the highest distribution coefficient obtained is 11,0 ± 0,9 at a complex composition of 0,36 F-.

The distribution coefficient for the studied enzymes is in the same order of magnitude. The maximum incorporation for each protein appears at different F- ratios. Intuitively, the maximum incorporation for PPL and CALB was expected to be at a complex composition where a more substantial amount of positively charged polyelectrolyte is present. This prospect is related to the charges of the lipases, presenting a net negative charge at a pH above their isoelectric point (PPL pI = 4,9, CALB pI =6). This expectation fits accordingly to the results obtained.

Regarding CALA at pH 7, the system is too close to its isoelectric point (CALA pI= 7,4) estimating that the enzyme at this conditions has a low net positive charge. However, its maximum incorporation takes place when positively charged polyelectrolytes are present, suggesting that the protein enrichment of the complex coacervate not only depends on the charges of the components present in the system.

Prior studies have noticed the importance of the complex composition for the incorporation of proteins in complex coacervates, demonstrating that there is an optimal complex composition for the concentration of proteins inside complex coacervates[7,58]. Lindhoud & Claessens (2016), for example, found an optimal complex composition of 0,63 F- for the incorporation of lysozyme in a complex coacervate form by PPA and poly(N,N dimethylaminoethyl methacrylate). Furthermore, these studies suggest that the partitioning of this proteins is depenent on the properties of the component present in the system.

Now that the optimal composition for the incorporation of the enzymes is defined, selecting 0,26 F- for PPL and CALA and 0,36 for CALB, in the remainder of the report these optimal complex compositions will be used. However, before proceeding to examine the incorporation of the proteins in complex coacervates in a deeper extent; a brief estimation of the amount of water and polyelectrolytes that form the complex coacervate at the optimal composition is carried out, as can be seen in the following section.

4.1.2. Water content of PEI/PAA complex coacervates

Previous studies have explored the relationship between the type of polyelectrolytes used and the water content of the complex coacervates, reporting roughly from 60% to 80% of water content in different complex coacervates evaluated[50].

In this section the water content of the complex coacervate formed by PEI and PAA was determined experimentally by a thermogravimetric analysis (TGA), in which the mass loss of the samples is measured over time as it is exposed at elevated temperatures. The TGA measurement was conducted on Netzsch STA 449 F3 Jupiter using a sample holder with a k-type thermocouple in an air environment. The temperature was increased from 30 to 120°C at a constant rate of 5°C/min and then held at 120°C for 30 min to evaporate the water present in the samples. The weight of the samples was recorded along the heating procedure to obtain the mass loss that represents the evaporated water.

The measurements were carried out for a complex composition of 0,26 F- and 0,36 F-, at a total polyelectrolyte concentration of 10 g/L and a salt concentration of 10mM. The total sample volume was 1,2 mL and at these conditions, 27 mg of complex coacervate is formed at a composition of 0,36F- and 16 mg at a composition of 0,26 F-.

Figure 4.4 shows the weight fraction of the complex coacervate during the heating process as a function of time. As can be seen, the complex coacervate at a composition of 0,36F- contained the more substantial amount of water, calculating a water content of 73,5 wt.%. Meanwhile, the complex at 0,26 F- presented a water content of 51,9wt%.

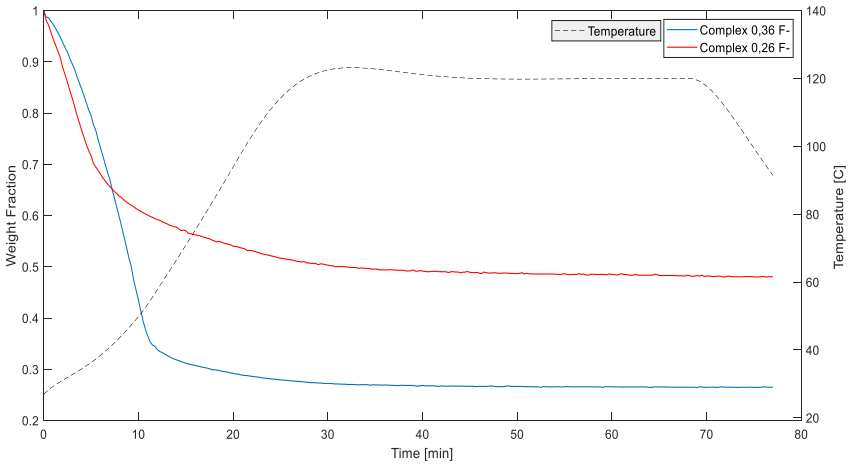

Figure 4.4 Weight losses of the complex coacervate at a composition of 0,36F- (blue line) and 0,26F- (red line) during the heating process as function of time. The temperature is plotted as function of time (dotted line).

The amount of polyelectrolyte that forms the complex coacervate can be estimated taking into account the water content of this dense-polyelectrolyte phase, the weight of the complex coacervate and the amount of polyelectrolytes that was added to the solution before the complex formation takes place. At 0,36 F- composition the 61% of the added polyelectrolytes constitute the complex coacervate, the remaining 39% stay in the supernatant phase. Furthermore, at the composition of 0,26F- the 68% forms the complex coacervate, with 32% of the polyelectrolytes added stand in the supernatant.

It is interesting to see the alterations in the complex coacervates created at different compositions. From the results, it can be inferred that a change in the complex composition affects the amount of polyelectrolytes that will interact to create the phase separation, including the water uptake of the complex coacervate. In the next section, the effect of the ionic strength will be evaluated for the accumulation of the lipases inside the complex coacervate.

4.1.3. Protein incorporation as function of salt concentration

Ionic strength is an important parameter that affects the interactions within the polyelectrolyte complexes. In this section, the effect of the salt concentration on the partitioning of the enzymes is evaluated.

The experiments were done at a neutral pH with a total polyelectrolyte concentration of 5g/L at the optimal complex composition.

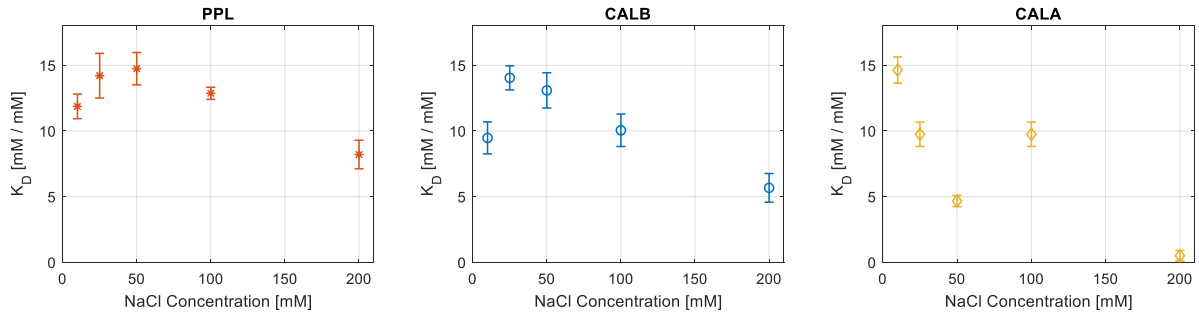

Figure 4.5. Lipases distribution coefficient [K_D] as function of the salt concentration [mM].]. All enzymes were studied at an Initial concentration of 0,067 mM. PPL (left). CALB (middle). CALA (right). The error bars show the standard deviation over three experiments.

In Figure 4.5, the distribution coefficient of the studied enzymes is plotted as a function of the salt concentration. A higher distribution coefficient is observed at low salt concentrations for the different lipases. However, 10mM is preferred for CALA, while for PPL and CALB 25mM and 50 mM are the most suitable values to achieve higher incorporation of the enzymes inside the complex coacervate.

What is interesting is the decrease of the lipase incorporation in the complex coacervate at a higher salt concentrations, which indicates the importance of electrostatic interaction on the partitioning of the enzyme in the complex coacervate. It can thus be suggested that at higher salt concentration the charges of the components are being screened by the presence of the counterions, minimizing the interaction between the protein and the charged polyelectrolytes.

These results corroborate the findings of a great deal of the previous reports[7,9,48], showing that the ionic strength is a critical factor for the distribution of the enzymes inside complex coacervates. For example, Lindhoud et al. (2009), studied the incorporation of lipases in polyelectrolytes micelles. They observed that at a salt concentration around 120mM lipases molecules are released from the core of the polyelectrolyte complex micelles, while the micelles remain intact at up to 500 mM of salt concentration[9]. The addition of extra charges shifts the equilibrium of the system in the form of small ions, allowing the release of the protein molecules, because these have a lower charge density than the polyelectrolytes.

4.1.4. Protein incorporation as function of total polyelectrolytes concentration

Another interesting factor to analyse is the total polyelectrolyte concentration that may affect the partitioning of the proteins in complex coacervates. The experiments were carried out at low salt concentration (10mM), and neutral pH at the optimal complex composition. The results are presented in Figure 4.6 in terms of the distribution coefficient of the lipases (K_D) as a function of the total polyelectrolyte concentration [g/L].

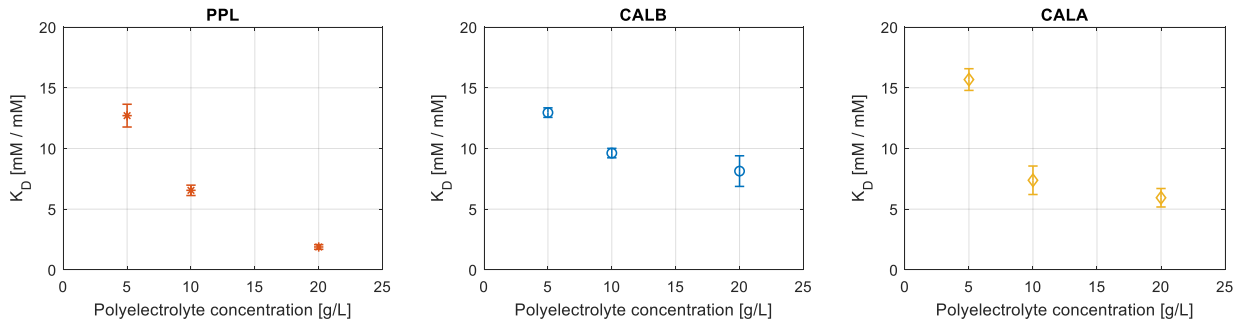

Figure 4.6. Lipases distribution coefficient [K_D] as function of the total polyelectrolyte concentration[g/L]. All enzymes were studied at an Initial concentration of 0,067 mM. Salt concentration 10mM. PPL (left). CALB (middle). CALA (right). The error bars show the standard deviation over three experiments.

As shown in Figure 4.6, an increase in the total polyelectrolyte concentration reduced the lipase enrichment of the complex coacervate. The results were consistent between the three enzymes studied, which indicates that the total polyelectrolyte concentration has a significant effect on the incorporation of the proteins in the complex coacervate.

Previous studies have noted the importance of this variable in the coacervation phenomena. Burgess (1994) studied a system formed by two biopolymers. They observed that a high concentration of the polymers has a negative effect on the coacervation. This behavior was explained in terms of the proximity of the molecules, suppressing its free movement, and finally reducing the interactions[72]. Furthermore, Li et al. (2018) reported that an increase in the concentration of the total polyelectrolyte reflects an increase in the concentration of salt counterions next to the polymer chains inducing a stronger screening. Thus, a reduction of the polymer content in the complex coacervate is observed[73].

4.1.5. Overview – Proteins partitioning

The partitioning of the lipases in the complex coacervate formed by PAA and PEI under the variation of 3 significant factors was investigated: complex composition, salt concentration and total polyelectrolyte concentration. A maximum distribution coefficient of ~18 was achieved for PPL and CALA at a complex composition of 0,26 F-, 10 mM of salt concentration and 5g/L of total polyelectrolyte concentration. Other factors such as the order of addition of the components for the formation of the complex coacervate and the mixing time were studied only for one lipase (PPL), concluding that when the system reaches the equilibrium, these factors do not affect the partitioning of the lipases (details can be seen in Appendix B.4)

Regarding the measurements of the concentration of the lipases by the spectrophotometer, the complex concentration was evaluated to avoid an interference with the absorbance measurements. (Appendix B.5). Nonetheless, a source of uncertainty is present in all the measurements done in this study. Impurities, such as other proteins present in the samples, contribute to the absorbance measurements at 280 nm. Thus, while measuring the concentration of the enzymes in the supernatant, not only the incorporation of the specific enzyme in the discussion is determined, but also the other proteins incorporation is taken into account. In further investigations, it might be possible to purify the commercial products beforehand, and in this way eliminate this source of error in the determination of the enzymes incorporation into the complex coacervates.

The interactions between these three components (lipases, PAA and PEI) have not been previously reported. When it is compared with studies evaluating the partitioning of proteins in complex coacervates the magnitude of the distribution coefficient of the proteins in this study is small. McCall et al. (2018), assessed the partitioning of actin into coacervate droplets formed by poly-L-Lysine and poly-glutamic acid reporting distribution coefficient up to ~30 [10]. Furthermore, Black et al. (2014) reported a distribution coefficient of ~240 for the incorporation of bovine serum albumin within complex coacervates containing poly(allylamine hydrochloride) (PAH) and PAA[54]. Mctigue & Perry (2019) investigate the encapsulation of three enzymes with different properties: bovine serum albumin, human hemoglobin and hen egg white lysozyme into a complex formed by poly(L-lysine) and poly(D,L-glutamate), concluding that there is a higher uptake for proteins that includes cluster of like-charged residues on the protein surface[74]. This substantial differences between several systems suggest the dependence of the distribution coefficient of the proteins on the physicochemical and structural properties of the components present in the systems.

The findings regarding the lipase incorporation, while preliminary, indicate that the lipases PPL, CALB, and CALA can be incorporated into complex coacervates. Nonetheless, to develop a full picture of this partitioning behavior, additional studies will be needed. For example, the pH effect could be explored, to have a stronger influence over the charges in the system. PEI, PAA, and the proteins are weak polyelectrolytes. Therefore, a modification on the pH will significantly variate its net charge, that could be reflected in the incorporation of the enzyme.

Besides, to enhance the protein incorporation, an indication related to the charges present in the system will provide a better understanding of the interactions. In this study, the amount of charges was determined based on estimations according to the structure of the molecules. However, the charges could be measured via zeta potential to have a more accurate description of the system and elucidate pathways to accumulate the proteins inside the complex coacervate effectively.

In the case of considering complex coacervates for a potential application, taking into account the incorporation of the proteins, one possible limitation is related to its dependency on the electrostatic interactions. The conditions of the solutions containing the proteins and the compositions will affect the incorporation. Thus, the success of the uptake of the proteins will strongly rely on system conditions.

The results in this section indicate that the relation between polyelectrolytes and proteins is dominated by electrostatics interactions, meaning that factors such as complex composition and ionic

strength present a significant effect on the partitioning of the enzyme within the complex coacervated. Furthermore, it can be suggested that this interactions are unique for every system studied, depending on the structure and physicochemical properties of the components contained in the mixture. The next section moves on to the discussion of the incorporation of smaller molecules in the complex coacervate, evaluating the lactic acid incorporation into these dense-polymer phases.

4.2. Partitioning of lactic acid in complex coacervates

The recovery and purification of LA is an area of interest to obtain lactic acid at high purity implementing a cost-efficient process; to ultimately, take advantages of this acid as a feedstock for environmentally friendly chemicals such as biodegradable polyesters PLA or green solvents. In this section, the potential use of complex coacervates as an extraction media is evaluated, considering its partitioning a critical parameter to identify the viability of complex coacervates as a reaction media for the concentrated LA.

The LA accumulation in complex coacervates is studied, taking into account the effects of different parameters that could play a significant role in this process. Method 1 (Section 3.2.1) is followed for this evaluation. The standard conditions for the experimental section on LA incorporation are neutral pH with a total polyelectrolyte concentration of 5g/L and a salt concentration of 10mM.

It is known that several variables affect the incorporation of small molecules in complex coacervates such as polyelectrolyte concentration, ionic strength and pH[46,52,75]. Five variables were assessed in this section: complex composition, total polyelectrolyte concentration, salt concentration, initial concentration of LA and temperature. The results for the composition dependence, total polyelectrolyte concentration, salt dependence and initial LA concentration are presented in Figure 4.7 in terms of the distribution coefficient (Equation 4.2), defined as the ratio between the concentration of LA in the complex coacervate and LA in the supernatant.

$$K_D = \frac{[LA]_{coacervate}}{[LA]_{supernatant}} \qquad 4.2$$

Figure 4.7 (a) presents the distribution coefficient as a function of the complex composition. The initial LA concentration used in this set of experiments was 100mM. No significant differences can be seen between the compositions regarding the partitioning of the lactic acid in the complex coacervate, achieving a maximum distribution coefficient of 3,2 ± 0,6 at 0,16F-.

Concerning, the effect of the total polyelectrolytes concentration (Figure 4.7 (b)) a higher distribution coefficient is obtained at lower polyelectrolytes concentration. Similar results were observed for the protein's incorporation; thus, it may be the case, that the less saturated the sample is with polyelectrolytes, the easier is to incorporate molecules inside the complex.

In the case of the effect of salt concetration in the LA distribution; Figure 4.7 (c) shows that between 25 and 100mM of salt concentration a larger amount of LA molecules go inside the complex coacervate, resulting in a distribution coefficient of 7,5± 0,5. This outcome, indicates that the salt concentration is a variable that has a larger effect on the lactic acid accumulation in comparison to

the variables studied here, by doubling the distribution coefficient at the proper ionic strength. It can thus be suggested that the introduction of salt at low concentrations in the mixture shift the equilibrium of the components in the two phases in favor of migration of LA inside the complex coacervate. A small increase in the ionic strength could screen some interaction between the polyelectrolytes present in the complex coacervate, making it more permeable for the incorporation of the molecules.

Furthermore, the distribution coefficient of LA was evaluated at low LA initial concentrations as can be seen in Figure 4.7 (d). An average distribution coefficient of 4,6 ± 0,6 is observed in the range of initial lactic acid concentration studied, excluding results at the lowest concentration (25mM). According to these data, it can be inferred that an increase in LA concentration does not enhance the LA partitioning, which indicates that the system of LA with a PEI/PAA complex is at equilibrium at around 4,6 ± 0,6 distribution at the conditions evaluated.

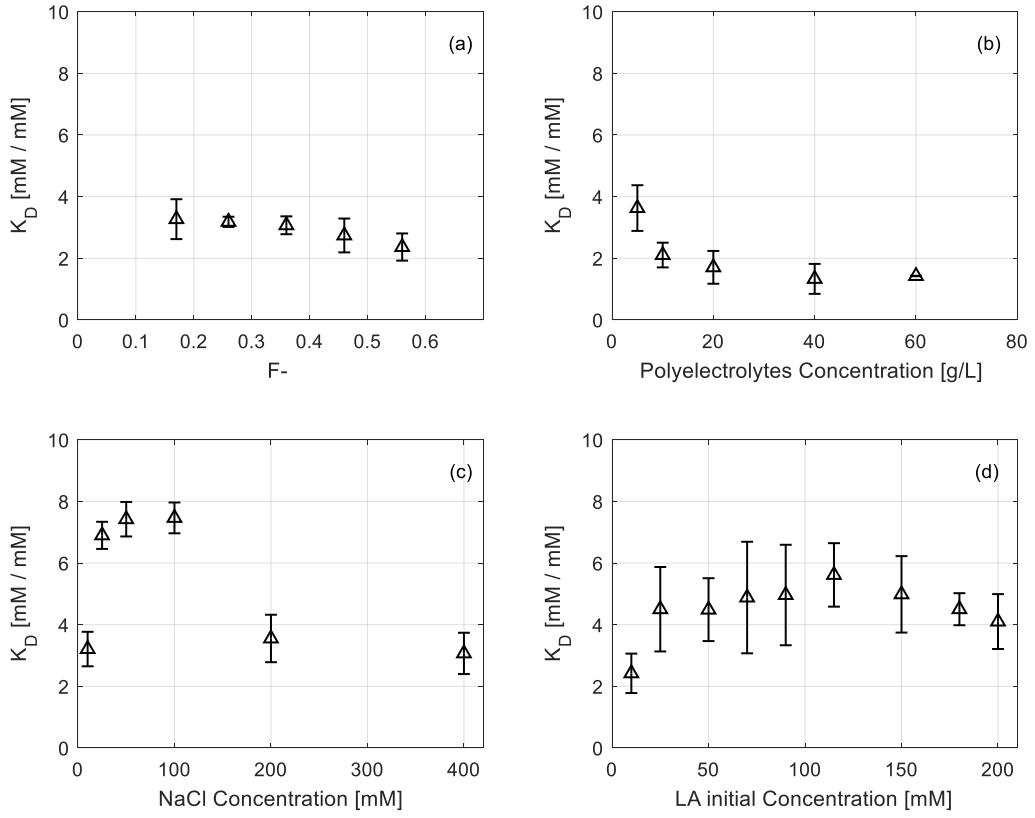

Figure 4.7. Lactic acid distribution coefficient (K_D) as a function of (a) Complex composition (F-) (b) total polyelectrolytes concentration [g/L] (c) salt concentration [mM] (d) Initial lactic acid concentration [mM]. The error bars show the standard deviation over three experiments.

In a fermentation broth, during LA production, the LA concentration can variate roughly from 50mM up to 1000mM [77]. Therefore, in Figure 4.8, the distribution coefficient of LA is presented as a function of initial LA concentration in a higher range than the formerly studied, between 200 and 1200 mM. It is interesting to note that above 400mM a marked decrease on the distribution coefficient can be observed, presumably because the complex coacervate becomes saturated with LA.

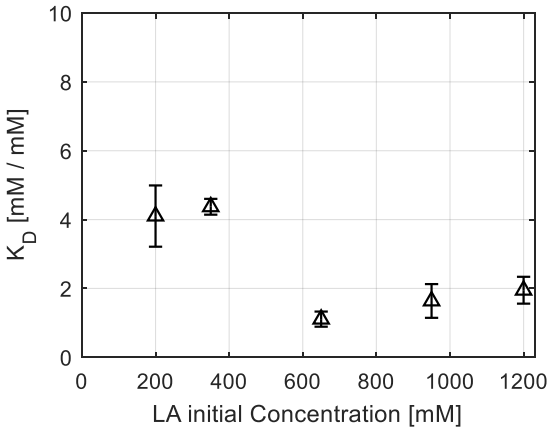

Figure 4.8. Lactic acid distribution coefficient (K_D) as a function of Initial lactic acid concentration [mM].

Additionally, the effect of the temperature on the distribution coefficient of LA is shown in Figure 4.9. Three temperatures were evaluated room temperature (~20°C), 50 and 70 °C at the optimal complex composition. A slight increase in the distribution coefficient of LA can be seen when the temperature increases, which indicates that the temperature has a weak effect on the partitioning of LA inside the complex coacervates.

A relationship between the formation of complex coacervates and temperature has been reported in literature[44,75,78]. It has been suggested that the effect of the temperature is much smaller than the ionic strength for the phase behavior of complex coacervate systems[50]. Nonetheless, with a temperature increase, the hydrophobic interactions are enhanced by weakening the hydrogen bonds of the components in the mixture[79]. Thus it may be the case that the electrostatic interactions play a more substantial role in LA accumulation.

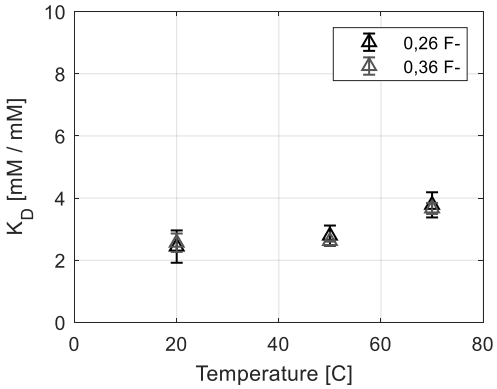

Figure 4.9. Lactic acid distribution coefficient (K_D) as a function of temperature [°C]. Initial concentration LA 100mM, NaCl 10mM.

Previous studies have been focused their investigation in the incorporation of small molecules in complex coacervates with dyes, such as methylene blue, taking into account different interactions due to the aromatics rings in their structures[46,80]. However, in reviewing the literature, no information was found on the interaction between complex coacervates and lactic acid. In this study a maximum distribution coefficient of 7,5± 0,5 is obtained at 100mM initial LA concentration, 100mM NaCl and 5g/L total polyelectrolyte concentration.

Different extraction media have been considered for the removal of lactic acid from aqueous solution. Alamine 336, is reported as one of the most promising extractants due to the affinity of nitrogen atoms

towards the acid, achieving distributions coefficients between ~8 and 26[23,26,28], and around 13 for low lactic acid concentrations[27]. When these values are compared with the ones obtained in this research for the complex coacervate, it can be seen that the distribution of the lactic acid is rather small, which suggest that electrostatic interactions or the affinity of the LA towards the polyelectrolytes dense phase environment are not enough to efficiently remove the LA from the water-rich phase.

Having observed the interactions of LA with the polyelectrolytes at different conditions, becomes interesting to evaluate whether the presence of the enzyme inside the complex coacervate will modify significatively the effects previously observed. Consequently, the evaluation of the incorporation of the lactic acid in enzyme-filled complex coacervates is presented in Figure 4.10. This accumulation is evaluated at 2 different temperatures room temperature (~20°C) and 50°C and at different levels of salt concentration, from 25mM to 100mM. The results are expressed in terms of the distribution coefficient.

These experiments were done following method 2 (section 3.2.1) as an initial step for the accumulation of the enzymes, consecutive to method 1 (section 3.2.1) for the incorporation of LA. The initial concentration of LA was 100mM and a total polyelectrolyte concentration of 5g/L was used.

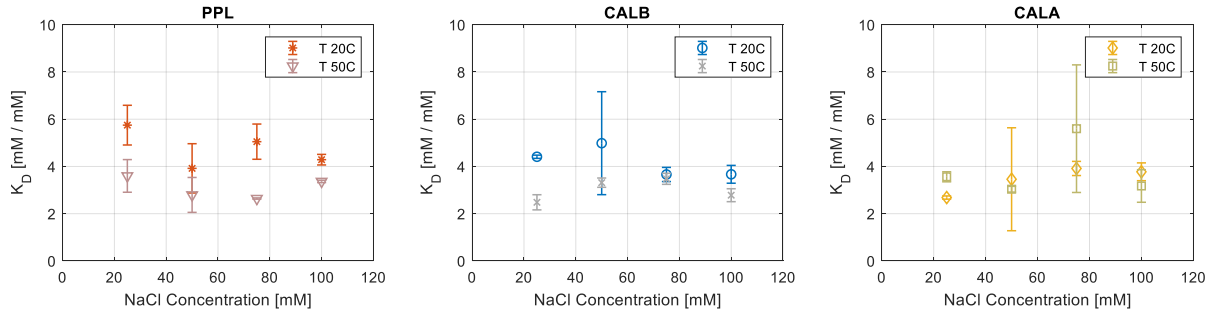

Figure 4.10. Lactic acid distribution coefficient [K_D] in enzyme-filled complex coacervates as function of salt concentration[mM]. The error bars show the standard deviation over three experiments.

As shown in Figure 4.10, LA can be incorporated in the complex coacervate with the presence of a lipase. The distribution coefficients obtained are in the same order of magnitude for the different enzyme-complex systems studied, and no significant differences were found between the levels of salt concentration evaluated. Interestingly, the enzymes accumulated inside the complex coacervates reduce the incorporation of LA at low salt concentrations, which indicates that the lipases slightly change the equilibrium of the system.

Besides, the effect of the temperature is more significant in complex coacervates containing PPL and CALB than for CALA, obtaining higher LA distributions coefficients at room temperature. This result is not in accordance with the previously observed for a mixture containing LA and polyelectrolytes, where the incorporation of LA seems to be slightly larger at higher temperatures. Nonetheless, it can be seen that the presence of the enzymes inside the complex coacervates has a minor effect on the incorporation of LA, obtaining distributions coefficients between the range of 2 up to 7.

4.2.1. Overview – Lactic acid partitioning

Overall, in this section, the potential of using the two-polyelectrolytes coacervate system to concentrate effectively lactic acid across a range of solution conditions was explored. While, electrostatic considerations, such ionic strength demonstrated to have a substantial effect on the incorporation of LA, temperature, that could be associated with molecular interactions, such as hydrogen bonding and hydrophobic interactions, presented no significant impact for the accumulation of LA inside the complex coacervate.

It was observed that LA could reach concentrations up to ~2500mM in the complex coacervate when high initial concentrations of LA are used, exemplifying 1200mM in this case. Interesting levels of LA concentrations can be obtained in the complex coacervates, nonetheless, when the distribution coefficients are analysed the values are within the range of some commonly used extractants, such as Tri-n- octylamine[25] or Alamine 336 [26]. The magnitudes obtained for the distribution coefficients could be attributed to the high affinity that LA has towards water, exposing a limitation to reach higher levels of LA distributions. Therefore, the directly possible application of complex coacervates for LA extraction from a fermentation broth is not appealing.

Further research should be undertaken to investigate the effect of the pH in the LA incorporation. In the production of LA, the pH of the fermentation broth has to be maintained around 5 - 6 to protect microorganisms and promote the formation of the acid. However, new developments on yeast that can perform under lower pH to produce LA are used[14], avoiding the addition of bases to keep the desired levels of pH. Therefore, it becomes interesting to investigate whether, at a lower pH, LA will reach higher concentrations inside the complex coacervate.

Additionally, the attribution of the accumulation of molecules inside complex coacervates relies on the properties and interactions of the individual components that are present in the mixture. For example, Mctigue and Perry (2019) evaluated the incorporation of 3 different proteins: bovine serum albumin (BSA), human haemoglobin and lysozyme, into a complex coacervate and observed a distribution coefficients close to 1000 for lysozyme and close to 10 for haemoglobin, demonstrating the dependence of the partitioning in the components evaluated [74]. This study was limited to one pair of polyelectrolytes for the formation of the complex. Thus, a further investigation could explore other combination of polyelectrolytes and perhaps achieve a higher distribution coefficient of LA.

The results in this section indicate that LA can be concentrated inside complex coacervates, conditions such as complex composition, temperature, total polyelectrolyte concentration, and ionic strength were evaluated to enhance the incorporation of LA, concluding that the ionic strength is the factor that presents the highest effect on its distribution. The next chapter moves on to discuss the partitioning of butanol inside the complex coacervates, considering different conditions to have an insight regarding the interaction between butanol and the polyelectrolyte-dense phase.

4.3. Partitioning of Butanol in complex coacervates

One of the aims of this research is to evaluate the possible formation of butyl lactate inside complex coacervates. Thus, now that it has been demonstrated that lactic acid and the lipases: PPL, CALB and CALA; can be incorporated inside complex coacervates, the evaluation of the partitioning of butanol in this dense-polyelectrolyte phase becomes essential. This study will provide information concerning whether butanol can be accumulated inside the complex coacervate to study the ester formation in a later section.

The incorporation of butanol in the complex coacervate was studied as a function of complex composition and temperature at neutral pH. Method 1 from section 3.2.1, was the base for the development of these experiments, employing a total polyelectrolyte concentration of 5g/L, a salt concentration of 10mM and an initial butanol concentration of 400mM.

The results are presented in Figure 4.11 in terms of the distribution coefficient of butanol, defined as the ratio between the concentration of butanol in the complex coacervate and the concentration in the supernatant (Equation 4.3).

$$K_D = \frac{[Butanol]_{coacervate}}{[Butanol]_{supernatant}} \qquad 4.3$$

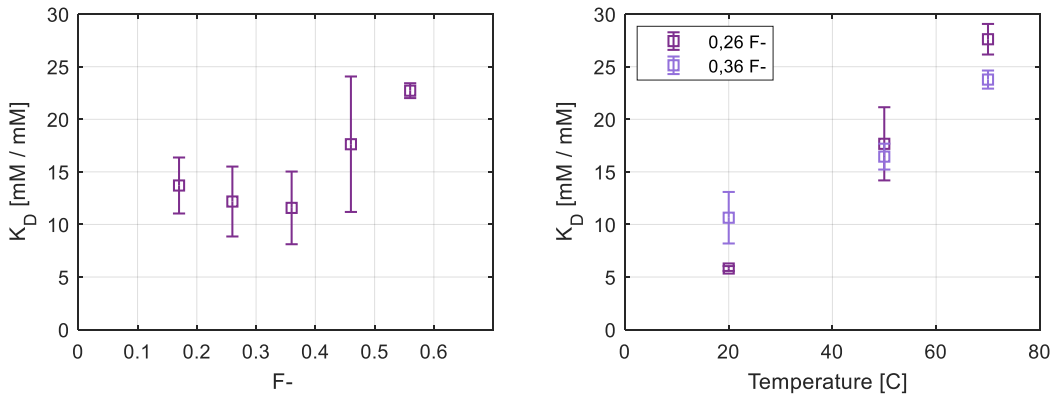

Figure 4.11. Butanol distribution coefficient (K_D) as a function of (a) Complex composition (F-) (b) Temperature. The error bars show the standard deviation over three experiments.

As can be seen from Figure 4.11 , enriched butanol complexes can be obtained when they are in contact with butanol aqueous solution. The complex composition of interest, in this case, is 0,26 and 0,36 F-defined as the optimal composition for the lipases incorporation, and between these two no significant differences are observed, achieving a distribution coefficient of ~12 ± 3.

Furthermore, what stands out from the results is the considerable effect of the temperature in the incorporation of butanol inside the complex coacervate, the distribution coefficient of butanol changes from 5,8 ± 0,2 up to 27,6 ± 1,4 when the temperature increases from 20 to 70 degrees, which indicates a strong dependency of butanol interaction with the complex coacervate on the temperature.

The experiments dependent on the complex composition were carried out at room temperature(~20°C); this variable was not controlled during this set of experiments. Thus, a possible

explanation for the discrepancy presented in the results at a composition of 0,26F- between the two independent experiments could be a difference in temperatures.

Several reports have studied the extraction of butanol from aqueous solutions, evaluating polymers, organic solvents, and ionic liquids as possible extractants[81]. Cockrem *et al.* (1986), identified useful solvents for the extraction of butanol from dilute solutions, achieving a distribution coefficient of 25 with 4-n-butyl-phenol as the extraction media[82]. Domańska and Królikowski (2012) evaluated the potential of ionic liquids, such as 1-hexyl-3-methylimidazolium tetracyanoborate, for butanol extraction obtaining very encouraging results with distributions coefficients from 25 to 48[83]. Accordingly, the partitioning obtained in this study for butanol in complex coacervates is promising, reaching similar distributions ratios when it is compared to other preferred extractants.

Turning now to the experimental results of the incorporation of butanol in enzyme-filled complex coacervates, the effect of the temperature and salt concentration can be seen in Figure 4.12. The experiments were done following the methods described in section 3.2.1, where initially the lipase is incorporated inside the complex coacervate and then the supernatant is replaced with a butanol solution of 400mM concentration. The total polyelectrolyte concentration used was 5g/L.

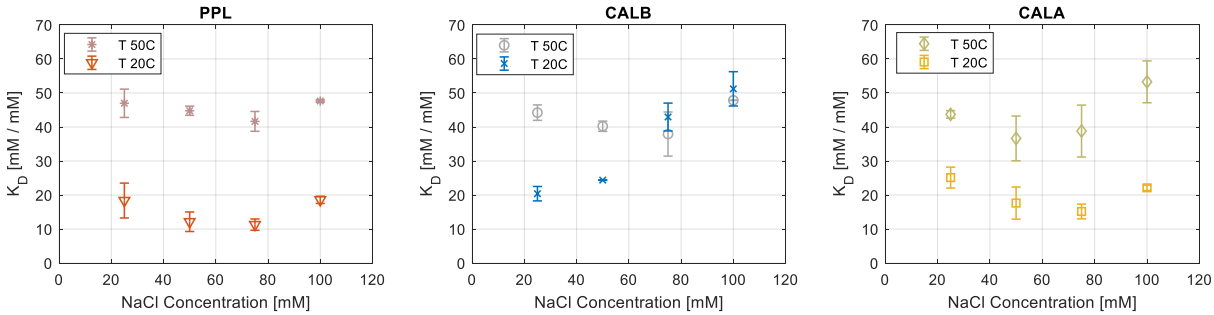

Figure 4.12. Butanol distribution coefficient [K_D] in enzyme-filled complex coacervates as function of salt concentration[mM]. The error bars show the standard deviation over three experiments.

As shown in Figure 4.12, the effect of the temperature in the butanol incorporation is significant. This finding is consistent with the previous results (Figure 4.11). Nonetheless, the presence of the enzyme inside the complex maximizes the effect of the temperature obtaining a distribution coefficient roughly ~2,4 times higher. Regarding the ionic strength effect, slightly differences can be noticed from 25mM to 100mM, observing that the highest incorporation is obtained at the edges of the range evaluated.

Furthermore, the distribution coefficients achieved for the different systems containing PPL, CALB and CALA are in the same order of magnitude, which indicates that the lipases present a similar effect over the distribution of the butanol in these enzyme-rich complex coacervates.

4.3.1. Overview – Butanol partitioning

The partitioning of butanol into complex coacervates was evaluated in this section, taking into account the influence of the complex composition and temperature over its distribution. An evident strong effect of the temperate could be noticed from the results reaching high distributions coefficients for

this component, at 70°C distribution of 27,6 ± 1,4 was achieved at a total polyelectrolyte concentration of 5g/L and 10mM of NaCl. Besides, when the enzymes are present inside the complex coacervates, higher distributions are reached, at 50° degrees a maximum distribution of ~50 is obtained for the different system studied.

The usage of complex coacervates to concentrate butanol seems encouraging. Further research should be undertaken to have a better understanding about their interaction. It may be the case, that the distribution of butanol inside the complex coacervate is not only attributed to electrostatic interactions but as well to the hydrophobicity of butanol, having a stronger affinity towards a more hydrophobic surroundings, such as the complex coacervate phase.

These findings suggest that butanol have an attraction towards the dense polyelectrolyte phase migrating from the aqueous phase to a more dense phase. In comparison with lactic acid, butanol is more propense to accumulate inside the complex coacervate at the conditions evaluated. Nevertheless, one of the aims of this research was to assess the possibility of concentrate butanol and lactic acid inside the complex coacervate. According to the results, it is possible to accumulate these components in the PEI/PAA complex. Before proceeding to study the formation of an ester inside the complex coacervates, it is pertinent to observe whether it prefers the coacervate phase or the supernatant phase. Therefore, in the following section, the partitioning of butyl lactate will be briefly evaluated for the optimal complex composition.

4.4. Partitioning of Butyl lactate in complex coacervates

Previous researches have studied the formation of esters of lactic acid with low molecular weight alcohols, such as methanol, ethanol and butanol, in the presence of lipases[11,42,84]. These esters have become an area of interest in the chemical industry due to their properties as green solvents, having the potential to replace toxic petroleum-based solvents[85] In this section, the interaction of butyl lactate with complex coacervates is studied and, the detailed results are presented in Appendix B.6.

This representative study intended to determine the affinity of butyl lactate towards one of the phases in the system tested at the optimal complex composition with and without enzymes previously incorporated in the complex. The initial concentration of butyl lactate and the concentration after being in contact with the complex coacervate were very close, even slightly higher concentrations after the interaction were obtained. This result may be explained by an error in the preparation of the samples perhaps in the replacement of the supernatant or the dilution with acetone (1:1) for the measurements of the concentration. Nonetheless, this findings provide an indication that the preferred phase for butyl lactate is the supernatant, demonstrating no incorporation of butyl lactate in the complex coeacervates. Furthermore, the presence of the enzymes did not modify the possible accumulation of butyl lactate, showing still a preference of butyl lactate to remain in the supernatant.

So far this report has focused on the accumulation of molecules inside the complex coacervate including macromolecules such as enzymes and small molecules including lactic acid and butanol. The following section will discuss the possibility of producing an ester from lactic acid and butanol catalyzed by lipases. Initially, testing the activity of the lipases in an organic solvent and then followed by the evaluation inside complex coacervates.

Chapter 5. Esterification reaction – Production of butyl lactate

PLA is a polyester produced by the direct polycondensation of lactic acid or by ring opening polymerization of lactides of the acid[86]. In condensation reactions, esters are produced by the reaction of a carboxylic acid and an alcohol generating water as a by-product. The rate of the esterification reaction along with the extent of the equilibrium are determined by the structure of the molecules and functional substituents of the acids and alcohols[87]. This section explores the production of an ester of lactic acid, butyl lactate, in the presence of an enzyme-filled complex coacervate.

In the previous chapter it has been determined that is feasible to concentrate lipases, lactic acid and butanol inside the complex coacervate, therefore, the possible formation of an ester inside the complex coacervate enzymatically catalyzed will contribute to consider a potential application of these dense-polyelectrolytes phases.

This chapter is divided in two sections, initially the activity of the lipases will be tested in a conventional experiment, an esterification reaction in the presence of an organic solvent. To finalize with the evaluation of the usage of complex coacervates as a reaction media for the production of butyl lactate.

5.1. Lipase Activity

Generally, commercial activities of the lipases are reported in terms of hydrolytic activity. However, this information is not suitable when the desired reaction is the esterification, as the reaction rates for these two mechanisms are entirely different[68]. Therefore, in this segment a method proposed by Kiran *et al.* (2000)[68] is followed to determine the activity of the commercial lipases, creating a framework to initially, validate the activity of the enzyme at a small scale in an esterification reaction, to further, be able to compare the performances of the lipases.

The reaction model selected for the evaluation of the esterification activity of the lipases is the synthesis of butyl lactate in the presence of an organic solvent (Figure 5.1), as was introduced in section 3.2.2.

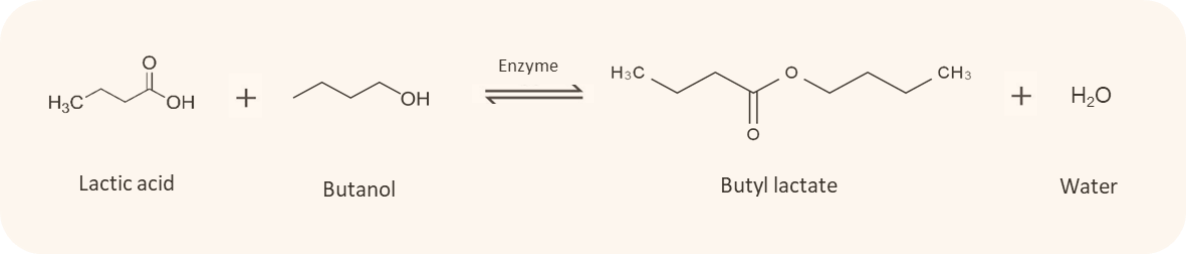

Figure 5.1. Synthesis of Butyl lactate from lactic acid and butanol.

The reaction of lactic acid with 1-butanol to produce butyl lactate was evaluated at 50°C for PPL, CALA, and CALB. The esterification activity unit was defined based on the measurement of initial rates, and it is expressed as one µmol of butanol consumed per mg catalyst per minute [µmolBn/min*mg lipase]. The results are shown in Table 5.1 , with a graphic representation in Figure 5.2. Control samples were

exposed at the same conditions observing no significant reduction on the butanol concentration (control butanol conversion 0,9 ± 0,5%).

Table 5.1. Esterification activities lipases

Enzyme	Esterification Activity [µmolBn/min*mg lipase]
PPL	0,038 ± 0,012
CALB	0,106 ± 0,018
CALA	0,087 ± 0,012

*The esterification activities were determined at 50°C and the result are presented as an average with the standard deviation of 4 experiments.

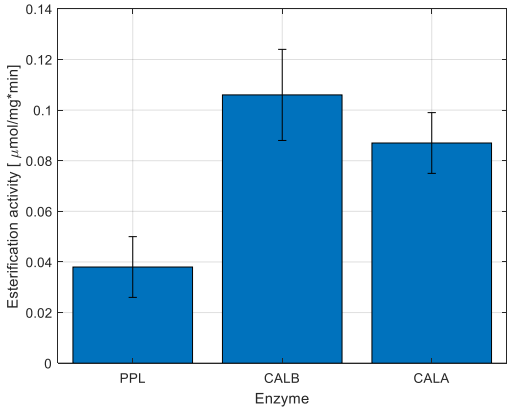

Figure 5.2. Esterification activity of the lipases studied.

Overall, these results indicate that the lipases evaluated were able to catalyse the reaction between lactic acid and 1-butanol under the conditions studied. As can be seen from Figure 5.2, the enzyme with the highest catalytic activity is CALB achieving a butanol conversion of 13,7 ± 2,9%; followed by CALA with a butanol conversion of 11,7 ± 2,1% and finally PPL with a butanol conversion of 5,0 ± 1,6%.

An statistical analysis was performed using a two-tailed student's t-test to compare the conversion of butanol for PPL and CALB with a significant level of p = 0,05. A significant different in the scores of PPL and CALB conditions were observed (p = 0,015). These results suggest that the enzyme used to catalyse the esterification reaction affects the butanol consumption for the production of the ester of lactic acid. Thus a difference would be observed while using PPL or CALB as a bio-catalyst in an organic solvent. The same analysis was performed for PPL and CALA observing a significant difference between these two enzymes as well (p = 0,0094). However, for CALB and CALA, no significant difference was obtained (p = 0,601), suggesting that around the same level of conversion of butanol will be obtained using CALB or CALA.

The results are in line with previous data reported in the literature for PPL[68], showing a low catalytic activity for esterification reactions in comparison with other lipases. This outcome could be attributed at the low purity of the commercial crude used, meaning that a small amount of PPL is employed in the experiments. Therefore, to achieve higher conversion, a more significant amount of this biocatalyst should be used. On the other hand, CALA and CALB present a higher catalytic activity, showing their potential for applications concerning esterification reactions as it has been demonstrated in previous studies[39].

It can be suggested from this section that the commercial lipases selected for the development of this project are suitable and capable to catalyse an esterification reaction in the presence of an organic solvent, producing butyl lactate from lactic acid and 1-butanol. The next chapter, therefore, moves on to discuss whether these lipases are able to perform the same function in a different environment, inside the complex coacervate, being a more challenging setting with higher water content.

5.2. Esterification reaction in the presence of complex coacervates

Having defined the activity of the lipases to catalyse an esterification reaction, the evaluation of its performance inside complex coacervates is one following purpose. This section aims to evaluate the possible application of a complex coacervate for the formation of an ester from lactic acid previously concentrated inside the complex coacervate.

5.2.1. Esterification reaction at small scale

In this experimental section, the methodology described in section 3.2.3 was followed. Initially, the accumulation of the enzymes inside complex coacervates takes place. The enzymes were incorporated at the optimal complex composition (0,26F- and 0,36F-) at low salt concentration (10mM). The initial concentration of the enzymes was 0,0057 mM, achieving a distribution coefficient of 11,87 ± 0,93 for PPL, 9,77 ± 1,17 for CALB and 12,90 ± 1,47 for CALA. Then, the supernatant is replaced for a solution containing LA (100mM) butanol (300mM) and NaCl (10mM) and the samples are placed at 50°C for 98 hours. Control samples, complex coacervates without the enzymes were placed at the same conditions . The results of this experiments are presented in Table 5.2

Table 5.2. Results esterification reaction inside complex coacervate. Results as presented as the mean and standard deviation of two independent experiments.

Sample	Concentration enzyme Complex [mM]	Butanol Conversion [%]	Butyl lactate yield [%]	Estimated conversion LA [%]
Control 0,26 F-	-	3,63 ± 0,89	0,013 ± 0,003	0,036
Control 0,36 F-	-	3,35 ± 1,02	0,013 ± 0,001	0,036
PPL	0,067 ± 0,93	7,11 ± 1,24	0,748 ± 0,007	2,050
CALB	0,055 ± 1,17	6,83 ± 0,55	0,741 ± 0,004	2,042
CALA	0,073 ± 1,17	4,17 ± 0,54	0,744 ± 0,001	2,061

As can be seen from Table 5.2, the production of butyl lactate is low for the three different systems studied. One unanticipated finding was that in the control samples a consumption of butanol is perceived, even though no significant formation of butyl lactate was observed, which indicates that butanol presented an interaction with some other components in the mixture. Besides, after 98 hours of reaction, exposing the samples at 50°C conversions below 7% were reached.

Statistical analysis was performed using an unpaired, two-tailed student's t test with a significance level of 0,05. This study aimed to detect differences of butanol conversion between the control and the samples containing the lipases. A significant difference was obtained comparing CALB and the control at 0,36F- complex composition ($p = 0,0011$). This result indicates that CALB performs its catalytic function in the samples, enhancing the conversion of butanol. Similar results were observed for CALA and the control at 0,26F- complex composition, obtaining a significant difference ($p= 0,0022$) for the results analysed. Furthermore, comparing PPL and the control 0,26F- no statistically significance was observed for the conversion of butanol ($p = 0,064$). Which suggest that the presence of the enzyme does not enhance the conversion of butanol. However, in the presence of PPL, a higher amount of butyl lactate is being produced. Thus, even though there is not a difference in the butanol conversion, it is evident that PPL is enhancing the formation of butyl lactate when the product formation is observed.

As it is known from previous studies esterification reactions are limited by thermodynamic equilibrium[85]. Water, a product from this reaction, is present in a large amount inside the complex coacervates between 50 and 70wt%, thus, its high initial concentration in the complex may be hindering the formation of the ester inside the complex coacervate. To elucidate the possible formation of butyl lactate under the conditions tested in the previous experiments a back of the envelope calculation was performed.

Qu *et al*. (2009) studied the esterification reaction of lactic acid and n-butanol catalyzed by ion-exchange resins, they develop a catalytic model and reported a maximum equilibrium conversion of 35% at a molar ratio 3:1 butanol lactic acid[87]. Bankole & Aurand (2014), studied the kinetics for uncatalyzed esterifications reactions of carbocilyc acids. They reported for esterification reaction of lactic acid at 50°C an equilibrium constant of or 0,07[88]. Based on this value and with an estimation of the concentration of LA, butanol and water inside the complex coacervate according the distribution coeffcients from previous experiments, the concentration of butyl lactate at equilibrium is calculated through Equation 5.1. The values employed for this rough approximation are presented in Table 5.3.

$$LA + Butanol \Leftrightarrow Butyl\ lactate + water$$

$$K_{eq} = \frac{[LA][Butanol]}{[Butyl\ lactate][Water]} \qquad 5.1$$

Table 5.3. Results back of the envelope calculation for equilibrium concentrations inside the complex coacervate.

	KD			Initial Concentration in Complex [mM]			Equilibrium Concentration in complex [mM]		
	PPL	**CALB**	**CALA**	**PPL**	**CALB**	**CALA**	**PPL**	**CALB**	**CALA**
LA	5,9	2,4	3,5	590,0	240,0	350,0	347,1	164,3	140,0
Butanol	18,0	20,0	25,0	1800,0	2000,0	2500,0	1557,1	1900,0	2314,3
Water [%wt]	52,0	73,0	52,0	28888,9	40555,6	28888,9	29131,7	40655,6	29074,6
Butyl lactate	-	-	-	0,0	0,0	0,0	242,9	100,0	185,7

It can be seen from the data in Table 5.3, that even though a high concentration of water is present in the system, a formation of butyl lactate can take place, achieving a lactic acid conversion between 40 and 50%. The base for these calculations does no accurately represent the process studied, however, this quick estimation could give us an indication in whether butyl lactate could be formed in the presence of water, which seems to be possible at the conditions evaluated. Nonetheless, the observation of the experimental results shows only around 2% of lactic acid conversion.

Prior studies have noticed the importance of the lactic acid concentration in an esterification reaction catalyzed by an enzyme, high concentration of this acid in polar solvents cause enzyme inactivation[89]. These inhibiting effects can be attenuated by reducing the quantity of the acid and increasing the amount of enzyme in the reaction media[84]. Furthermore, it has been reported that acid concentrations lower than 100mM could potentially enhace the product yield[84]. Thus, a possible explanation for the low yield of butyl lactate is the concentrations of LA and lipase present in the complex coacervate that does not favor the enzyme activity.

Temperature is reported to have a significant effect in the enzyme activity. Garcia *et al.* (2002) reported a positive influence of the temperature on the yield in esterification reactions, presenting the temperature as a more significant factor than the catalysts concentration[90]. However, Pirozzi & Greco (2004) observed that at low lactic acid concentrations (< 100 mM) in organic solvents CALB present no esterification activity when the temperature exceeds 60°C, with the highest yield towards butyl lactate at 45°C[11].

Regarding the effect of the initial molar ratio of butanol to lactic acid, studies have reported that the equilibrium conversion can be enhanced by using an excess of the alcohol[87]. Nonetheless, a large excess could affect the yield towards the ester formation. In the experiments reported in this project, it was estimated butanol: LA ratio around 8, and a ratio of 3 for PPL, inside the complex coacervates due to the high distribution coefficient of butanol at the conditions studied.

With this evidence, there is an indication that several aspects could be explored to enhance the formation of butyl lactate inside the complex coacervate. An increase in the concentration of the catalyst could improve the butyl lactate yield, or an increase in temperature could have the same effect. Furthermore, the concentration of the reagents could be adjusted to have alcohol: LA ratio closer to 3:1. Another factor that has not been mentioned earlier is the reaction time. Common reaction times employed for lipases-catalized esterification reactions can variate from 72 hours up to 200 hours[71,91]. An increase in the esterification reaction time could enhance the butyl lactate yield up to reaching the equilibrium concentrations.

5.2.2. Esterification reaction at longer reaction time

For the next experiment, some changes in the previous conditions were made with the expectation of producing a more substantial amount of butyl lactate. First of all, to have better observations in the complex coacervate phase It was decided to increase the volume of the samples. Therefore, the total size of the samples was set to 17 ml, with a total polyelectrolyte concentration of 80g/L. Additionally, the reaction time was set to 170 hours. Regarding the concentrations of the components, 0,067 mM of the enzyme was used as initial concentration. LA was kept at the same concentration as 100mM, and the butanol concentration was reduced to 60mM, to achieve a Butanol: LA ratio closer to 3. Besides, the amount of salt was set to 50mM to enhance the accumulation of the enzymes inside the complex coacervate.

Figure 5.3 ilustrate the two phases present in the samples to carry the esterification reaction. An amount of complex coacervate of 1,89 ± 0,04 g was obtained at a 0,36 F- composition and 1,26 ± 0,17 g at 0,26F-. As the previous experiments, once the complex was formed the supernatant was replaced with a solution with the desired concentrations of LA and Butanol and the samples were place in the oven (Laboratory incubators termaks series B9000) at 50°C.

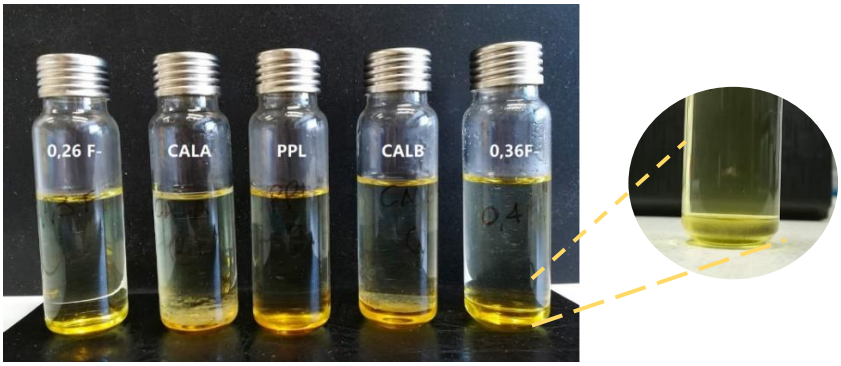

Figure 5.3. Samples of the esterification reaction at a longer reaction time.

The concentration of butanol and butyl lactate in both phases, supernatant and complex coacervate, were measured by gas chromatography. A small sample of the complex coacervate was taken (~0,2g) and previous the analysis was diluted in a 2,5 mM solution of NaCl at ~18 fold dilution.

The results obtained from the esterification reaction of LA at a longer reaction time are summarised in Table 5.4.

Table 5.4. Results esterification reaction at 170 hours. The values are presented as the average with the standard deviation of two experiments.

Sample	Butanol Conversion [%]	Butyl lactate yield [%]	Estimated conversion LA [%]
Control 0,26 F-	9,37 ± 2,51	2,89 ± 0,29	1,75
Control 0,36 F-	8,54 ± 2,28	0,00 ± 0,00	0,00
PPL	3,50 ± 1,02	0,00 ± 0,00	0,00
CALB	3,05 ± 0,94	9,84 ± 0,22	5,98
CALA	6,73 ± 1,53	7,56 ± 0,34	4,59

As shown in Table 5.4, a small formation of butyl lactate is observed for CALB and CALA being more significant in the samples that contained CALB as the catalyst. Furthermore, butyl lactate was not detected for the PPL experiments, indicating that no formation or a scarce formation of butyl lactate took place under these conditions. In the gas chromatography results (Figure 5.4), a peak is visible at the retention time of butyl lactate (10,3 min). However, it is not possible to accurately quantify the amount of butyl lactate present due to its appearance below the detection limit of the equipment (< 0,4 mM).

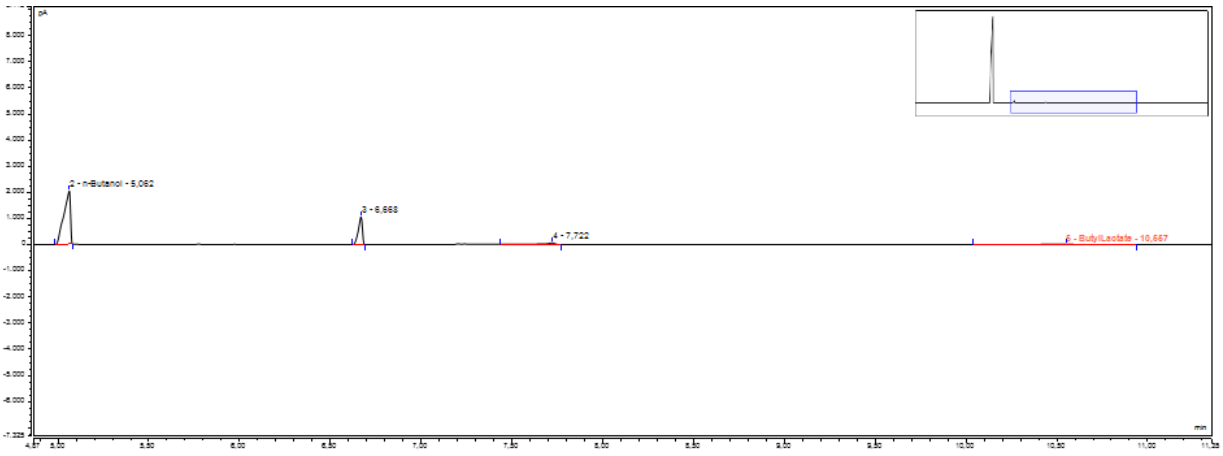

Figure 5.4. Gas chromatography results for the esterification reaction of butanol and LA with PPL as a catalyst.

Contrary to expectations, the butanol consumption was higher for the control samples in comparison with the samples containing the enzymes; and a detection of butyl lactate in the control samples at 0,26 F- was observed. Besides, butanol consumption is higher than the butyl lactate formation for all the experiments, a similar result was found in the first set of esterification experiments. These results are likely to be related to an interaction of butanol with another component present in the mixture, detecting a higher consumption of butanol without the presence of enzymes inside complex coacervates (control).

Prior studies have noted the ability of polyamines to hydrolyse effectively adenosine 5-triphosphate (ATP), suggesting that hydrogen bonding between amino groups of the polyamine and some groups of ATP facilitate the hydrolytic attack of water molecules[92,93]. The catalytic activity of these macromolecules related to electrostatic and nucleophilic catalysis may suggest an explanation for the formation of butyl lactate at a complex composition of 0,26F-. At this composition, a more significant concentration of PEI is present in comparison to 0,36F-. Therefore, it could be possible that at the conditions studied PEI interacts with butanol or lactic acid promoting the formation of butyl lactate.

Overall, these results provide an insight into the formation of butyl lactate in the presence of enzyme-filled complex coacervates (CALA or CALB) from lactic acid and butanol, suggesting that butyl lactate can be produced in the presence of this dense-polyelectrolyte phase at low yields. Thus, from this initial results it seems that the enzyme-filled complex coacervates are not a promising reaction media for the esterification of lactic acid at the conditions studied. Nonetheless, the results are preliminary, other factors with a higher impact towards butyl lactate formation could be explored to enhance its production in the system.

It is essential to bear in mind that the samples evaluated are a complex mixture containing different chemicals: PEI, PAA, LA, NaCl, enzymes, lactic acid, and butanol that could interfere with the catalyst activity to efficiently promote the formation of butyl lactate. Follow up studies are required to verify the disappearance of butanol in the samples, taking into account other products that could take place in the presence of the different components in the mixture. Thus, there are still unanswered questions about the possible applications of complex coacervates as a reaction media for an esterification reaction.

The next section is created following a different approach with the intention of encouraging the production of butyl lactate in the presence of complex coacervates, an organic solvent will be used as secondary phase containing butanol and lactic acid, and it is placed in contact with the complex coacervates.

5.2.3. Esterification reaction including an organic phase

From the previous experiments it can be seen that the yield obtained towards butyl lactate is not very encouraging. Thus, to try to promote its formation, the supernatant from the previous experiment is removed and replaced with an heptane solution containing 100mM of lactic acid and 30mM of butanol. The idea behind this change of environment, from water to an organic solvent, was based on the hydrophobicity of butyl lactate, therefore, it could be possible that the product will migrate from the complex coacervate phase towards the organic phase, pulling the equilibrium towards the formation of the ester. Besides, the reactants will be available in the interphase between the complex coacervate and the organic phase, and the lipases have the tendency of presenting a higher activity at interphases[94].

Table 5.5 shows the experimental data of the esterification reaction with heptane as the organic phase in contact with the complex coacervate. The results are presented in terms of the amount of butanol that was incorporated at the system with the heptane layer plus the butanol that was inside the complex coacervate (Initial Butanol [mM]) and the final amount of butanol that is present after 170 hours of the samples placed in the oven at 50°C (final Butanol [mM]). Besides, the amount of butyl lactate existing initially, due to the previous experiments inside the complex coacervate and after the reaction time is presented as well.

Table 5.5. Results esterification reaction with heptane as organic phase. The values are presented as the average with the standard deviation of two experiments.

Samples	Initial Butanol [mmol]	Final Butanol [mmol]	Initial Butyl lactate [mmol]	Final Butyl Lactate [mmol]
Control 0,26 F-	0,309	0,492	0,020	0,0094
Control 0,4 F-	0,315	0,531	0,000	0,0094
PPL	0,316	0,288	0,000	0,018
CALB	0,331	0,521	0,062	0,015
CALA	0,314	0,504	0,053	0,017

Interestingly, as can be seen from Table 5.5, in most of the samples, a higher amount of butanol at the end of the reaction time than at the addition of extra butanol to the system can be observed. Besides, a reduction in the amount of butyl lactate is detected for CALB and CALA, the most active lipases in the production of the ester. A possible explanation for these results is that the equilibrium of the reaction has shifted towards the formation of the acid and alcohol. Hydrolysis of butyl lactate is taking place, increasing the amount of butanol molecules in the samples. Other possible explanation could be the migration of butanol from the complex coacervate phase towards the organic phase, having a major affinity to the heptane phase.

These results suggest that the heptane phase added to enhance the formation of butyl lactate did not have a positive effect on the production of butyl lactate. Thus, not compelling indications were observed to follow the addition of an organic phase to change the conditions and promote the reaction. Therefore, it is more convenient to explore the possible formation of butyl lactate in complex coacervate by studying other factors that are more promising to enhance the activity of the lipases.

5.2.4. Overview – Esterification reaction

In this chapter the viability of the production of butyl lactate in complex coacervates was studied. Initially, the activity of the lipases was tested in the presence of an organic solvent, concluding that CALA and CALB present the highest activity when all the enzymes are used at the same amount (grams). Then, the enzyme-filled complex coacervates were place in contact with a solution of butanol and lactic acid, expecting the incorporation of the molecules and the production of butyl lactate. At small scale (500µL samples) a low yield towards butyl lactate was detected, providing indications about the production of these component.

Consequently, the following set of experiments was carried out at a longer reaction time, 72 hours more, and larger volumes (17 mL). Butyl lactate production was detected in the samples, but then again at a low yield. Besides, butanol is consumed in a higher amount than butyl lactate is produced.

Thus, it seems that the butyl lactate production is limited in the system. A possible explanation for this could be attributed to LA; LA tends to form oligomers that can be esterified, leading to the production of undesired side products[85]. Another possible explanation may be related to a reduction in the stability of the lipases by the presence of the acid and water in the system, or some additional interaction are taking place associated to butanol and other components present.

This study was limited to estimations regarding the lactic acid concentration, the small sample size did not allow to measure the concentrations of the LA, butanol and butyl lactate, due to the different techniques used for the individual analysis. Thus, it was decided to detect the product in the samples and make an estimation on the consumption of LA.

These preliminary experiments offer an insight about the production of butyl lactate in the presence of a complex coacervates. A low butyl lactate yield (7-9%) is obtained at a reaction time of 170 hours and at 50°C. The yield of butyl lactate may be improved by an increase of enzyme's concentration inside the complex coacervate, or an increase in the concentration of the reagents. Furthermore, it would be interesting to evaluate the reaction at higher temperatures, that tends to increase the activity of the enzymes.

Chapter 6. Conclusions

The aim of the present research was to examine whether lactic acid could be concentrated inside complex coacervates and to assess the possible formation of an ester from the acid accumulated inside the enzyme-filled complex coacervate. To give a perspective to this research, associated studies were carried out, including the incorporation of the enzymes and butanol, as the other reactant, in the complex coacervates.

In the first section of this study, the partitioning of specific molecules inside complex coacervates composed of PEI and PAA was evaluated. The distribution of these molecules was analysed by studying the effect of complex composition, total polyelectrolyte concentration, ionic strength, and temperature. For the enzymes, the results suggested that its interaction with the complex coacervate was dominated by electrostatic forces, observing an optimal complex composition for its incorporation at 0,26F- for PPL and CALA and 0,36F- for CALB. For lactic acid, its incorporation at low polyelectrolyte concentration (5g/L) and low ionic strength (25-100mM) was preferable, obtaining a maximum distribution coefficient of 7,5± 0,5. The distribution coefficient reached for LA at low concentrations (<100mM) is comparable to values of other common solvents used for the extraction of LA from aqueous solutions, such as Alamine 336. However, higher distributions have been achieved at higher concentrations for the same extractant, minimizing the application of complex coacervates as a possible extractant. Furthermore, high distributions coefficients (>20) were obtained for butanol, noticing that the temperature played a significant role to enhance its incorporation.

The first section offers insight into complex coacervates applications. It has been demonstrated that molecules with different properties, macromolecules to small organic acids, have the tendency to accumulate inside these dense polyelectrolyte phases and that variations in the solution conditions can enhance or deteriorate its incorporation. These results suggest that with a further exploration about conditions in the system, better distributions can be achieved, introducing the usage of complex coacervates as possible extractants.

In the second section, the production of butyl lactate in the presence of complex coacervates was investigated. This present study has been one of the first attempts to examine the formation of an ester inside complex coacervates. The results indicate that butyl lactate can be produced in complex coacervates at a low yield. Several reasons could be attributed to these results including a reduction on lipase stability or limited concentrations available to promote the reaction. These empirical findings provide an initial approach towards further investigations to enzymatically catalyzed reactions inside complex coacervates. More work will need to be done to assess the viability of using enzyme-filled complex coacervates to concentrate and esterify LA.

6.1. Recommendations for upcoming research

The research presented in this thesis has opened a number of research lines that should be explored in the future. For instance, for the incorporation of the molecules, the most exciting factor to investigate will be the pH. Working at low pH, with the enzymes and LA incorporation would be interesting from a process point of view, as this more accurately resembles the conditions of

fermentation broth in the production of LA. Furthermore, taking into account the effect that the temperature presents on butanol incorporation, a broader study on the impact of this variable over the interactions between complex coacervates and butanol, including higher temperatures, seems promising.

Besides, future research could explore the incorporation of the enzymes in the complex coacervate in closer detail. The commercial product (PPL) could be purified beforehand to have a more accurate indication about the accumulation of this enzyme in the complex coacervate, due to the impurities present in the crude as can be seen in the electrophoresis results (Figure 4.1). Besides, it has been established that lipases are accumulated inside complex coacervates, suggesting they are equally distributed in this phase. Nonetheless, to have an understanding regarding its distribution inside this dense-polyelectrolyte media, an approach could be using microscopy with fluorescently label proteins. Using microscopy, the identification of the location of the enzymes inside the complex coacervate would be possible, studying the morphology and dynamics of the complex-protein interactions.

All the experiments carried out in this project follow a batch method, placing the components in contact and allowed phase separation, waiting for the system to reach equilibrium. However, to perhaps go one step ahead regarding a future application of complex coacervates in a continuous process another variables should be studied. Thus, the characterization of the complex, measuring its density, viscosity, among other properties would give us an insight regarding the viability of application in a continuous process, such as liquid-liquid extraction.

Considering the esterification reaction in complex coacervates, several questions still remain to be answered. Even though the results until now are preliminary, different actions can be followed to explore this possible application of complex coacervates. A progression of this work would be to analyse higher concentrations of the enzyme to promote the production of butyl lactate, and to avoid problems with detection limits in the analytical analysis, higher concentrations of the reagents should be considered. Moreover, the temperature is a factor that influences the activity of the enzymes, thus, higher temperatures (60 or 70°C) should be tested.

Regarding the analytical analysis, ion chromatography presented an excellent performance for the identification and quantification of LA. Nonetheless, 4mL are required to be able to make one analysis. Therefore, working with 500µL samples, dilution is necessary to measure its concentration in ion chromatography. For some samples, the LA couldn't be detected since the concentration was below the detection limit of the equipment.

In gas chromatography, a similar situation was observed with the diluted complex coacervate phase. Samples with a high concentration of polyelectrolytes and salts required an extra dilution to form one phase solution with acetone (necessary for the analysis). In future investigations, another analytical technique could be used to avoid some of these issues, such as liquid chromatography (HPLC). In this technique, a small volume sample (min 400µL) is required for the analysis, and the dilution with acetone is not necessary. Furthermore, the three molecules of interest: LA, butanol, and butyl lactate can be measured with the same technique, avoiding to make estimations.

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Appendix A - Calibration Curves

Appendix A.1. Calibration curve lactic acid concentration – Ion chromatography

A calibration curve was made to be able to determine the concentration of lactic acid throughout the different set of experiments. For this calibration curve, a stock solution of lactic acid [100mM] was prepared, followed by a series of dilution. Each sample was filtered using a 0,2µm pore size syringe filter before being injected to the separation column.

The ion chromatography analysis was done using a Grom Resin H+ IEX column (8 µm, 250 x 8 mm), 1 mM H_2SO_4 solution as mobile phase, column temperature of 45 °C and a flow rate of 0.6 mL/min, on a Metrohm 850 Professional IC; where the lactic acid presented a retention time of ~ 11,9 min as can be seen in Figure A. 1

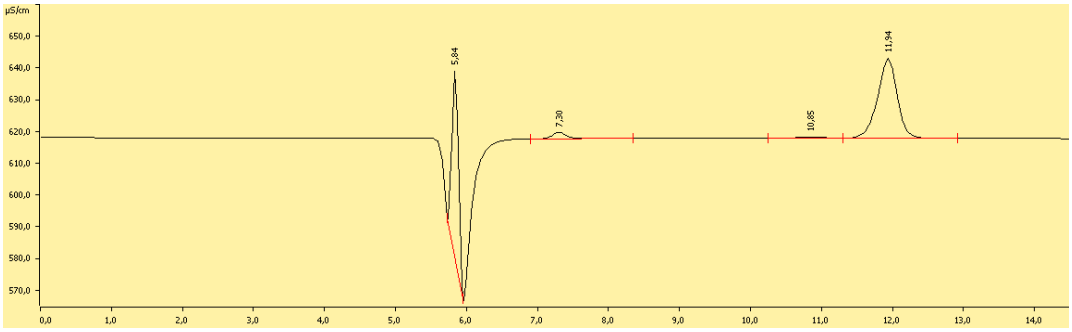


Figure A. 1. Ion chromatography result of a lactic acid sample

The concentration of lactic acid (c) and the peak area (A) of the ion chromatography presented a linear relation described by Equation A. 1 with a coefficient of determination of 0,9985, as can be seen in Figure A. 2, where the calibration curve is shown.

$$A = 0{,}6834c + 1{,}3085 \qquad A.\ 1$$

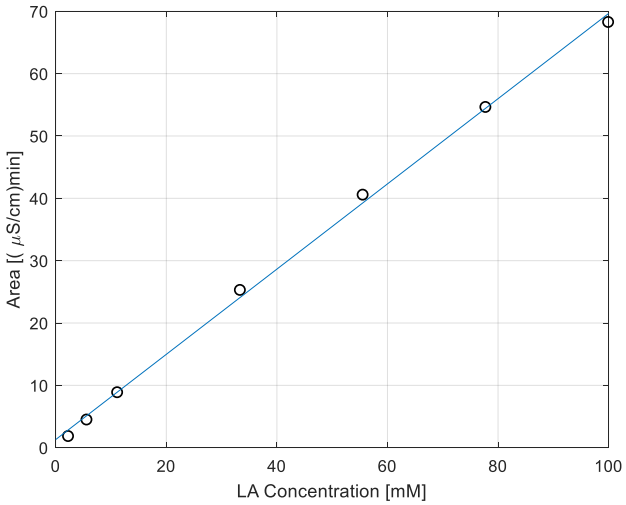


Figure A. 2. Calibration curve lactic acid concentration Ion chromatography

Appendix A.2. Calibration curve 1-Butanol Concentration – Gas Chromatography

The esterification activity of the enzymes was estimated by terms of the butanol and butyl lactate concentration. The butanol concentration was measured by a Thermo Scientific Trace 1300 gas-chromatograph equipped with a flame ionisation detector. The separation column used for the butanol detection was Agilent DB-1MS (length 60m, outer diameter 0,25mm and inner diameter 0,25 µm) with hydrogen as a carrier gas. The injection volume was 1µL and the detector temperature was maintained at 330°C; giving a retention time of 1-butanol of ~5,1min.

A concentrated stock solution of 1-Butanol [5M] was prepared, followed by a series of dilution. Each sample was mixed with acetone in a ratio 1:1 and filtered through a 0,2µm pore size syringe filter before being injected to the column.

The butanol concentration [c] and the peak area [A] presented a linear relation expressed by the following equation with a coefficient of determination of 0,9996, as can be seen in the calibration curve in Figure A. 3

$$A = 0{,}428c - 22{,}145 \qquad (6.1)$$

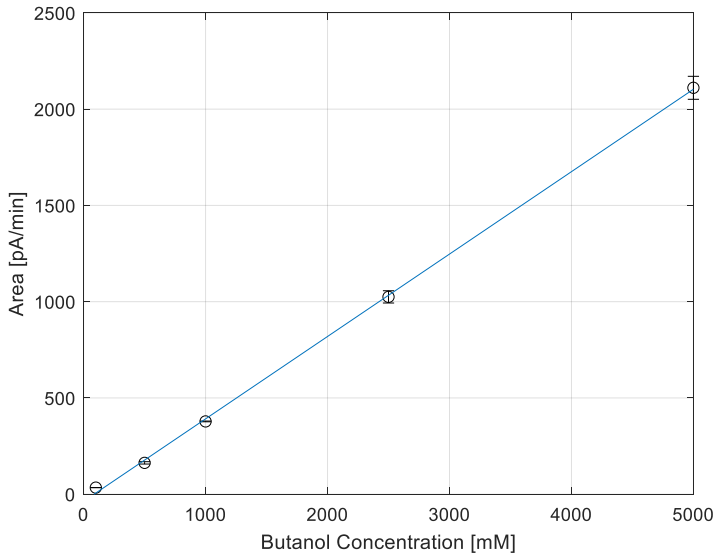


Figure A. 3. Calibration curve butanol concetration – Gas chromatograph. The error bars represent the standard deviation between 3 measurements on the same sample.

Appendix A.3. Calibration curve Butyl lactate Concetration – Gas Chromatography

The butyl lactate concentration was measured by a Thermo Scientific Trace 1300 gas-chromatograph equipped with a flame ionization detector. The separation column used for the butyl lactate detection was Agilent DB-WAX (length 60m, outer diameter 0,25mm and inner diameter 0,25 µ) with hydrogen as a carrier gas. The injection volume was 1µL and the detector temperature was maintained at 330°C; giving a retention time of butyl lactate of ~10,3 min, as it is shown in Figure A. 4.

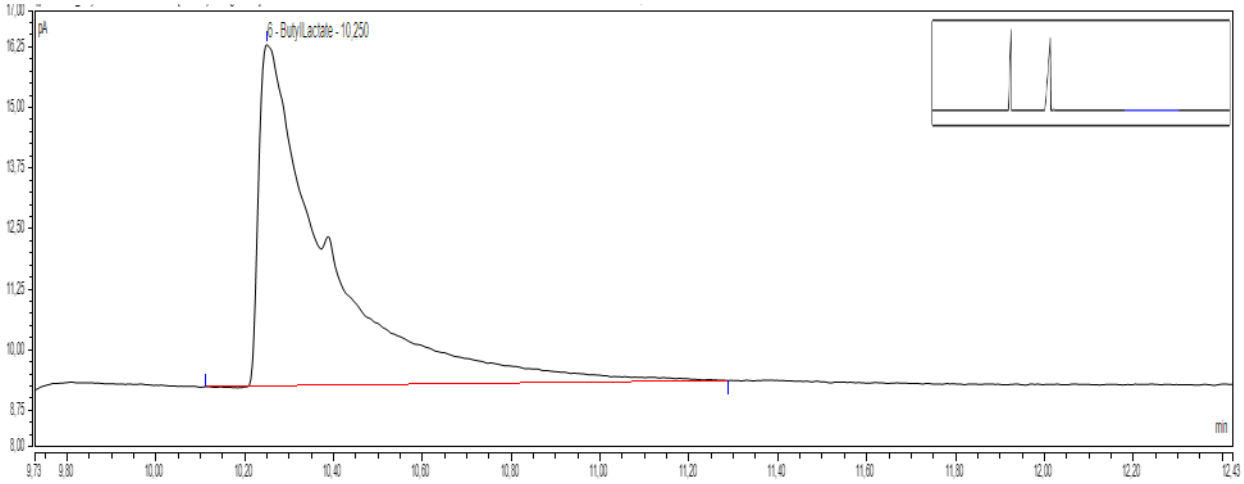


Figure A. 4. Gas chromatography results – Butyl lactate.

A stock solution of Butyl lactate [0,13 M] was prepared, followed by a series of dilution. Each sample was mixed with acetone in a ratio 1:1 and filtered through a 0,2µm pore size syringe filter before being injected to the column. The calibration curve shown in Figure A. 5 represents the linear relation between the butyl lactate concentration [c] and the peak area [A] with a coefficient of determination of 0,9996 represented in Equation A. 2.

$$A = 0{,}5822c - 0{,}9395 \qquad A.\ 2$$

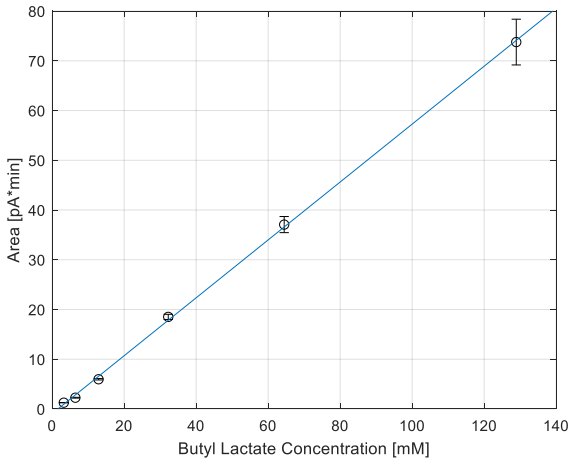


Figure A. 5. Calibration curve butyl lactate concetration – Gas chromatography. The error bars represent the standard deviation between 3 measurements on the same sample.

Appendix A.4. Protein analysis on polyacrylamide gel (SDS-page gel)

Polyacrylamide gel electrophoresis was used to evaluate the purity of the enzymes and to observe variances qualitatively in the incorporation of the proteins at different complex composition. In this appendix, the standard procedure is presented, where according to the molecular weight of the samples, a percentage of polyacrylamide gel is selected. Protein electrophoresis in acrylamide gel is used as a size-selective sieve, with the aid of an electrical field the gel's pore structure allows a faster migration of the smaller proteins in comparison to the larger ones.

1. Casting the gel
    a. The glass plates and spacers are assembled.
    b. A resolving polyacrylamide 12% gel is prepared: 4ml of acrylamide/bis 30%, 2,5ml of 0.15M (hydroxymethyl)aminomethane (Tris) (Merck, 1.08382.0500) adjusted to pH 8,8, 0,1ml of 10% sodium dodecyl sulphate, 0,1ml of 10% ammonium persulfate (Bio-Rad, 1610700), 0,004ml Tetramethylethylenediamine (Sigma-Aldrich, T7024) and 3,3ml of milliQ water.
    c. The resolving gel is poured into the gel plates up to 2cm below the top of the glass plate, adding a layer of demi water to prevent the inhibition of polymerization by the diffusion of oxygen into the gel.
    a. After the polymerization is completed (between 30 -40 minutes) the remaining water is removed from the top of the gel.
    b. A stacking gel is prepared: 0,67ml of acrylamide/bis 30%, 0,5ml of 0.15M (hydroxymethyl)aminomethane (Tris) (Merck, 1.08382.0500) adjusted to pH 6,8, 0,04ml of 10% sodium dodecyl sulphate, 0,04 ml of 10% ammonium persulfate (Bio-Rad, 1610700), 0,004ml Tetramethylethylenediamine (Sigma-Aldrich, T7024) and 2,7ml of milliQ water.
    c. The staking gel is poured into the gel plates on top of the resolving gel, followed by the insertion of the plastic comb to from the wells for the sample application.
    d. Left the staking gel to polymerize between 30 to 40 min.

2. Preparation of the samples
    a. Undiluted supernatant was mixed 1:1 with sample-buffer consisting of 10ml of 1.5 M tris(hydroxymethyl)aminomethane (Tris) (Merck, 1.08382.0500) ph 6.8, 6ml 20% glycerol (Merck, 356350) and 1.8 mg bromophenol blue (Bio-Rad, 161-0404) adjusted to 100ml with ultrapure water.
    b. 30µL of the mixture is placed in the slots of the gel. The electrophoresis was done at 50V for 40min and then the voltage was increased to 100V for 1 hour and 30 minutes.

3. After the electrophoresis, the gel was left to stain in a Coomassie blue staining solution overnight (2,5g of Coomassie Brilliant Blue R-250, 450 mL of methanol, 100 mL of acetic acid and 400 mL of water. Volume adjusted to 1 litre with ultrapure water). Following by the destaining with a solution of methanol/acetic acid /water (45/10/45 %volume) four times for 2 hours.

4. The gel was imaged with a ProteinSimple Fluorchem

Appendix B - Additional results

Appendix B.1. Peptide sequence of PPL and CALA

The extinction coefficient used for PPL and CALA was calculated according to the peptide sequence of the enzymes, while the extinction coefficient used for CALB is reported by Rabbani *et al*. (2015)[62], therefore its sequence is not implemented for the calculation of the extinction coefficient.

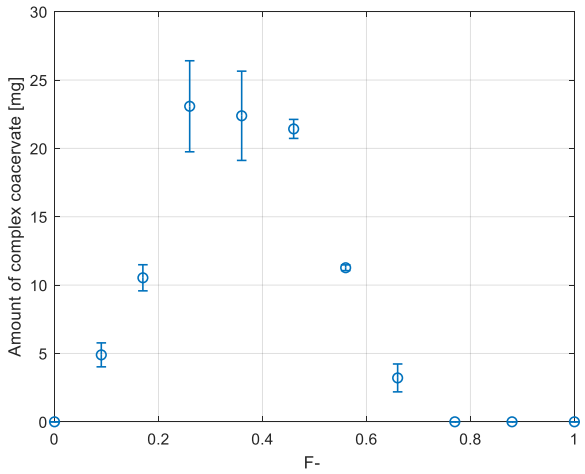
The peptide sequences of PPL and CALA were obtained from the Uniprot database (*www.uniprot.org)* and are presented below:

Table B. 1. Peptide sequence of PPL and CALA.

PPL	CALA
UniProtKB - F1S4T9 (F1S4T9_PIG) Protein: Triacylglycerol lipase Extinction coefficient: 68715 $M^{-1}cm^{-1}$ Molecular weight: 51579 Da	UniProtKB - W3VKA4 (LIPA_PSEA5) Protein: Lipase A Extinction coefficient: 54570 $M^{-1}cm^{-1}$ Molecular weight: 49263 Da
10 20 30 40 50 MLLIWTLSLL LGAVLGSEVC FPRLGCFSDD APWAGIVQRP LKILPWDPKD 60 70 80 90 100 VNTRFLLYTN ENQDNYQELV ADPSTITDSN FRMDRKTRFI IHGFIDKGEE 110 120 130 140 150 DWLSNICKNL FKVESVNCIC VDWKGGSRTG YTQASQNIRI VGAEVAYFIE 160 170 180 190 200 VLKSSLGYSP SNVHVIGHSL GSHAAGEAGR RTNGTIERIT GLDPAEPCFQ 210 220 230 240 250 GTPELVRLDP SDAKFVDVIH TDAAPIIPNL GFGMSQTVGH LDFFPNGGKE 260 270 280 290 300 MPGCQKNILS QIVDIDGIWE GTRDFVACNH LRSYKYYADS ILNPDGFAGF 310 320 330 340 350 PCDSYNVFTA NKCFPCPSEG CPQMGHYADR FPGTNGVSQV FYLNTGDASN 360 370 380 390 400 FARWRYKVSV TLSGKKVTGH ILVSLFGNEG NSRQYEIYKG TLQPDNTHSN 410 420 430 440 450 EFDSDVEVGD LQKVKFIWYN NVINPTLPRV GASKITVERN DGKVYDFCSQ 460 ETVREEVLLT LNPC	10 20 30 40 50 MRVSLRSITS LLAAATAAVL AAPATETLDR RAALPNPYDD PFYTTPSNIG 60 70 80 90 100 TFAKGQVIQS RKVPTDIGNA NNAASFQLQY RTTNTQNEAV ADVATVWIPA 110 120 130 140 150 KPASPPKIFS YQVYEDATAL DCAPSYSYLT GLDQPNKVTA VLDTPIIIGW 160 170 180 190 200 ALQQGYYVVS SDHEGFKAAF IAGYEEGMAI LDGIRALKNY QNLPSDSKVA 210 220 230 240 250 LEGYSGGAHA TVWATSLADS YAPELNIVGA SHGGTPVSAK DTFTFLNGGP 260 270 280 290 300 FAGFALAGVS GLSLAHPDME SFIEARLNAK GQQTLKQIRG RGFCLPQVVL 310 320 330 340 350 TYPFLNVFSL VNDTNLLNEA PIAGILKQET VVQAEASYTV SVPKFPRFIW 360 370 380 390 400 HAIPDEIVPY QPAATYVKEQ CAKGANINFS PYPIAEHLTA EIFGLVPSLW 410 420 430 440 450 FIKQAFDGTT PKVICGTPIP AIAGITTPSA DQVLGSDLAN QLRSLNGKQS 460 AFGKPFGPIT PP

Appendix B.2. Formation of complex coacervate as function of complex composition [F-]

The amount of complex formed between PEI and PAA is evaluated by mixing the polyelectrolytes at different complex composition [F-]. The experiments were done at a low salt concentration (10mM), neutral pH (pH=7), and a total polyelectrolyte concentration of 20g/L. The results are presented in



F-	Amount of complex [mg]
0,00	0,00 ± 0,00
0,09	4,89± 0,87
0,17	10,53 ± 0,95
0,26	23,08 ± 3,33
0,36	22,38 ± 3,26
0,46	21,42 ± 0,69
0,56	11,27 ± 0,21
0,66	3,21 ± 1,02
0,77	0,00 ± 0,00
0,88	0,00 ± 0,00
1,00	0,00 ± 0,00

Figure B. 1. Amount of complex coacervate as function of complex composition [F-] at 20g/L of total polyelectrolyte concentration.

From this data, it can be seen that the formation of complex coacervates between PAA and PEI takes place at a lower concentration of PAA, reaching a larger amount at a complex composition between 0,26 and 0,66 F-.

It is interesting to see that when the concentration of the negative polyelectrolyte exceeds the positive polyelectrolyte, there is no formation of the complex coacervate. This outcome could be attributed to the intricate structure of PEI. A more substantial amount of PAA can screen the exposed charges of the branched PEI, reducing further interactions between the two polymers with the hindered charges.

This experiment gives us an insight into the interactions between these two polyelectrolytes, detecting that at a low salt concentration a macro phase separation takes place at a complex composition between 0,09F- and 0,66F-. This range is used to evaluate further interactions between the complex coacervates with enzymes, lactic acid, butanol and butyl lactate as can be seen in chapter 4.

Appendix B.3. Protein partitioning as a function of the order of addition for the complex formation and mixing time

The formation of the complex coacervate is evaluated in the presence of an enzyme (PPL) as a function of the mixing time and the order of addition of the components of the system. All the samples contained 5 g/L of total polyelectrolyte concentration and 10mM of salt concentration. The mixing time was evaluated at a complex composition of 0,26 F-.

The results are presented in terms of the distribution coefficient [K_D] of PLL and can be seen in Figure B. 2

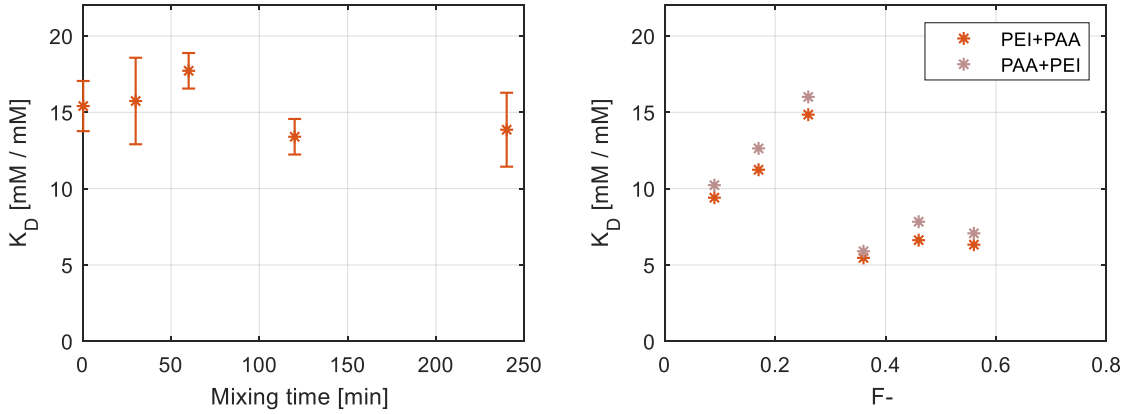


Figure B. 2. Lipases distribution coefficient [K_D] as function of the mixing time (left) and the order of addition (right) . PPL initial concentration 0,065 mM. The error bar represent the standard deviation of three experiments.

The mixing time was studied from 20 seconds up to 2 hours for the system at a constant mixing velocity of 1400 rpm in an Eppendorf Thermomixer. AS shown In Figure B. 2, the distribution coefficient of PPL is at the same order of magnitude for the different times evaluated, which indicates that the mixing time does not affect the partitioning of the lipases in the complex coacervate.

A similar result can be observed for the order of addition of the components in the mixture. For one of the experiments the positively charged polyelectrolyte is added first in a solution containing the negatively charged protein, then the solution is mixed, followed by the addition of the negatively charged polyelectrolyte. This variable was tested to verify if the possible formation of an intermediate polyelectrolyte between the lipase and PEI would enhance the incorporation of the enzyme. Nonetheless, when the experiments were conducted in the opposite order, no significant differences were observed.

Thus, it can be suggested from the results presented in this appendix that the partitioning of the lipases in the complex coacervate is nondependent of the order of addition and the mixing time when the system is evaluated at equilibrium.

Appendix B.4. Influence of PEI/PAA complexes on absorbance spectra

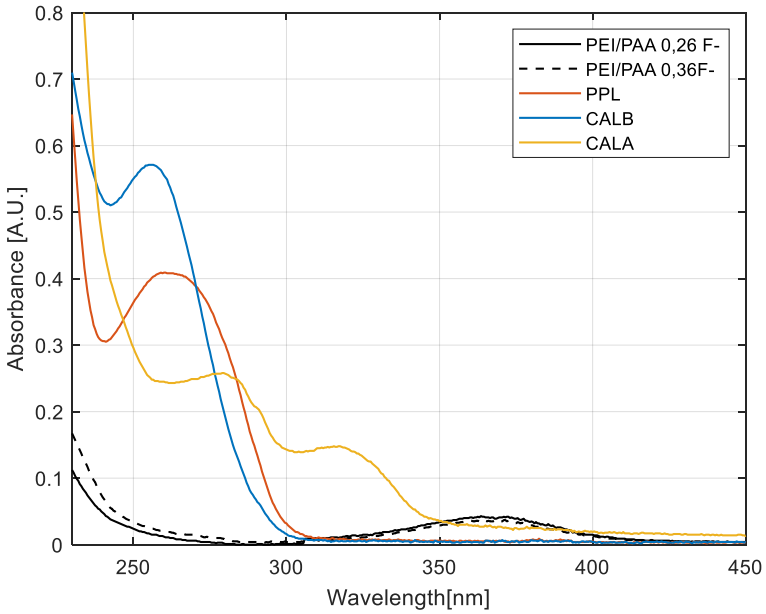


Figure B. 3. The absorbance spectrum of the supernatant of PEI/PAA complex coacervates compares to the absorbance spectrum of 0,065 mM of PPL, CALB and CALA. Lipases concentration is demined at 280nm, and all the samples were measured at a 13-fold dilution.

Absorbance measurement at 280nm is a stablish method to determine the protein concentration implementing a known extinction coefficient specific for a particular protein. The system studied in this project consists of PPL, CALA or CALB from (0,03 mM to 0,08mM), PEI, and PAA. In Figure B. 3 the absorbance of the supernatant of a complex coacervate formed at the highest concentration employed (20g/L) and at the optimal complex composition is presented, indicating that their absorbance does not significantly influence the protein concentration measurement at 280nm.

Appendix B.5. SDS-polyacrylamide gel electrophoresis (12%) at different complex compositions

In this appendix SDS-PAGE gel at 12% of polyacrylamide are presented for samples of the enzymes PPL, CALB and CALA incorporated at different complex composition. Here the supernatant is the representative sample, looking for a difference in the intensity of the lines when it is closer to the optimal complex composition. The first path on the left of each gel represents the ladder to relate the samples to the apparent molecular weight of proteins, followed by the supernatant of the experiments as a function of complex composition, from 0,2 to 0,6 F-.

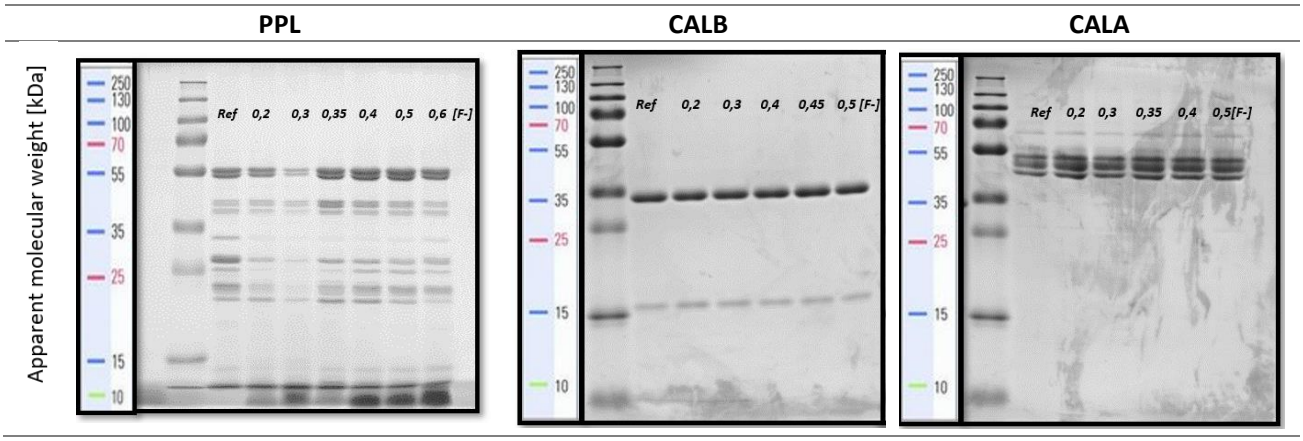


Figure B. 4. SDS-PAGE gel of the samples of the enzymes as function of the complex composition [F-]

Appendix B.6. Partitioning of butyl lactate in complex coacervates.

The results of section 4.4 are presented in this appendix. The initial concentration and final concentration, of butyl lactate after being in contact with the complex coacervate and its distribution coefficient can be seen in Table B. 2. The distribution coefficient of butyl lactate is defined as the concentration of butyl lactate in the complex coacervate divided by the concentration in the supernatant, as presented in Equation B. 1

$$K_D = \frac{[Butyl\ lactate]_{coacervate}}{[Butyl\ lactate]_{supernatant}} \qquad B.\ 1$$

For this experiments the method 2 from section 3.2.1 was followed. Neutral pH, a salt concentration of 10mM and a total polyelectrolyte concentration of 5g/L were the conditions studied. A butyl lactate initial concentration of 200mM was used to detect easily its concentration in the supernatant phase by gas chromatography.

Table B. 2. Results distribution of butyl lactate in complex coacervates. The values are presented as the average with the standard deviation of two experiments.

Complex Composition [F-]	**Enzyme**	**Initial Butyl Lactate [mM]**	**Final Butyl lactate [mM]**	K_D
0,26	None	195,57 ± 4,64	201,25 ± 14,61	-0,98 ± 4,41
0,26	PPL	195,57 ± 4,64	200,52 ± 0,22	-1,23, ± 0,09
0,26	CALA	195,57 ± 4,64	195,44 ± 8,23	0,55 ± 2,00
0,36	None	195,57 ± 4,64	210,10 ± 3,14	-3,06 ± 0,72
0,36	CALB	195,57 ± 4,64	204,46 ± 0,03	-1,60 ± 0,01

Appendix B.7. Partitioning of proteins – Supplementary results

Protein incorporation as function of F- (Section 4.1.1.)

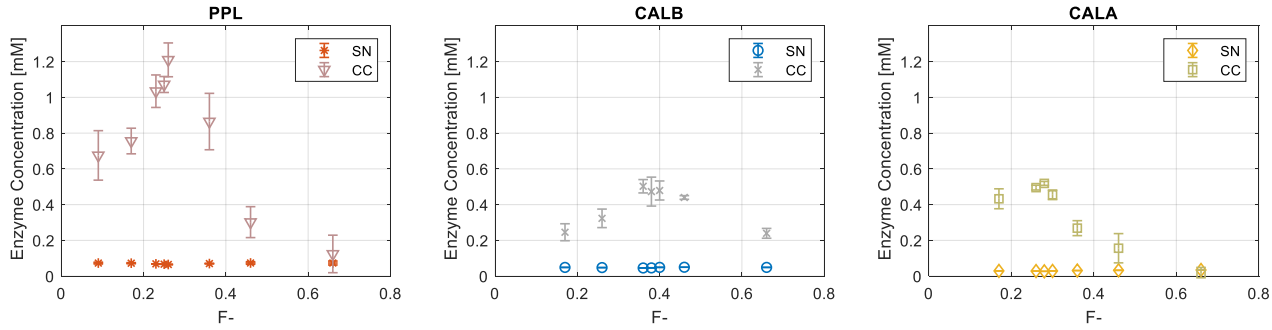


Figure B. 5. Lipases concentration in supernatant [SN] and complex coacervate [CC] as function of the polyelectrolyte composition F-. PPL initial concentration 0,083 mM. CALB Initial concentration 0,052 mM. CALA Initial concentration 0,035 mM. The error bars show the standard deviation over three experiments.

Protein incorporation as function of salt concentration [section 4.1.3]

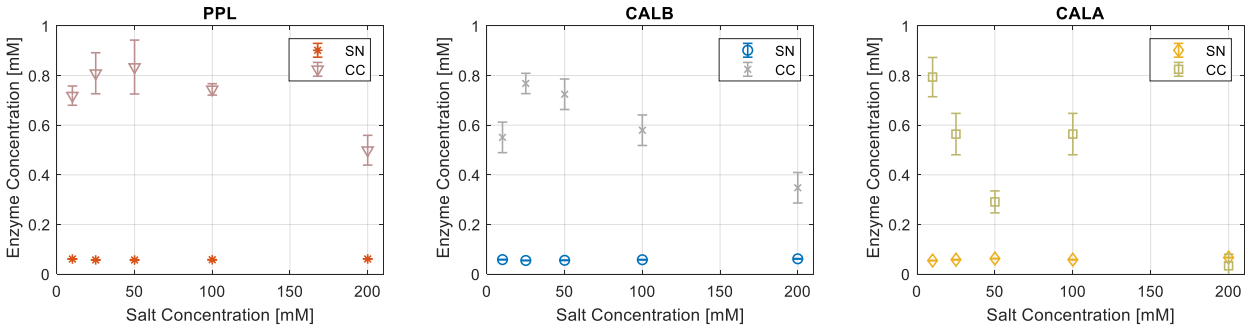


Figure B. 6 Lipases concentration in supernatant [SN] and complex coacervate [CC] as function of the salt concentration [mM].]. All enzymes were studied at an Initial concentration of 0,067 mM. The error bars show the standard deviation over three experiments.

Protein incorporation as function of total polyelectrolytes concentration [section 4.1.4]

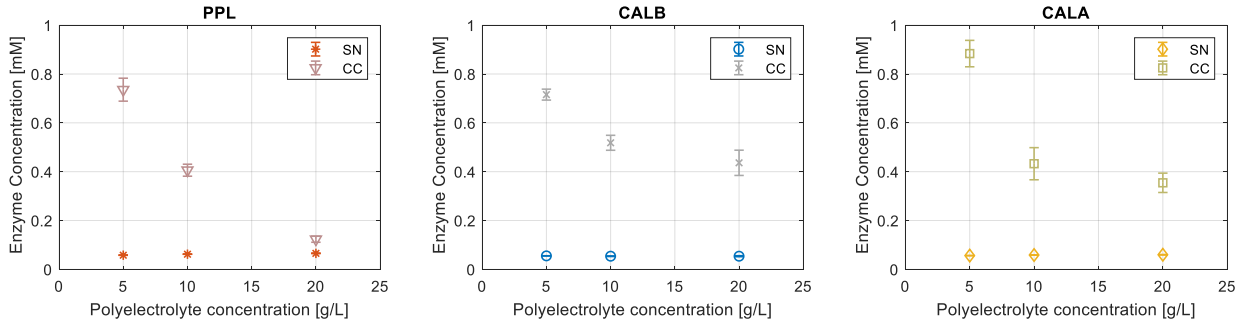


Figure B. 7. Lipases concentration in supernatant [SN] and complex coacervate [CC] as function of the total polyelectrolyte concentration[g/L]. All enzymes were studied at an Initial concentration of 0,067 mM. Salt concentration 10mM.The error bars show the standard deviation over three experiments

Appendix B.8. Partitioning of Lactic acid – Supplementary results

Partitioning of lactic acid in complex coacervates [Section 4.2]

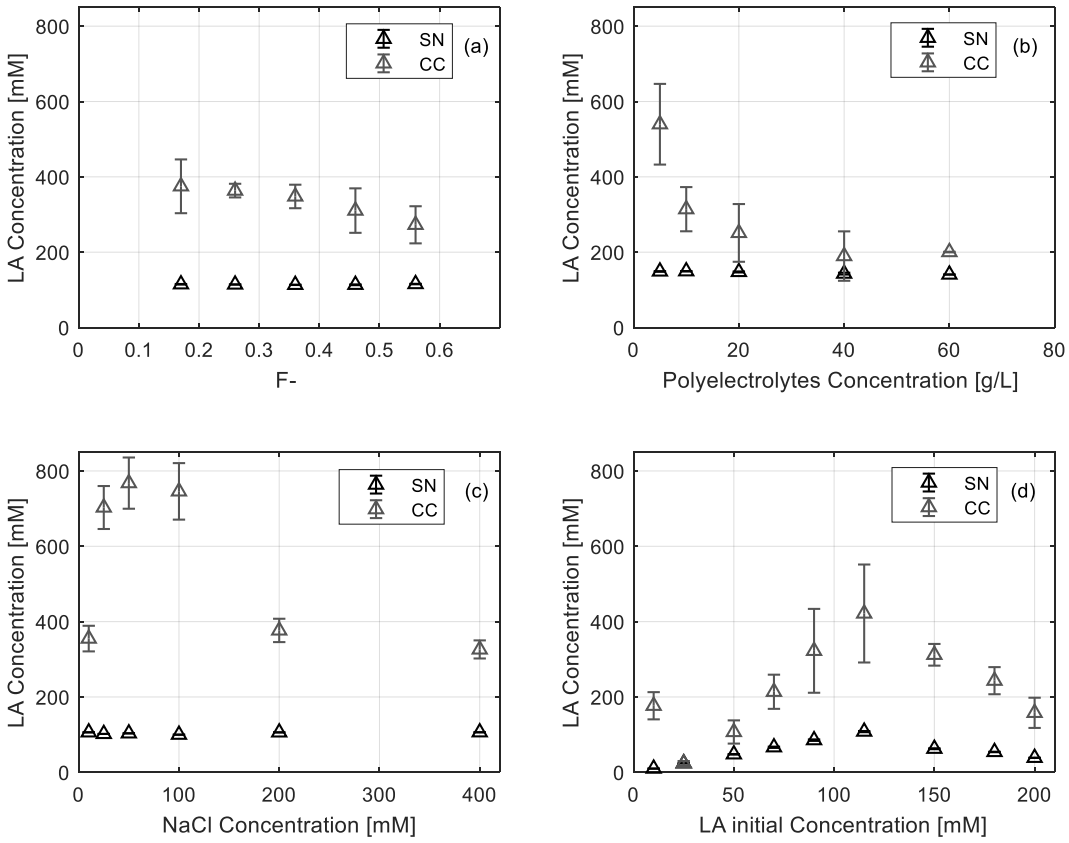


Figure B. 8. Lactic acid concentration in supernatant [SN] and complex coacervate [CC] as a function of (a) Complex composition (F-) (b) total polyelectrolytes concentration [g/L] (c) salt concentration [mM] (d) Initial lactic acid concentration [mM]. The error bars show the standard deviation over three experiments.

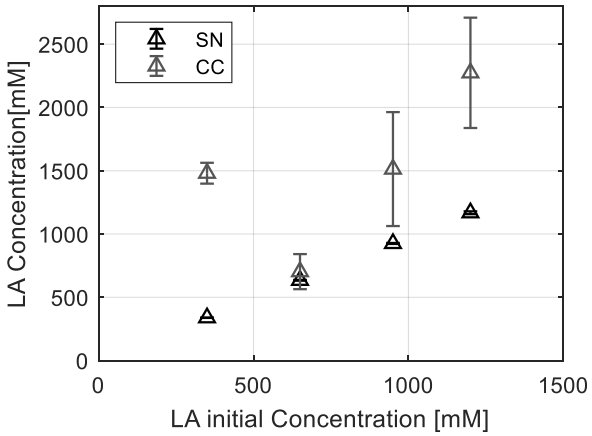


Figure B. 9. Lactic acid concentration in supernatant [SN] and complex coacervate [CC] as a function of Initial lactic acid concentration [mM].

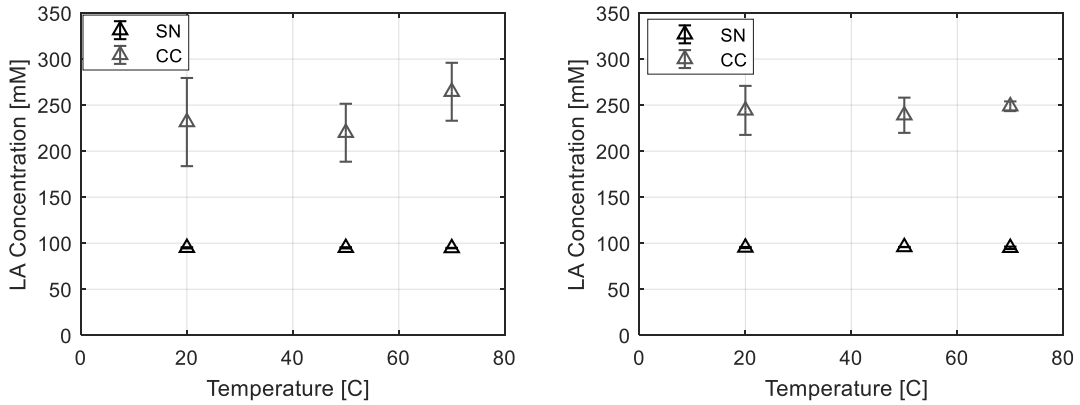


Figure B. 10. Lactic acid concentration in supernatant [SN] and complex coacervate [CC] as a function of temperature [°C]. At 0,3 F- complex composition (left). At 0,4 F- Complex composition (right). Initial concentration LA 100mM.

Appendix B.9. Partitioning of Butanol - Supplementary results

Partitioning of Butanol in complex coacervates [Section 4.3]

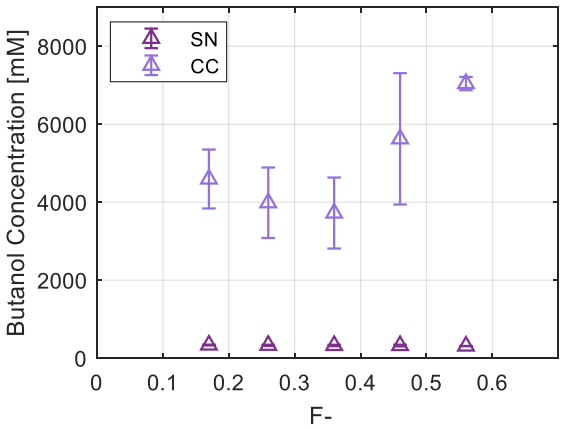


Figure B. 11. Butanol concentration in supernatant [SN] and complex coacervate [CC] as a function of Complex composition (F-).

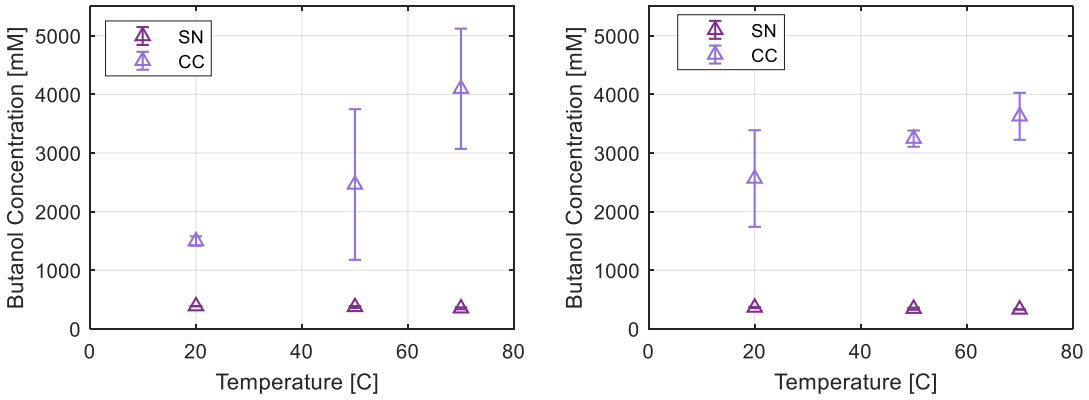


Figure B. 12. Butanol concentration in supernatant [SN] and complex coacervate [CC] as a function of temperature [°C]. At 0,3 F- complex composition (left). At 0,4 F- Complex composition (right) . Initial concentration butanol 400mM.