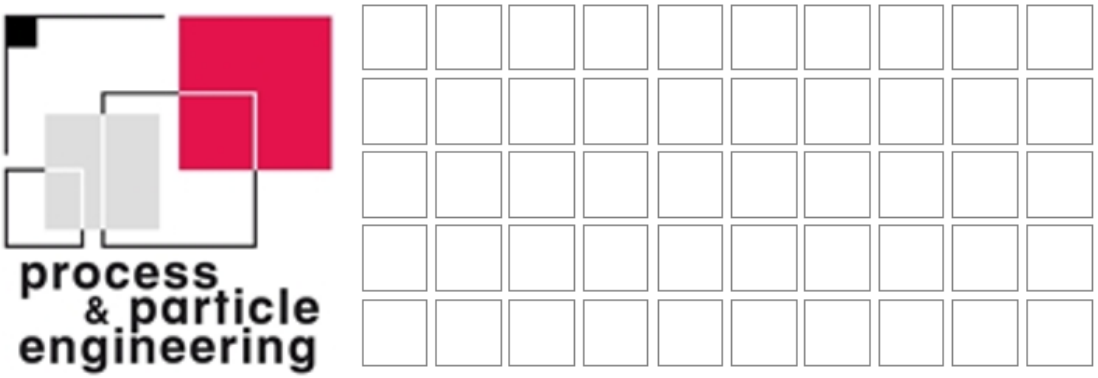
GRAZ UNIVERSITY OF TECHNOLOGY

Institute of Process and Particle Engineering



Qualitative and Quantitative Analysis of Crystals and Yield in Continuous Protein Crystallization

Project Thesis

Protein Crystallization

Written by FERNANDO MORENO LAVÍN

Advisor: PETER NEUGEBAUER

Graz, Austria at 30.06.2015

ABSTRACT

This essay is about the crystallization of proteins. Since the first published observation of crystallizing proteins about 160 years ago, protein crystals growth has developed into an extensive research field with many applications, for instance in the pharmaceutical industry. Nowadays, different fermentation, purification processes and recombinant technology produce potential protein drugs at acceptable cost which can be useful in various diseases through various routes like oral, transdermal, nasal, pulmonary, ocular, buccal, and rectal.

In this report, we focus on the continuous crystallization of proteins in a tubular plug-flow crystallizer. A solution of lysozyme from chicken egg was mixed with a crystallization solution and both of them went through three water baths at different temperatures along 13 meters of a silicone pipe. The different temperature of each water bath allows both nucleation and crystal growth, main steps in crystallization. The formed crystals are transported by air bubbles through the pipe to avoid the obstruction of the pipe.

Through the experiments, we have varied many parameters such as the temperature, the flow rate, the amount of Ionic Liquid in the crystallization solution or even the type of reactor (batch or continuous) to compare the yield in different crystallization conditions, as well as the crystal shape and the solubility.

CONTENTS

1. INTRODUCTION

1.1 MOTIVATION OF THE PROJECT

So, the question is: why would anybody want to spent efforts and time on the crystallization of proteins?

At the present, the main answer to the question stated above is provided by the fact that studies of the atomic structure of biological macromolecules (i.e. proteins, DNA, RNA, etc.) have proved of great value in revealing structure/function relationships that are of importance in our understanding of how enzymes, nucleic acids and other macromolecules operate in biological systems.

The elaborate information that can be obtained from the three-dimensional structure of a protein is useful in a variety of ways. Within the pharmaceutical industry, protein structure information can be helpful in the development of novel drugs. Since many pharmaceutical agents act by interacting with proteins, knowledge of the three-dimensional structure of a target protein can be used to design compounds that selectively bind to sites of this protein and thereby inhibit its activities.

Another highly promising application of protein crystallography is in protein engineering. Using molecular biology techniques, investigators can specifically alter protein molecules by site-directed mutagenesis. Furthermore, the protein crystallography is useful in the design of synthetic vaccines.

However, the crystallization of proteins is not only an excellent tool to obtain information about the three-dimensional protein structure, but also a very interesting subject for crystal growth studies in its own right. (Leunissen, 2001).

Concluding, Protein crystallization has two roles in the pharmaceutical industry: for structural biology applications involving in silico drug design and for controlled drug delivery. In the first application, protein crystallography ascertains the 3-D structure of a molecule. Producing better quality crystals will result in more accurate 3-D protein structures. This leads to a more precise understanding of biological function and improved drug design. (Lipp, 2006).

1.2 PROTEINS

Proteins are essential parts of living organism and participate in almost every cellular process. They are so called macromolecules, because the diameter is ~30-100 Å as compared to ~3 Å for most inorganic particles. Proteins can be considered as polymers of amino acids, linked together in a chain-like arrangement. The number of amino acids constituting one protein molecule ranges approximately from 100 to 27000. The specific sequence of amino acids in a protein is called its “primary structure”.

Natural proteins are built up by multiple numbers of twenty different amino acids. The general structure of an amino acid can be seen in figure 1.1. In each amino acid, the amino and carboxyl

groups make one bond each with the so called α-carbon atom, Cα (first carbon atom that attaches to a functional group). One of the other two bonds of Cα is occupied with a group, called the "side chain", which is different for each of the twenty amino acids, (Ratnaparkhi, Chaudhari, & Pandya, 2011).

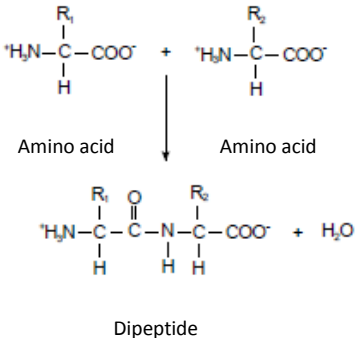


Figure 1.1. Primary structure of proteins

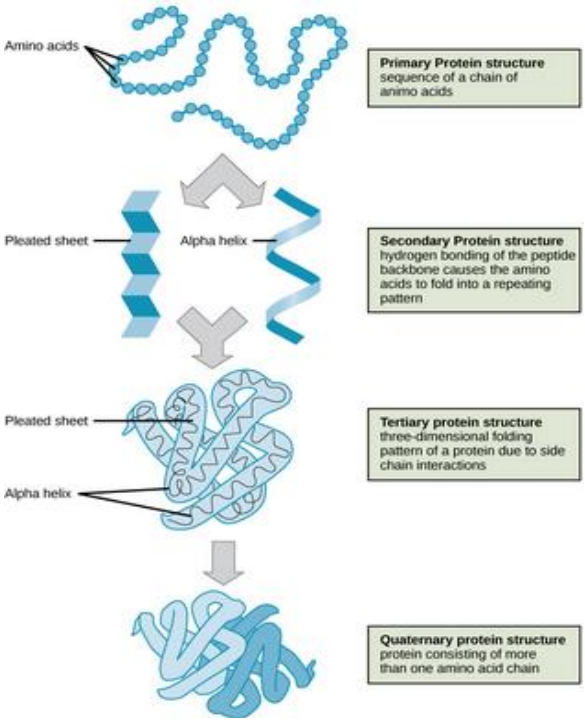


Figure 1.2. Protein structure

The same amino acids sequence (the primary sequence) can occur in different conformations through different azimuthal rotations of the side chain about peptide links. This determines the "secondary chain", folded into alpha-helices and beta-sheets. This secondary chain lowers its free energy by folding to a specific three-dimensional structure, the so called "tertiary structure". This three-dimensional structure represents the final folding of a polypeptide chain, but some proteins display another level of organization, the so called "quaternary structure". (Leunissen, 2001)

1.2.1 PROPERTIES OF PROTEINS IN SOLUTION

The amino acids side chains present on the protein surface play an important role in the way the protein behave in solution. In other words, the properties of proteins depend on those amino acid side chains. There are two classes of charged amino acids: acidic and basic. At a neutral pH, which centres the normal physiological range, the acidic amino acids are negatively charged and the basic amino acids carry a positive charge.

Like all polar side chains, the charged groups interact with water and tend to solvate the protein. Moreover, exposed hydrophilic groups on macromolecules can bind not only water molecules but also a variety of ions, both cations and anions. Lysozyme, for instance, has 10-12 positive charges at pH 4.5. As a consequence, under these solution conditions repulsion dominates the interactions between these macro-ions.

At the iso-electric point, (pH = 11.3 for lysozyme and between 5 and 7 for most other proteins) there are equal numbers of positively and negatively charges and as a consequence at this pH value the net surface charge vanished.

The net surface charge of a protein molecule changes with the pH of the solution and consequently, the solubility of the protein changes with the pH too. For a particular protein, with its own specific sequence of amino acids side chains, the solubility dependence on the solution pH is given by the following equation [1]: (Leunissen, 2001)

$$[P] = [P_{\pm}] + \sum_i [P_i] + \sum_j [P_j] = K_{\pm}[1 + \gamma_{\pm} \sum_i (K_i / [H^+]\gamma) + \gamma_{\pm} \sum_j ([H^+] / K_j \gamma)] \times \exp(-\alpha_0 s_0 / kT)$$

[P] = the total concentration of dissolved molecules
$[P_{\pm}]$ = the zwitterionic state of the protein molecule
$[P_i]$ = negatively charged states of the protein molecule
$[P_j]$ = positively charged states of the protein molecule
α_0 = the surface free energy of a protein molecule in solution
s_0 = the surface area of a protein molecule in solution
γ = activity coefficient
K_x = (de)protonation reaction constant side chain

Equation 1

1.2.2 PROTEIN SOLUTION

The protein solution must be prepared extremely exact in order to avoid unexpected results like precipitation, which takes place when the protein concentration is much higher than the equilibrium concentration. The main goal is to get the supersaturation (driving force for the generation of crystal nuclei and the growth of crystals) but to a certain extent, if it crosses the limit, precipitation occurs.

- At saturation $\mu_{solid} = \mu_{solution}$ (μ = chemical potential)

- At supersaturation $\mu_{solid} < \mu_{solution}$

1.3 CRISTALLIZATION

Crystallization is the process of formation of solid crystals originating from a solution, melt or more rarely deposited directly from a gas. Crystallization is also a chemical solid–liquid separation where the temperature plays an important role. Figure 1.3 illustrates a technique, in which mass transfer of a solute from the liquid solution to a pure solid crystalline phase occurs.

As we have seen during the experiment, the temperature plays an important role during the crystallization. Figure 1.3 illustrates the general thermodynamic phase behaviour of a solute-solvent system and the concepts of thermodynamic equilibrium and thermodynamic stability for a solid-liquid systems.

The solid line of the Figure 1.3 is the solubility curve, which represents thermodynamic equilibrium between the liquid and solid phase. So, any liquid with a composition and

temperature above the solubility curve exists as stable unsaturated liquid. On the other hand, any liquids composition and temperature below the solubility curve is not in thermodynamic equilibrium with respect to formation of a solid phase (it is supersaturated).

The region between the solubility curve and the limit of stability (the dashed line) is called the metastable zone. In this region, nucleation sites are necessary to initiate the formation of a solid phase from a supersaturated liquid.

Below the metastable zone boundary, however, the presence of nucleation sites is not a requirement for the formation of a solid phase. This is the unstable region, in which any supersaturated liquid is neither stable nor in equilibrium and therefore is subject to spontaneous nucleation and the formation of a solid phase, (Samant & O´Young, 2006), (Hekmat, Hebel, Schmid, & Weuster-Botz, 2007).

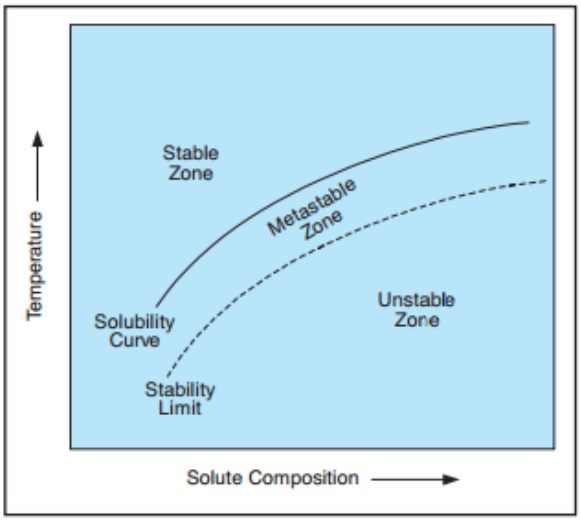


Figure 1.3. Solid-Liquid Phase Behaviour

1.3.1 PROPERTIES OF PROTEIN CRYSTALS

Protein crystals differ from crystals of most other compound in several respects. One of the major differences is the high solvent content of protein crystal, which is responsible for most of their unique features (Leunissen, 2001).

Depending on the conditions where the crystals are forming, they can crystalize, precipitate, aggregate or get denatured. There are many reason which show the difficulty of protein crystal growth. Apparently, protein molecules are very complex (large, flexible molecules often composed of several subunits), relatively chemically and physically instable (unfolding, hydration requirements, temperature sensitivity) and they have dynamic properties. If the solution changes, the molecules´ properties change too. So then, the external conditions and the protein properties influence the properties and the shape of the crystals. As already mentioned, the protein crystals could suffer:

- Denaturation: Non-proteolytic of a unique structure of a native protein that affects definite change in physical, chemical and biological properties. Several examples of denaturating agents are urea, alcohol, acetic acid.
- Aggregation and precipitation: the denatured, unfolded protein may rearrange in such a manner that hydrophobic amino acid residue of various molecules associate together to form the aggregates. If aggregation is on macroscopic scale, precipitation occurs.

1.3.2 PROTEIN CRYSTALLIZATION

In our case, the crystallization took place along a silicone tube, where supersaturation was controlled using water baths which divide the crystallizer into a nucleation zone and a growth zone. Both steps are illustrated in the Figure 1.4 according to his own free energy (ΔG), (Asherie, 2004).

- Nucleation (formation of a solid phase at a more or less constant supersaturation)
- Crystal growth (supersaturation decreases until solubility equilibrium is reached), (Mesters, 2007)

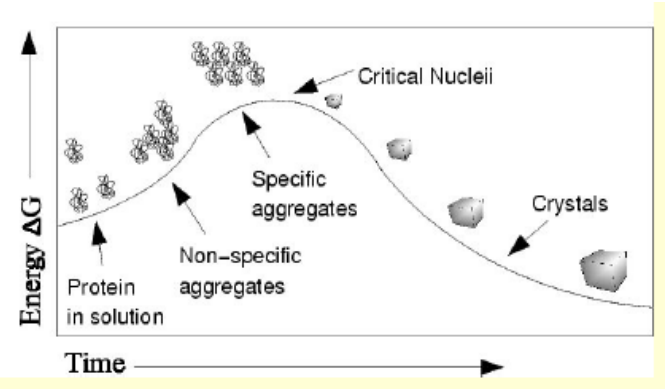


Figure 1.4. Nucleation and crystal growth

Both mechanisms were studied in the past for small molecules and also for proteins using kinetically and thermodynamically based models. Nowadays population balance models are used to predict and optimize crystallization processes.

Nucleation is the step where the solute molecules dissolved in the solvent start to gather into clusters, in the nanometre scale that become stable when they reach a critical size. This stable clusters constitute the nuclei. However, when the clusters are too small, they tend to dissolve again (see Figure 1.4). Therefore, the clusters need to reach a critical size in order to become stable nuclei. Such critical size is dictated by the operating conditions (temperature, supersaturation…). In this stage, the molecules arrange in a defined manner that defines the crystal structure. The crystal structure is related to the arrangement of the atoms, not to the properties of the crystals (size and shape).

The crystal growth is the subsequent growth of the nuclei that succeed in achieving the critical cluster size. Nucleation and growth tend to occur simultaneously depending on the level of supersaturation, (De Yoreo & Vekilov, 2003).

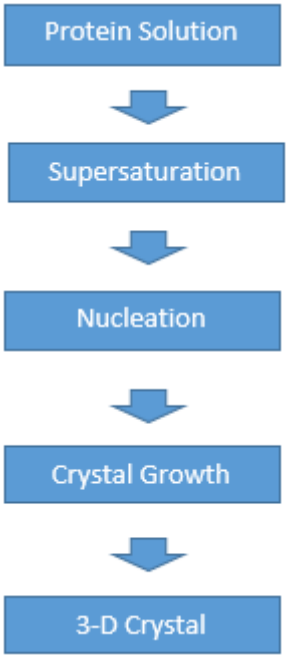


Fig.1.5. Crystallization process

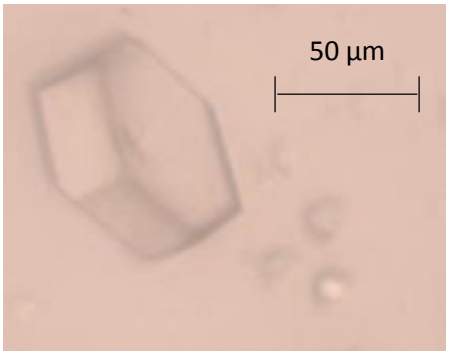


Figure 1.6. Protein crystal from discontinuous crystallizer at 10ºC and with 40 mg/ml of IL

As it is shown in the Figure 1.5, the production of crystals requires to reach the supersaturation area, which means that the solution contains more than the maximum amount of dissolved solute at the respective temperature.

By Figure 1.6 we can draw conclusions about the dimension of the crystals and their geometry. The picture was taken by the Leica Microscope with scale bar, 20x magnification. (Neugebauer & Khinast, 2015).

The next solubility diagram (Figure 1.7) shows how the supersaturation area can be reached by changing the crystallization agent concentration or the temperature or simply both of them at the same time.

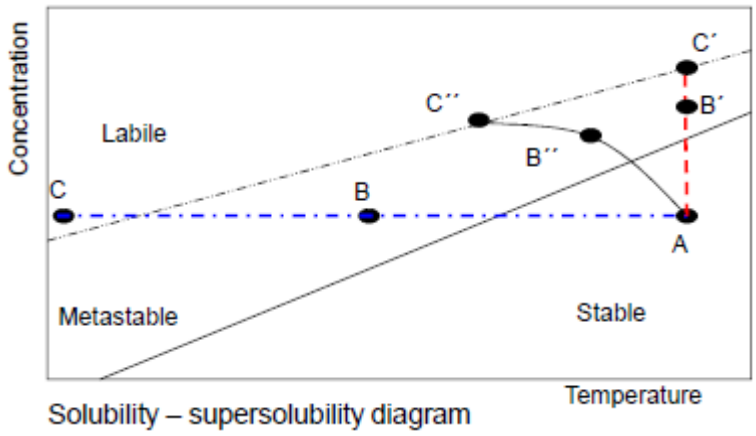


Figure 1.7. Different ways to achieve supersaturation area

Reaching the nucleation zone, which belongs to the supersaturation area, means that the first crystals could start appearing. However, it is very important to avoid the precipitation zone because there, the crystals lose their shape and their characteristic edges. All crystals have an external layer which allows them to keep their shape. However, at certain external conditions this layer could get broken, leading to precipitation (loss of the external layer).

Our crystallization process is represented clearly in the Figure 1.8, (Introduction to Protein Crystallization, 2011), (Huettmann, Zich, Berkemeyer, Buchinger, & Jungbauer, 2015). As we have already said, the first goal was to reach the supersaturation area as it´s described above. The protein concentration decreases along the process almost continuously due to the formation of crystals as the coloured lines show. Once in the metastable zone, the solution is still supersaturated but no new nuclei will form.

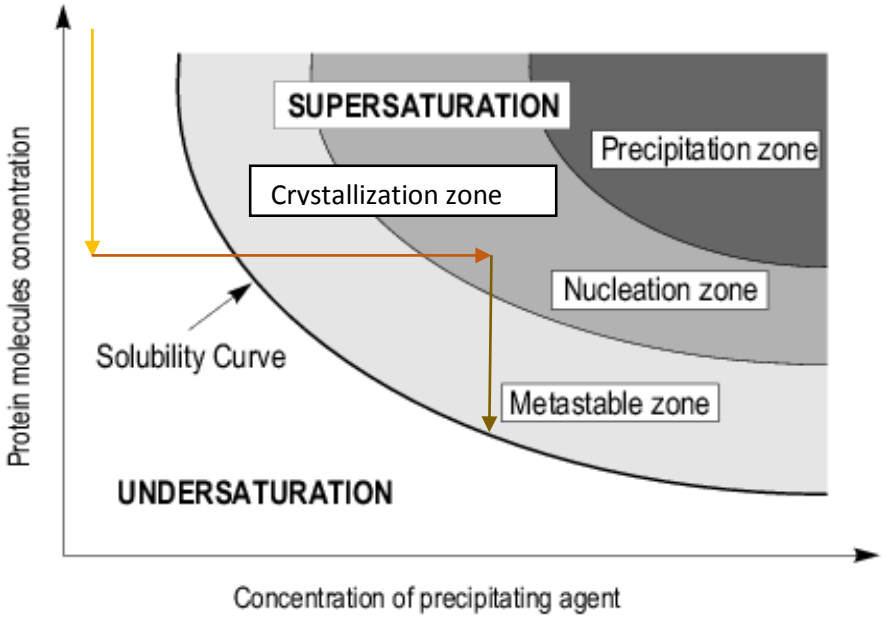


Figure 1.8. Solubility curve

1.4 IONIC LIQUID

Ionic liquids are salts consisting of ions, which exist in the liquid state at ambient temperature. They show reasonably high ionic conductivities. Although the first ionic liquid, ethylammonium nitrate was reported as early as 1914, ionic liquids have found great interest only recently.

Due to their high polarities, the ionic liquids are expected to be very suitable solvents for the reactions between organo-soluble and water soluble reagents, (Bicak, 2005).

The conductivity of the liquid at room temperature is 3.3 $mS \cdot cm^{-1}$, which is reasonable high. The ionic conductivity, σ, depends largely on temperature and rises exponentially as the temperature increases and reaches 40 $mS \cdot cm^{-1}$ at 92ºC. This can be ascribed to fast ion mobility at elevated temperatures.

Many experiments have been run after the IL discovery, and clearly, large crystals were obtained using the IL compared to the experiment without the addiction of IL. The mechanism of the influence of the IL on the protein crystals size distribution are quite complex and largely unknown. The strongest impact is attributed to the interaction of ions and counter ions at the protein solution interface, as the Figure 1.9 shows. Here, the degree of hydration of the various ion species plays a major role, (Judge, et al., 2009), (Wang, Dang, Han, Jiang, & Wei, 2010), (Pusey, Paley, Turner, & Rogers, 2007), (Bicak, 2005) .

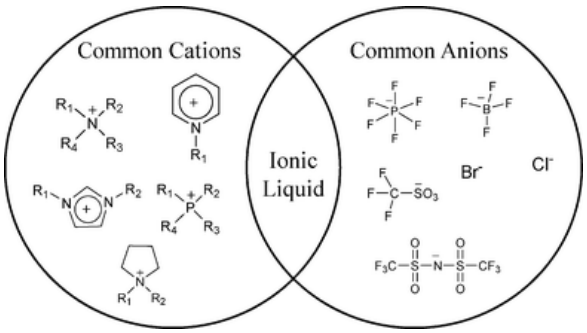


Figure 1.9. Most common Cations and Anions for the Ionic Liquid formation

1.5 LYSOZYME

Lysozyme is a single chain polypeptide of 129 amino acids cross-linked with four disulphide bridges. It hydrolyses β(1-4) linkages between N-acetyl-muraminic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrin (Sigma-Aldrich, n.d.), ((UniProt), 2015).

The commercial lysozyme was purified from chicken egg white, crystallized three times, dialysed and supplied as a lyophilized powder. The protein content by UV absorbance is >90% with the remainder (~10%) being buffer salts such as sodium acetate and sodium chloride.

Its molecular mass is 14.307 Da (amino acid sequence) and its isoelectric point is 11.35. Furthermore, the activity of lysozyme is a function of both pH and ionic strength. The enzyme is active over a broad pH range (6.0 - 9.0). At pH 6.2, maximal activity s observed over a wider range of ionic strengths (0.02 – 0.100 M) than at pH 9.2 (0.01 – 0.06 M).

The lysozyme from chicken egg must be stored between 0 -5ºC for its proper use.

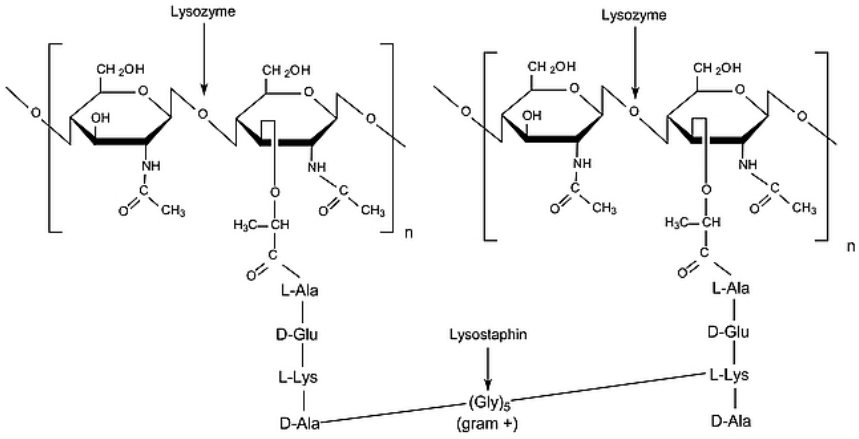


Figure 1.10. Lysozyme specificity: Peptidoglycans are polymers of β (1-4) –N-Acetyl-D-glucosamine units

1.6 SPECTROPHOTOMETRY

Spectroscopy is a technique that measures the interaction of molecules with electromagnetic radiation. Light in the near-ultraviolet (UV) and the visible (vis) range of the electromagnetic spectrum has an energy of about 150-400 KJ mol^{-1}. The energy of the light is used to promote electrons from the ground state to an excite state. A spectrum is obtained when the absorption of light is measured as a function of its frequency or wavelength. Molecules with electrons in delocalized aromatic systems often absorb light in the near-UV (150-400 nm), as the proteins, or the visible (400-800 nm) region, (Schmid, 2001).

This method has been used to measure the protein concentration of our samples either in the continuous process or in the discontinuous one. The concentration (C) of proteins in solution can be easily and accurately determined by absorbance (A) measurements according to the Lambert-Beer law [2], where (L) is the pathlength and (ε) is the molar absorption coefficient, with a value of 2.48 ($L \cdot mol^{-1} \cdot cm^{-1}$).

$$A = \varepsilon \cdot C \cdot L \qquad \text{[Equation 2]}$$

The measurement of absorbances higher than 2 should be avoided, because only 1% of the incident light is transmitted through a solution with an absorbance of 2.

Proteins usually show absorption maxima between 275 and 280 nm (Figure 1.11), which are caused by the absorbance of two aromatic amino acids tryptophan (Trp) and tyrosine (Tyr) and, to a small extent, by the absorbance of cysteine.

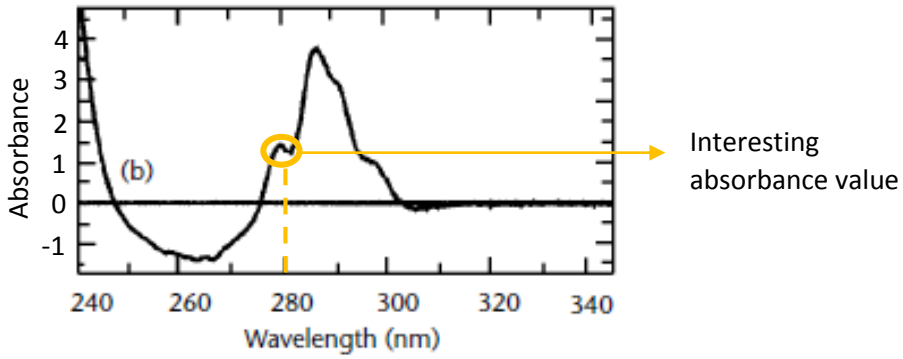


Figure 1.11: Ultraviolet absorption spectrum for proteins

2. MATERIALS AND METHODS

Different crystallization techniques have been used all over this work such as continuous or discontinuous reactor to compare their influence on the crystallization. Those techniques are:

- Batch/Discontinuous reactor: All the reactants are charged before the reaction takes place, and the products are discharged when the reaction is over
- Continuous reactor: Continuous charging and discharging of reactants and products respectively.

Furthermore, we have been varying many parameters in order to check out how the yield and the crystal´s shape change for different crystallization techniques. The flow rate, the temperature of the water baths, the NaCl concentration and the Ionic Liquid concentration are parameters which have a strong influence on the crystallization, so the variation of them gave us interesting results. Therefore, the variations of this parameters allowed us to study different points of the crystallization:

- Yield progression research for different process
- Solubility research and study of the crystal´s shape

To carry out the experiments, several solution were prepared accurately. Depending on the purpose of the experiment, the solutions were prepared in different ways. The following ones are useful for both kind of experiments:

- **BUFFER SOLUTION**
  This solution is used at several points all over the process. For instance, both to dissolve the samples which are taken after each experiment and for the preparation of the protein solution. Besides, as we discuss in the next page, the buffer solution is used as the reference sample in the spectrophotometer if the experiment was not carried out with Ionic Liquid.

In order to prepare it, I dissolved the following amounts in 1 litre of deionized Water

- 16 grams of NaCl
- 2.03 grams of NaOAc

At the beginning, the solution is a little basic, pH = 8.146, and we need it at a pH of 4.6. Therefore, is necessary to add HCl, drop by drop, until reach the goal value.

- **PROTEIN SOLUTION**
  Lysozyme from chicken egg is conserved daily at a constant temperature of 5ºC. One gram of lysozyme is weighed just before each experiment in a scale and it is dissolved to a total volume of 10 ml in buffer solution.
  The protein solution was filtered using a 0.2 µm filter to remove any contaminants possible acting as crystallization nuclei.
  Once it is prepared, the solution volume is a little smaller than 10 millilitres due to the filtration process and its concentration is 100 mg/ml.

- **REFERENCE SAMPLE/BLANK SOLUTION**
  The blank solution is essential for the measurement of the protein concentration in the spectrophotometer. The difference between the reference solution and the sample is that the concentration of substance that we are measuring is zero in the reference one. So, the reference tube transmits as much light as is possible. A sample tube with some concentration of the assayable substance absorbs more light than the reference, thereby transmitting less light to the photometer.
  The preparation of the reference solution depends on using of Ionic Liquid. If the experiment was run without IL, the blank consist just of the buffer solution. However, if we used IL, the blank solution must have the same IL concentration as the samples.

Now, we focus on the used methodology to achieve the different goals, either the study of the yield in batch and continuous reactor or the study of the solubility and the crystal shape.

2.1 YIELD EXPERIMENTS

For this experiments, the solutions which were mentioned above were required, but, apart from them, we needed to prepare the crystallization agent, which can be prepared in two different ways:

- **Crystallization agent**

3. 10 ml of NaCl with a concentration of 64 g/l.

4. NaCl solution plus Ionic Liquid

   - 8 ml of NaCl solution with a concentration of 80 g/l
   - 2 ml of Ionic Liquid

**The volume of the protein solution and the volume of the crystallization agent solution must be the same, mainly when we work at batch process.*

In the yield experiments we have worked with both kind of reactors, the continuous and the discontinuous reactor in order to compare them and check out which is their influence on the crystallization process.

2.1.1 Continuous reactor

The continuous reactor (Figure 2.1) is based on three water baths in series at different temperatures and with a certain pipe length for each one as the Table 2.3 shows. The different temperatures provide the perfect conditions for the crystallization performance. It means that the nucleation and the crystal growth take place in different tanks (B1, B2 and B3 respectively).
We have been working with two syringe as a pump mechanism, one (1) with the protein solution (100 mg/ml) and the other (2) with the crystallization agent solution, which could work with or without Ionic Liquid, as I described in the previous page.

At the reactor inlet (B4), the protein solution was mixed with the crystallization agent solution to create high supersaturation required for nucleation.

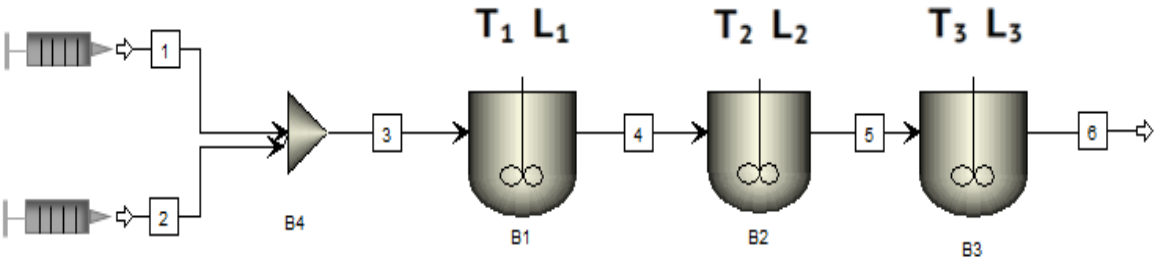


Figure 2.1. Set-up of the Continuous Crystallizer

The space between the mixer (B4) and the first water bath (B1) is crucial because here both solutions get mixed and the crystals, depending on the crystallization solution concentration as well as on the temperature, could start forming. Besides, just after the mixer is where the bubble maker (Figure 2.2) is located. This is a syringe filled with air, which provides air bubbles manually each 10 centimetres in order to transport the formed crystals along the tube, avoiding the pipe blocking.

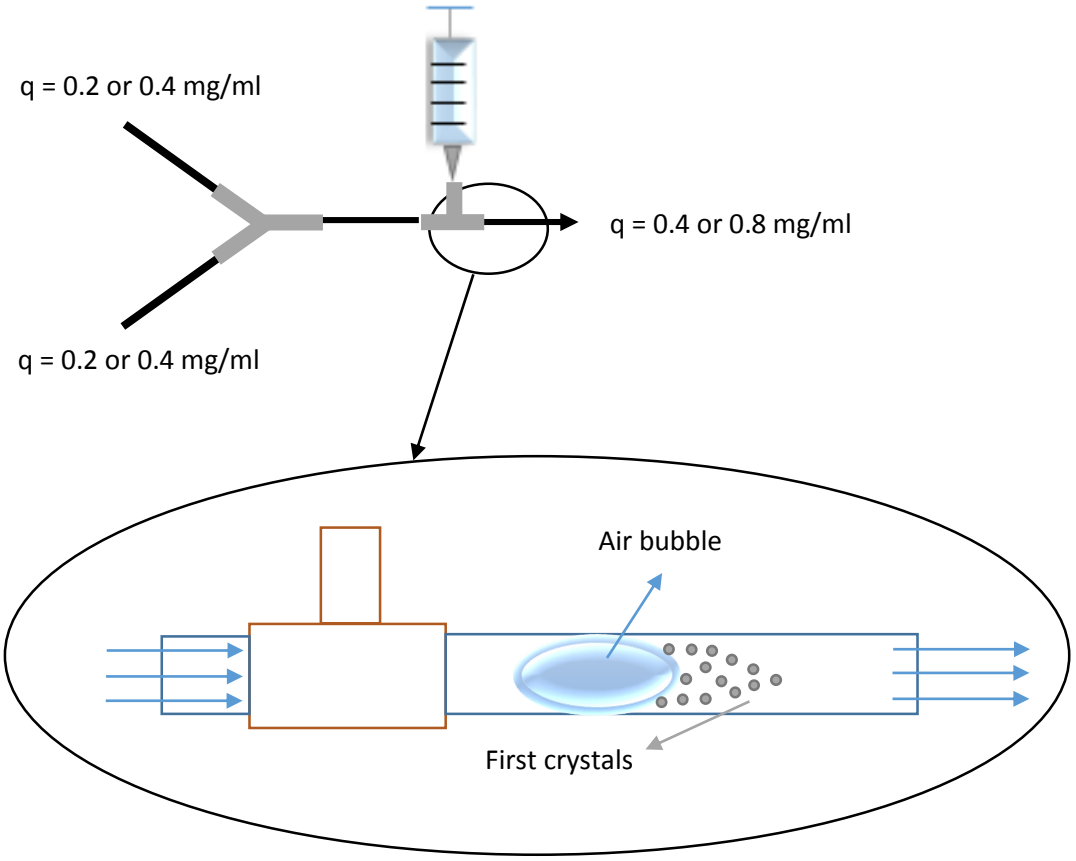


Figure 2.2. Bubble maker device

The temperature of the first water bath was determined by the supersaturation required for nucleation. In the next sections, supersaturation was chosen to be below the critical nucleation level in order to promote growth of nuclei to microscope crystals. The temperature in the third water bath was chosen on account of the low lysozyme concentration and to increase crystal growth. The next table, table 2.3, shows a summary of the temperature and the pipe length in each water bath.

	1º water bath	2º water bath	3º water bath
Temperature (ºC)	21.5	22	20
Length (m)	3	5	5

Table 2.3. Temperatures and lengths of each water bath in the continuous reactor

At a total flow rate of 0.4 ml/min, the overall residence time inside the crystallizer was approximately two hours. All over this experiments, we have been investigating the evolution of the yield along the three water baths, which involves the collection of samples at the end of the pipe. Usually, three samples of 200 µl each were enough. The samples were taken with Eppis (Volume = 1000 µl) and immediately were brought to the centrifuge trying to avoid touching or shaking them. The centrifuge works usually at 22ºC (depending on the characteristics of the experiment) and 15000 rpm during 2 minutes. The crystals get deposited in the bottom site as shows the Figure 2.4 and

carefully 100 µl of the liquid solution were taken by a graduated pipette and diluted with the buffer solution 1:100. For the measurement, the diluted samples (one at a time) and a reference sample are placed in a double beam spectrophotometer (Figure 2.5). The intensities of both light paths (through the test sample and through the reference sample) are measured and compared, giving the actual absorbance of the sample. From this results we could calculate the amount of protein crystallized. (For further information check previous chapter).

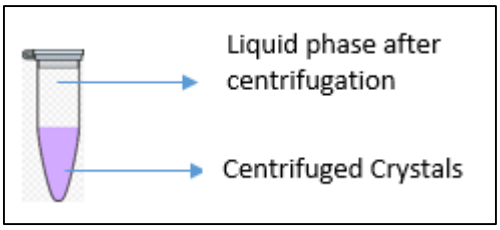


Figure 2.4. Eppi

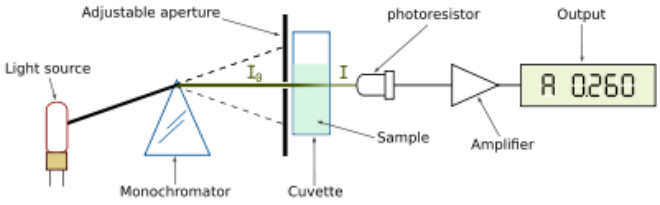


Figure 2.5. Spectrophotometer mechanism

2.1.2 Batch/Discontinuous reactor

The discontinuous reactor is illustrated in the Figure 2.6 where we just work with one water bath at 22ºC. The vessel where the crystallization was carried out was placed within the water bath.

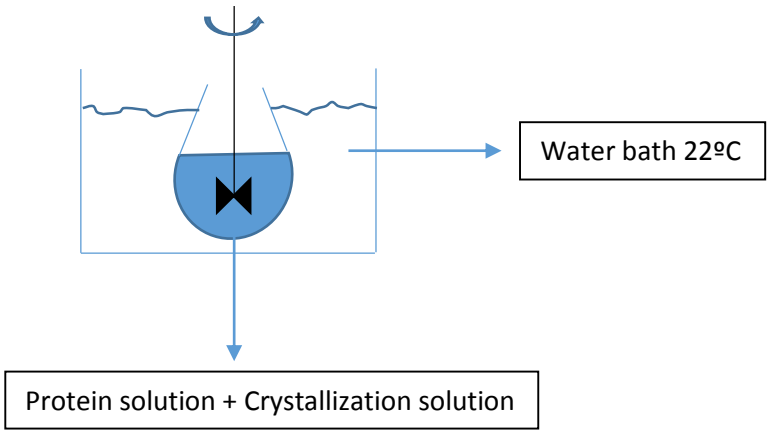


Figure 2.6. Batch reactor

For this experiment, it was really important to mix the same amount of each solution. So, we prepared 10 ml of the protein solution and 10 ml of the crystallization solution. However, the protein solution must be filtrated before using it, and during that process, some volume was always lost. So, after knowing the exact volume of the protein solution, the preparation of the crystallization solution could be carried out.

The protein solution and the crystallization solution were mixed at the very beginning of the process, and the mixture was stirred continuously all over the experiment. After around 24 hours the yield of the process didn´t increase anymore, so we could start taking samples with a graduated pipette directly from the solution. Three or four samples with a volume of 200 µl were taken and centrifuged. The following procedure (measurement) was exactly the same as the continuous one, which was explained above.

2.2 SOLUBILITY EXPERIMENTS

For this experiments, we have varied parameters such as the NaCl concentration, the Ionic Liquid concentration or the temperature both for the continuous experiment or batch in order to find out the behaviour of the lysozyme in presence of IL at different temperatures.

2.2.1 Continuous reactor

In this case, we worked with a continuous reactor which contains just one water bath at several temperatures (depending on the case we work at), Figure 2.7. However, the length of the tube remained the same, 3 metres. As in the yield experiments, we worked with two different solutions, the protein solution (100mg/ml) and the crystallization agent (depends on the Experiment, it has different IL concentration, see Table 2.9).

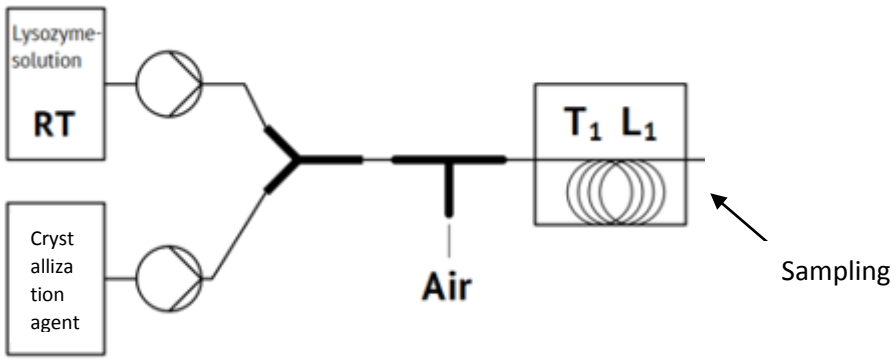


Figure 2.7. Set-up of the Continuous reactor. Just with one water bath

The different temperatures used in the experiments are shown in the Table 2.8

TEMPERATURE (ºC)	LENGTH (m)
20	3
16	3
14	3
10	3
7	3

Table 2.8

Both the protein solution syringe and the crystallization agent syringe contain the same volume inside (10 ml for each experiment).

So, to find out the position of the precipitation area and the crystallization area for the solubility diagram of the lysozyme, we run several experiments, as the Table 2.9 shows, with different crystallization agent composition (NaCl + Ionic Liquid).

	NaCl (mg/ml)	IL (mg/ml)	Temperature (ºC)	Flow rate (ml/min)
Experiment 1	40	0	15	0,4
Experiment 2	40	0	10	0,4
Experiment 3	40	0	10	0,8
Experiment 4	40	0	14	0,8
Experiment 5	40	0	16	0,8
Experiment 6	40	0	20	0,8
Experiment 7	40	20	20	0,8
Experiment 8	40	20	16	0,8
Experiment 9	40	20	14	0,8
Experiment 10	40	20	10	0,8
Experiment 11	40	40	20	0,8
Experiment 12	40	40	16	0,8
Experiment 13	40	40	14	0,8
Experiment 14	40	40	10	0,8
Experiment 15	40	100	20	0,8
Experiment 16	40	100	16	0,8
Experiment 17	40	100	14	0,8
Experiment 18	40	100	10	0,8
Experiment 19	40	0	7	0,8
Experiment 20	40	20	7	0,8
Experiment 21	40	100	7	0,8
Experiment 22	40	40	7	0,8
Experiment 23	40	10	20	0,8
Experiment 24	40	10	16	0,8
Experiment 25	40	10	14	0,8
Experiment 26	40	10	10	0,8

Table 2.9. Different continuous experiments where the IL concentration, the temperature and the flow rate were changed in order to find out the position of the precipitation zone and the crystallization zone for the solubility diagram of lysozyme

The analysis of the protein crystals was done after the first water bath by a microscope (Leica Microscope). Samples were taken just at the end of the pipe and brought to the microscope to check their shape. Crystallization or precipitation could take place depending on the working conditions.

2.2.2 Batch reactor

In the discontinuous process we worked just with one water bath as we have done in the continuous one. Twenty-one different samples (each one in a different Eppi), spent 24 hours inside the tank at a certain temperature which varied for each experiment.
Each Eppi, illustrate in the Figure 2.10, contained the same volume of the protein solution (250 µl) with a concentration of 100 mg/ml and the same volume of NaCl (100 µl) with a concentration of 40 mg/ml or 60 mg/ml, depending on the experiment. However, the amount of Ionic Liquid varied from 0 µl to 100 µl in each Eppi, modifying obviously its concentration, as the Table 2.10 shows. The Eppis are filled with ionized water until reach 500 µl.

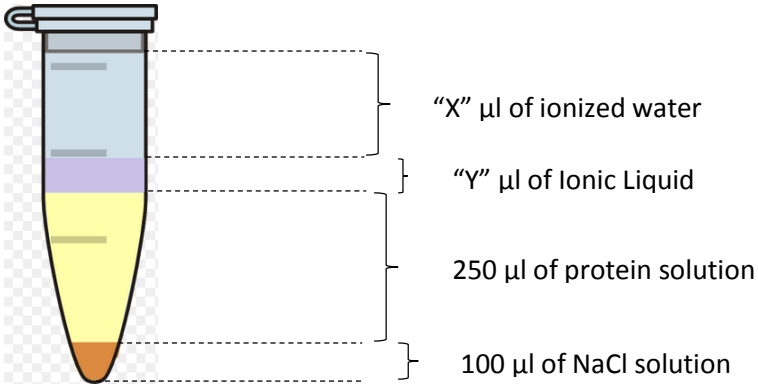


Figure 2.10: Components and their respective amount in each Eppi

$$X + Y = 150\ \mu L \qquad \text{[Equation 3]}$$

The Table 2.11 shows the concentration of each solutions inside the twenty-one Eppis. We worked both with 40 mg/ml and with 60 mg/ml of NaCl, and we modified the IL concentration in each Eppi. Besides, this twenty-one Eppi were placed at different temperature in the water bath to check out the influence of the lysozyme, as we have already explained above.

	Protein concentration (mg/ml)	NaCl solution (mg/ml)	Ionized water (µl)	IL (mg/ml)
Eppi 1	100	40 or 60	150	0
Eppi 2	100	40 or 60	145	10
Eppi 3	100	40 or 60	140	20
Eppi 4	100	40 or 60	135	30
Eppi 5	100	40 or 60	130	40
Eppi 6	100	40 or 60	125	50
Eppi 7	100	40 or 60	120	60
Eppi 8	100	40 or 60	115	70
Eppi 9	100	40 or 60	110	80
Eppi 10	100	40 or 60	105	90
Eppi 11	100	40 or 60	100	100
Eppi 12	100	40 or 60	95	110
Eppi 13	100	40 or 60	90	120
Eppi 14	100	40 or 60	85	130
Eppi 15	100	40 or 60	80	140
Eppi 16	100	40 or 60	75	150
Eppi 17	100	40 or 60	70	160
Eppi 18	100	40 or 60	65	170
Eppi 19	100	40 or 60	60	180
Eppi 20	100	40 or 60	55	190
Eppi 21	100	40 or 60	50	200

Table 2.11. Concentration of each component inside the Eppis.

3 EXPERIMENTAL RESULTS

3.1 YIELD COMPARISON

The yield is one of the most important parameters to take into account in chemistry because it express the amount of product obtained in a chemical reaction. In the experiments, the protein solution should crystallize as much as possible. The higher the crystallization the higher the yield, which means that the protein concentration in the final samples is the lowest possible. As it has been explained above, the yield experiments were carried out both in batch reactor and in continuous reactor.

As we said in the introduction, the selected absorbance value is 280 nm because there, proteins show an absorption maxima. This maximum absorbance value is caused by three different aromatic amino acids, quoted in the introduction contents.

- **Batch Process**

1. Batch crystallization without ionic liquid
   The yield progression in a batch experiment is shown in the Figure 3.3. A batch crystallization was run while taking samples during 24 hours. The samples consist on an "initial" and a "final" one (taken at the very beginning of the process and just at the end respectively) and 5 more samples (sample 1,2,3,4,5) which show the yield progression during the first 3 hours.

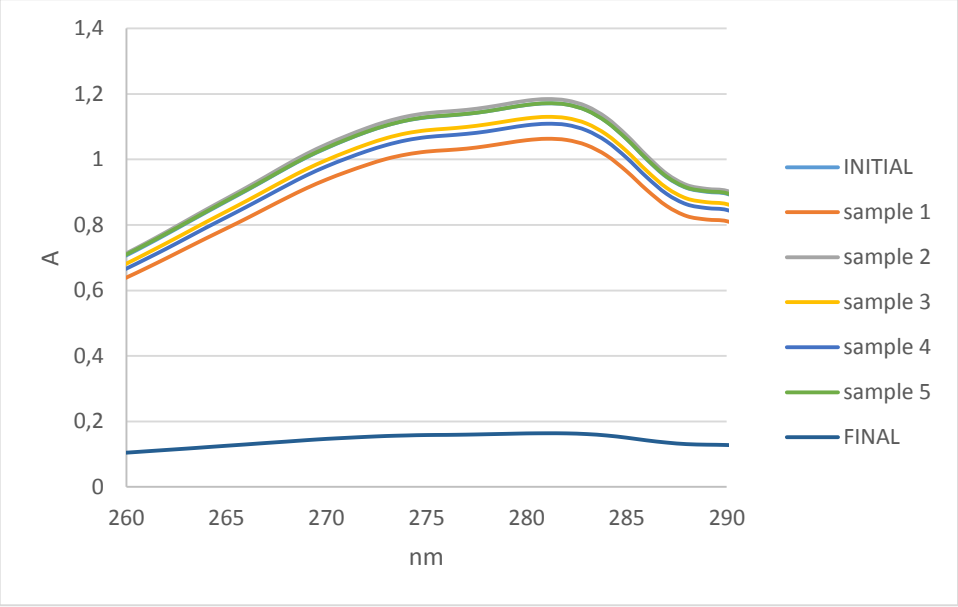


Figure 3.1. Absorbance spectrum (260-290 nm), bath, without IL

Time (min)	Sample	Absorbance (at 280 nm)	Protein concentration (mg/ml)	Yield [%]
0	INITIAL	1,17	47,0	
30	sample 1	1,06	42,7	9
70	sample 2	1,18	47,6	1
120	sample 3	1,13	45,4	4
170	sample 4	1,10	44,5	5
210	sample 5	1,17	47,1	0
1 day	FINAL	0,16	6,9	86

Table 3.2. Yield progression in batch reactor without IL

The yield progression all over the bath process without using Ionic Liquid is shown graphically in the next plot.

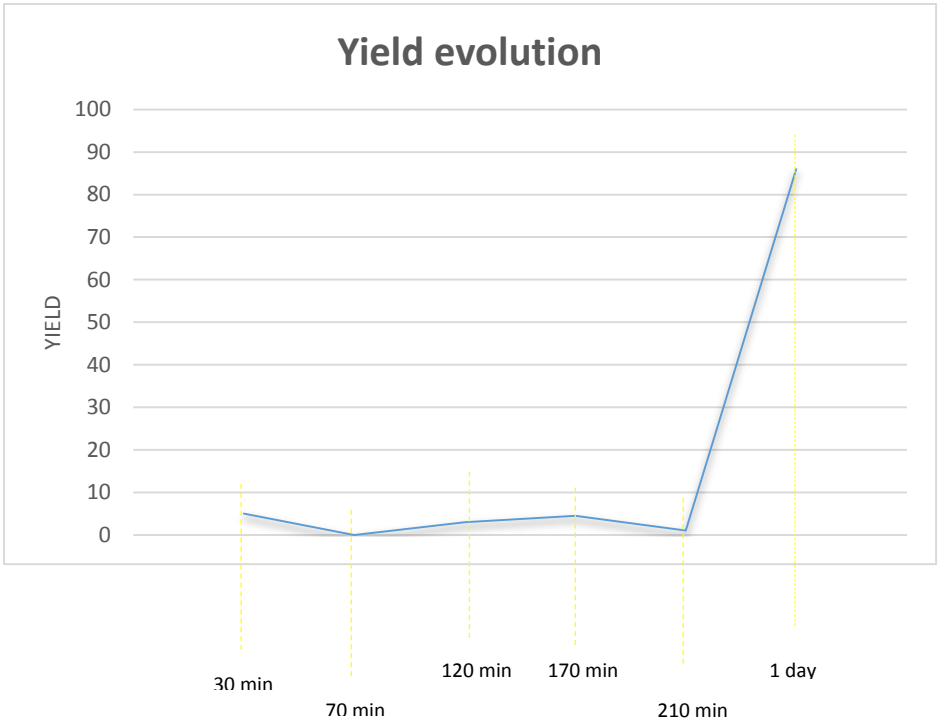


Figure 3.3. Yield evolution in batch reactor

2. Batch crystallization with Ionic Liquid

The results of the crystallization process in batch reactor are shown below, but this time, we use Ionic Liquid in the experiment. For the measurement, we took samples all over the process, meaning that there is a sample from the very beginning (just after mixing both solution, INITIAL 1) and other from the end of the process (Final A). Besides, we took samples (1A, 2A, 3A…) during the first 2.5 hours each 30 minutes in order to analyse the yield evolution.

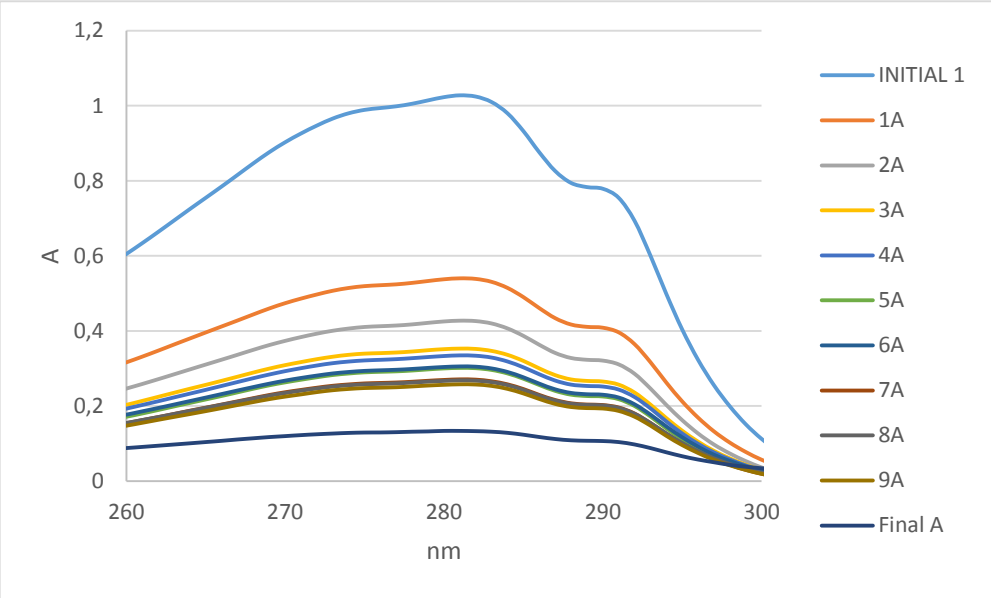


Figure 3.4. Absorbance spectrum (260-300 nm), batch reactor, with IL

Time (min)	Samples	Absorbance (at 280 nm)	Protein concentration (mg/ml)	Yield [%]
0	INITIAL 1	1,02	41,2	
13	1A	0,54	21,7	47
26	2A	0,43	17,1	58
39	3A	0,35	14,2	66
52	4A	0,33	13,4	67
72	5A	0,30	12,1	71
92	6A	0,30	12,3	71
112	7A	0,27	10,9	74
132	8A	0,26	10,8	74
152	9A	0,26	10,3	75
1 day	FINAL A	0,13	5,4	87

Table 3.5. Yield progression in batch reactor with IL

➢ **Continuous Process**

The continuous crystallization has been run varying two main parameters:

- Use or not of Ionic Liquid
- The flow rate

In order to check the progression of the yield all over the process, we have done several experiments in which the sampling was done after the first, second and third water bath.

1. Continuous crystallization without Ionic Liquid and a flow rate of 0.4 ml/min. Samples were taken at the end of the first water bath

   Here, we took and INITIAL sample (for the measurement of the protein concentration at the very beginning). Besides, 6 more samples (1A, 1B, 2A…) were taken to analyse the yield at the end of the first water bath. The temperature of the water bath was 21.5ºC and the residence time was 26 minutes.

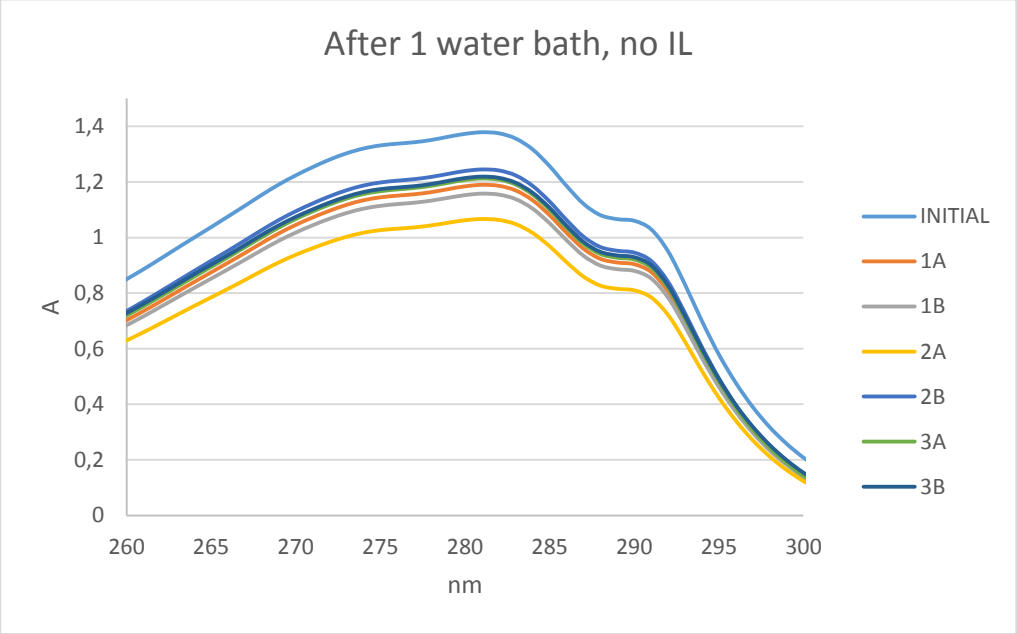


Figure 3.6. Absorbance Spectrum (260-300 nm), first water bath, without IL, 0.4 ml/min

Sample	Absorbance (at 280nm)	Protein Concentration (mg/ml)	Yield [%]
INITIAL	1,37	55,4	
1A	1,18	47,8	14
1B	1,15	46,5	16
2A	1,06	42,8	23
2B	1,24	50	10
3A	1,21	49	12
3B	1,21	49	12
Average			14

Table 3.7. Yield after the first water bath, without IL, 0.4 ml/min

2. <u>Continuous crystallization without Ionic Liquid and a flow rate of 0.4 ml/min. Samples were taken at the end of the second water bath</u>
   As in the former experiment, an INITIAL sample was taken and 6 more samples (1A, 1B, 2A…) were taken at the end of the second water bath each 10 minutes. The temperatures in the first and second water bath were 21.5ºC and 22ºC respectively. The total residence time was 66 minutes.

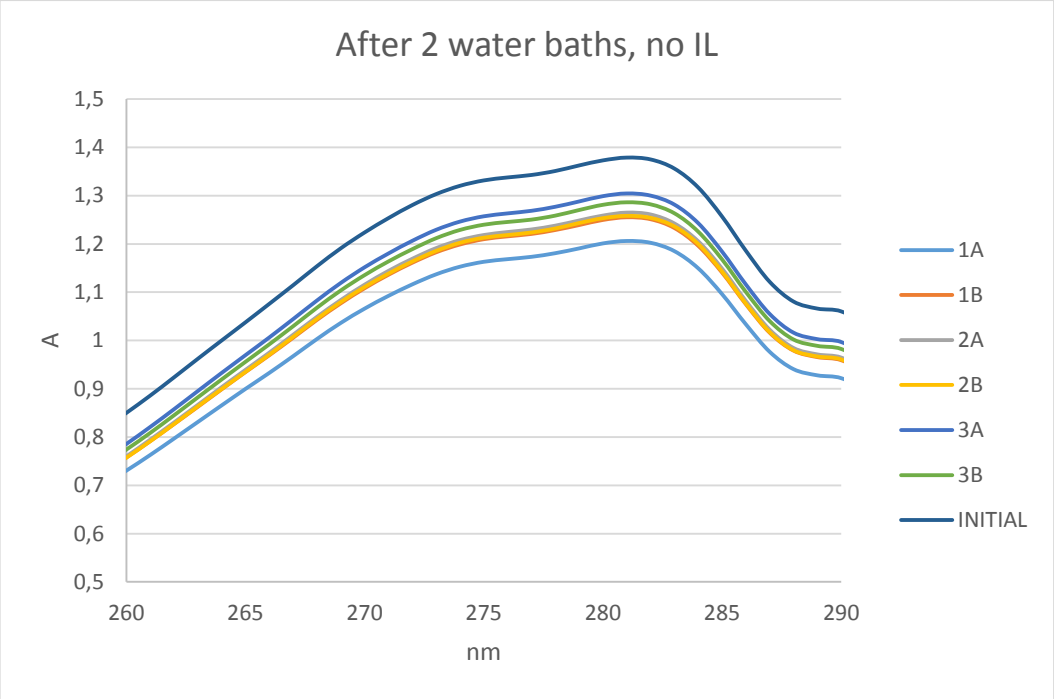


Figure 3.8. Absorbance Spectrum (260-300 nm), second water bath without IL, 0.4 ml/min

Samples	Absorbance (at 280 nm)	Protein Concentration (mg/ml)	Yield [%]
INITIAL	1,37	55,4	
1A	1,20	48,4	13
1B	1,25	50,4	9
2A	1,26	50,8	8
2B	1,25	50,5	9
3A	1,30	52,4	5
3B	1,28	51,7	7
Average			8

Table 3.9. Yield after the second water bath, without IL, 0.4 ml/min

3. <u>Continuous crystallization without Ionic Liquid and a flow rate of 0.4 ml/min. Samples were taken at the end of the third water bath</u>
   One initial sample (INITIAL 1) and 6 more samples (1A, 1B, 2A…) were taken. Those 6 samples were taken at the end of the third water bath each 10 minutes. The total residence time were 2 hours and the temperatures of the first, second and third water bath were 21.5ºC, 22ºC and 20ºC respectively.

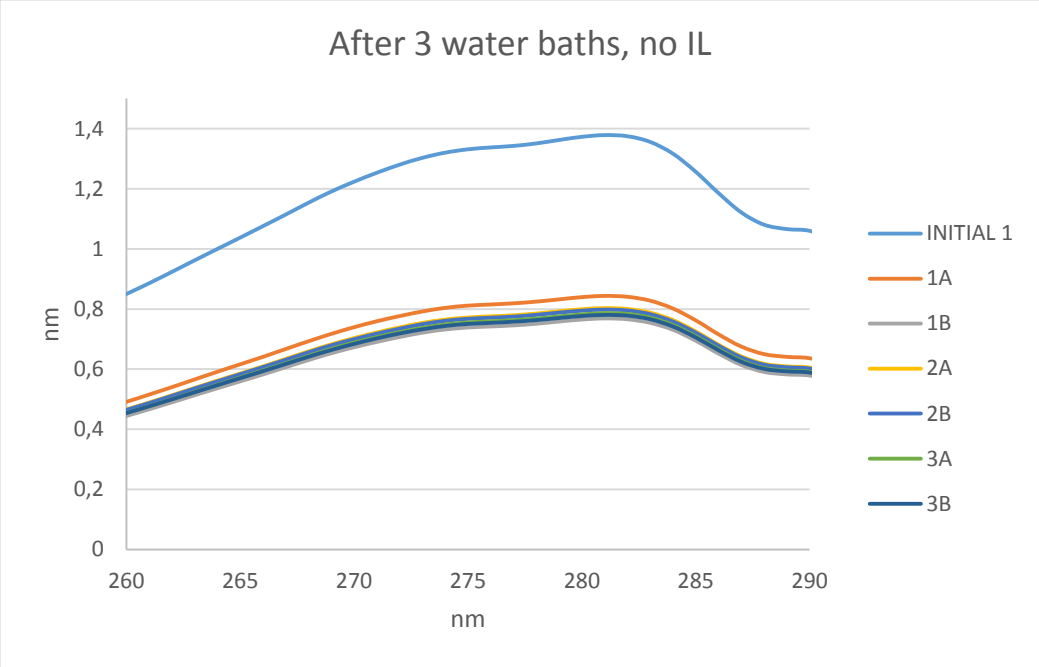


Figure 3.10. Absorbance Spectrum (260-290 nm), third water bath without IL, 0.4 ml/min

Samples	Absorbance (at 280 nm)	Protein Concentration (mg/ml)	Yield [%]
INITIAL	1,37	55,4	
1A	0,84	33,9	39
1B	0,77	30,9	44
2A	0,80	32,2	42
2B	0,79	32,0	42
3A	0,78	31,5	43
3B	0,78	31,3	43
Average			42

Table 3.11. Yield after the third water bath, without IL, 0.4 ml/min

4. <u>Continuous crystallization with Ionic Liquid and a flow rate of 0.4 ml/min. Samples were taken at the end of the first water bath</u>
   In this experiment, and initial sample (INITIAL) and 4 more samples (2A, 2B, 3A and 3B) were taken. The last four samples were taken at the end of the first water batch each 10 minutes. The water bath was operated at a temperature of 21.5ºC, and the total residence time was 26 minutes.

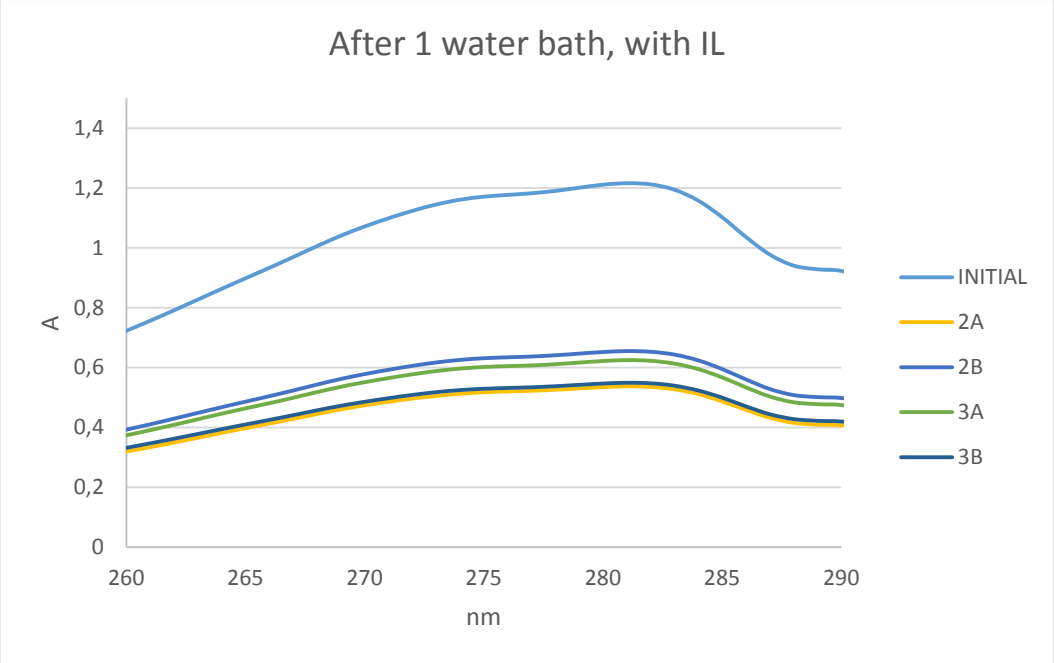


Figure 3.12. Absorbance Spectrum (260-290 nm), first water bath with IL, 0.4 ml/min

Samples	Absorbance (at 280 nm)	Protein Concentration (mg/ml)	Yield [%]
INITIAL	1,21	48,8	
2A	0,53	21,6	56
2B	0,65	26,3	46
3A	0,62	25,1	49
3B	0,55	22,0	55
Average			51

Table 3.13. Yield after the first water bath, with IL, 0.4 ml/min

5. <u>Continuous crystallization with Ionic Liquid and a flow rate of 0.4 ml/min. Samples were taken at the end of the second water bath</u>
   One initial sample (INITIAL) was taken at the very beginning of the process and 6 more samples (1A, 1B, 2A…) were taken at the end of the second water bath each 10 minutes. The temperatures of the first and second water bath were 21.5ºC and 22ºC respectively. The residence time in the first water bath was 24 minutes, and the whole experiment time was 68 minutes.

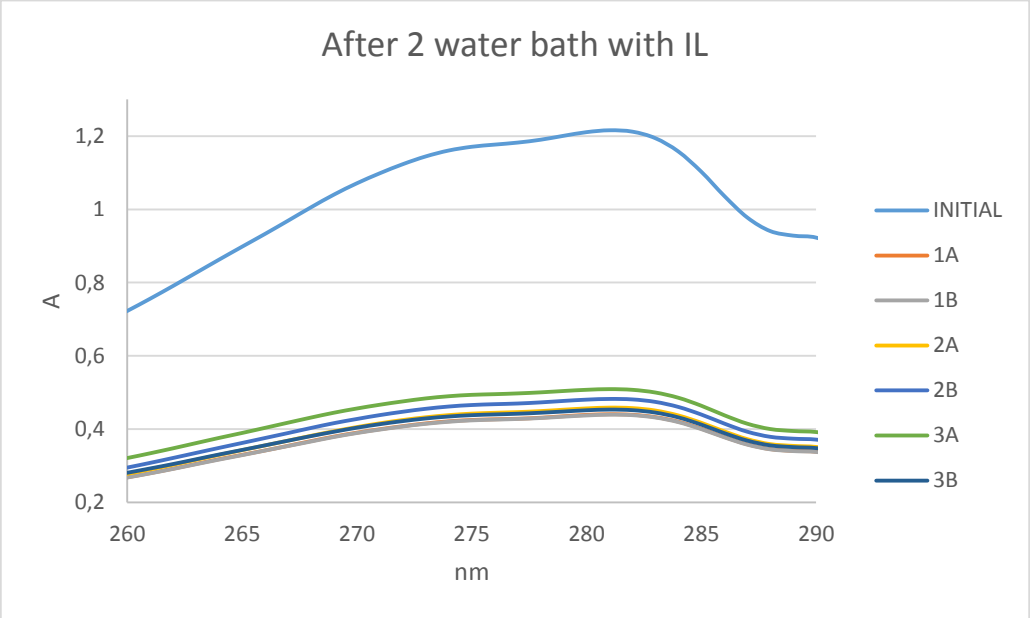


Figure 3.14. Absorbance Spectrum (260-290 nm), second water bath with IL, 0.4 ml/min

Samples	Absorbance (at 280 nm)	Protein Concentration (mg/ml)	Yield [%]
INITIAL	1,21	48,8	
1A	0,44	17,7	64
1B	0,44	17,6	64
2A	0,46	18,4	62
2B	0,48	19,4	60
3A	0,51	20,5	58
3B	0,45	18,2	63
Average			62

Table 3.15. Yield after the second water bath, with IL, 0.4 ml/min

6. <u>Continuous crystallization with Ionic Liquid and a flow rate of 0.4 ml/min. Samples were taken at the end of the third water bath</u>
   One initial sample (INITIAL) was taken at the very beginning of the process and 6 more samples (1A, 1B, 2A…) were taken at the end of the third water bath each 10 minutes. The temperatures of the first, second and third water bath were 21.5ºC, 22ºC and 20ºC respectively. The whole process took 2 hours.

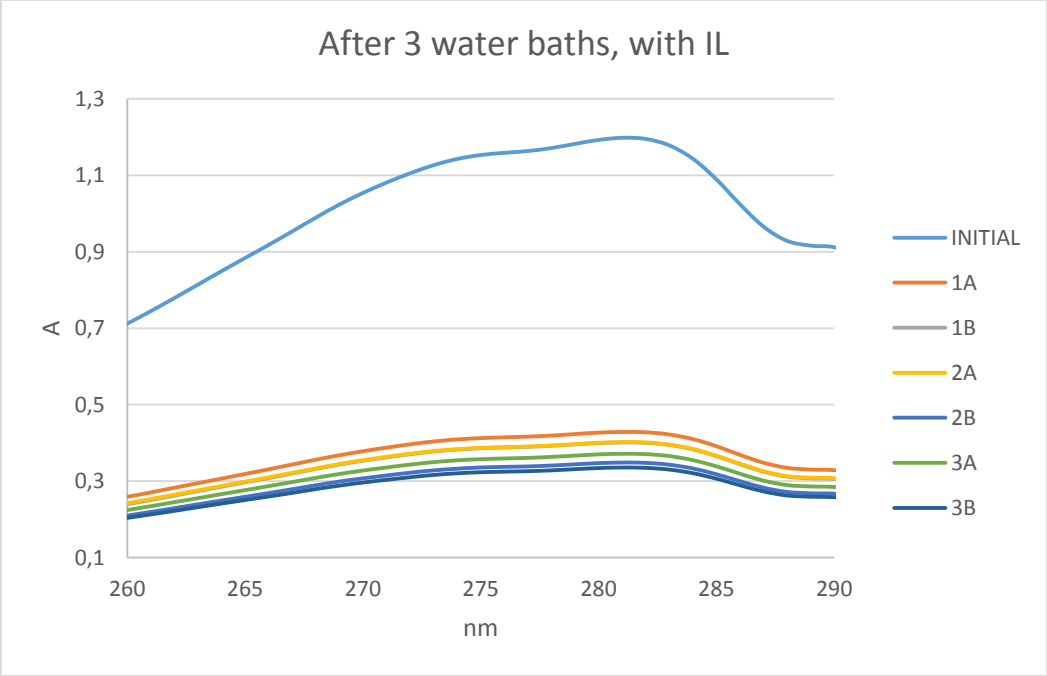


Figure 3.16. Absorbance Spectrum (260-290 nm), third water bath with IL, 0.4 ml/min

Sample	Absorbance (at 280 nm)	Protein Concentration (mg/ml)	Yield [%]
INITIAL	1,19	48,1	
1A	0,43	17,2	64
1B	0,40	16,1	67
2A	0,40	16,1	66
2B	0,35	14,0	71
3A	0,37	14,9	69
3B	0,33	13,5	72
Average			68

Table 3.17. Yield after the third water bath, with IL, 0.4 ml/min

7. <u>Continuous crystallization with Ionic Liquid and a flow rate of 0.8 ml/min. Samples were taken at the end of the first water bath</u>
   In this experiment, we took the initial sample before the starting (INITIAL) and 4 more samples (1A, 1B, 1C and 1D). The last four samples were taken at the end of the first water batch each 10 minutes. The water bath worked at a temperature of 21.5ºC, and the total residence time was 12.5 minutes.

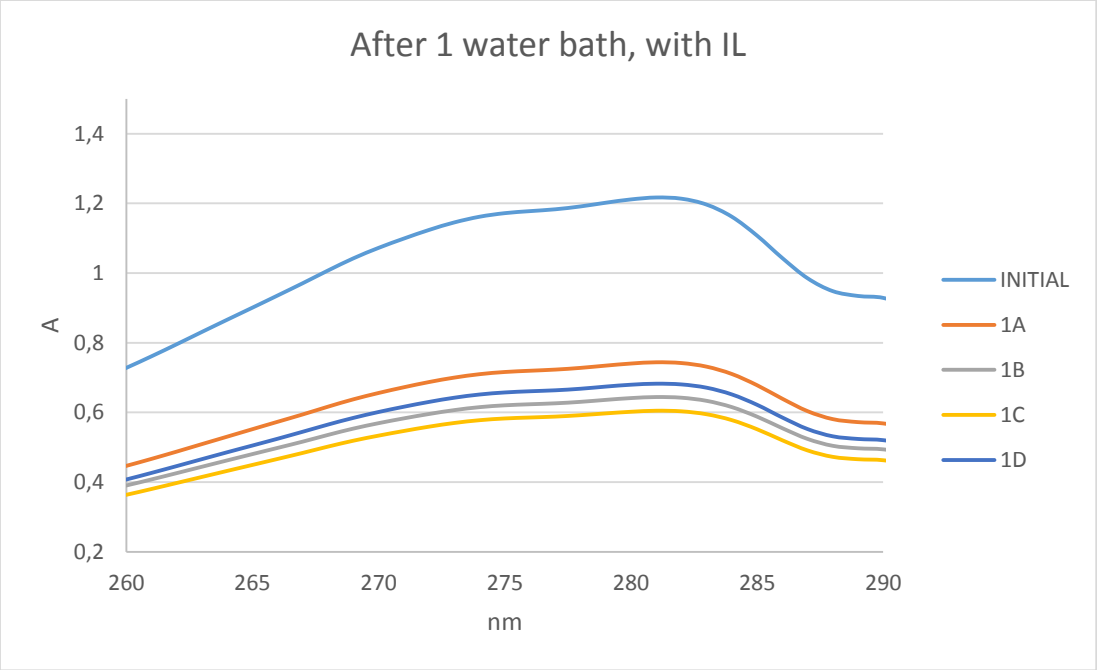


Figure 3.18. Absorbance Spectrum (260-290 nm), first water bath with IL, 0.8 ml/min

Sample	Absorbance (at 280 nm)	Protein Concentration (mg/ml)	Yield [%]
INITIAL	1,21	48,8	
1A	0,74	29,9	39
1B	0,64	25,9	47
1C	0,60	24,3	50
1D	0,68	27,4	44
Average			45

Table 3.19. Yield after the first water bath, with IL, 0.8 ml/min

8. <u>Continuous crystallization with Ionic Liquid and a flow rate of 0.8 ml/min. Samples were taken at the end of the second water bath</u>
   One initial sample (INITIAL) was taken at the very beginning of the process and 4 more samples (1A, 1B, 1C and 1D) were taken at the end of the second water bath each 10 minutes. The temperatures of the first and second water bath were 21.5ºC and 22ºC respectively. The residence time in the first water bath was 12.5 minutes, and the whole experiment time was 37 minutes.

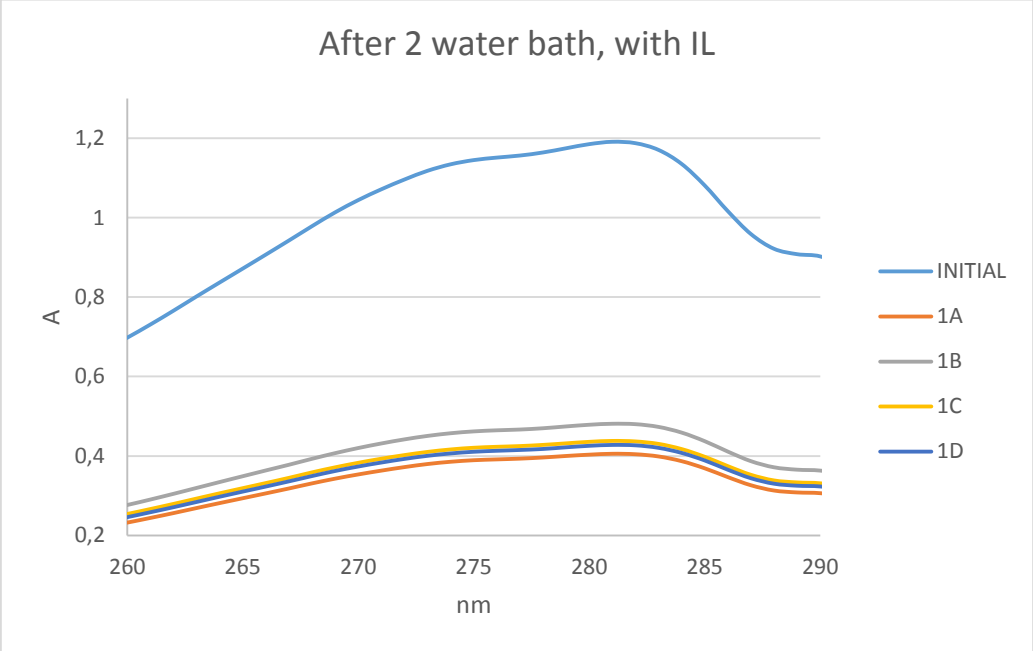


Figure 3.20. Absorbance Spectrum (260-290 nm), second water bath with IL, 0.8 ml/min

Sample	Absorbance (at 280 nm)	Protein Concentration	Yield [%]
INITIAL	1,19	47,8	
1A	0,40	16,3	66
1B	0,48	19,3	60
1C	0,44	17,6	63
1D	0,43	17,2	64
Average			63

Table 3.21. Yield after the second water bath, with IL, 0.8 ml/min

9. <u>Continuous crystallization with Ionic Liquid and a flow rate of 0.8 ml/min. Samples were taken at the end of the third water bath</u>
   One initial sample (INITIAL) was taken at the very beginning of the process and 6 more samples (1A, 1B, 2A, 2B and 3A) were taken at the end of the third water bath each 10 minutes. The temperatures of the first, second and third water bath were 21.5ºC, 22ºC and 20ºC respectively. The whole process took 57 minutes to finish.

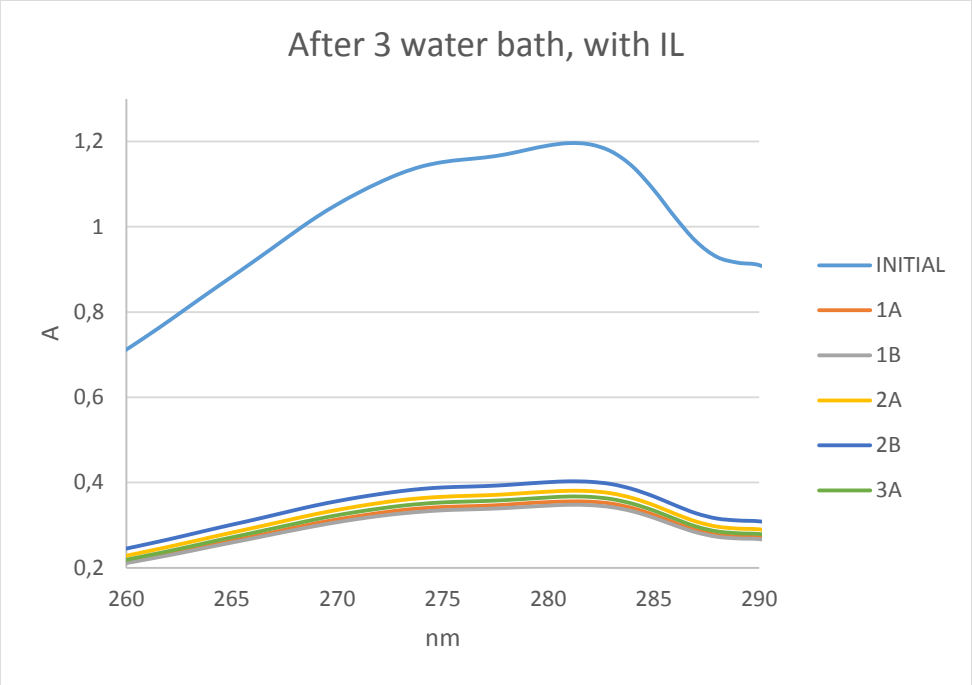


Figure 3.22. Absorbance Spectrum (260-290 nm), third water bath with IL, 0.8 ml/min

Samples	Absorbance (at 280 nm)	Protein Concentration (mg/ml)	Yield [%]
INITIAL	1,19	48,0	
1A	0,35	14,3	70
1B	0,35	13,9	71
2A	0,38	15,3	68
2B	0,40	16,2	66
3A	0,36	14,7	69
Average			69

Table 3.23. Yield after the third water bath, with IL, 0.8 ml/min

3.2 CRYSTAL´S SHAPE AFTER CRYSTALLIZATION

➢ **Batch Process**

- First experiment

Temperature (ºC)	Protein (mg/ml)	NaCl (mg/ml)	IL (mg/ml)
22	0,1	40	0-200

Table 3.24

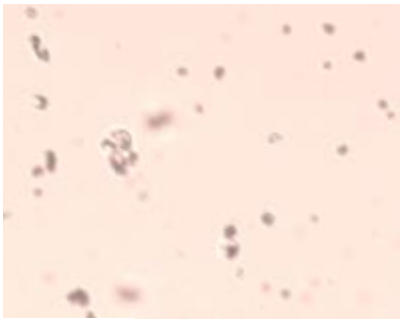


Figure 3.25

Figure 3.26

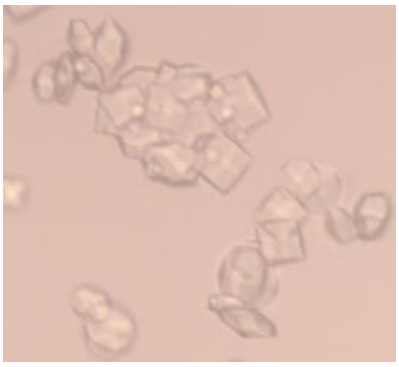


Figure 3.27

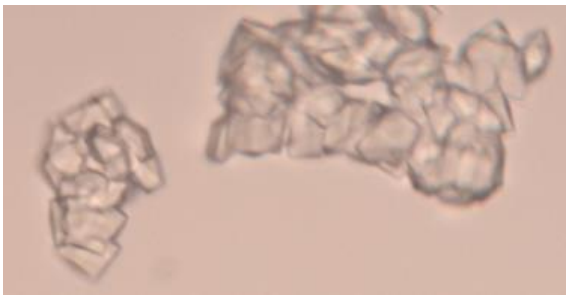


Figure 3.28

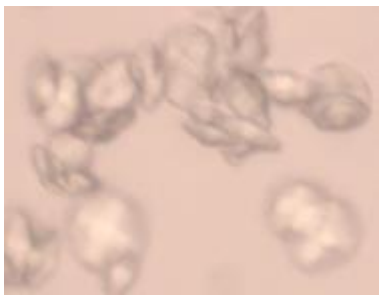


Figure 3.29

	IL (µl)	water (µl)	IL (mg/ml)
Figure 3.25	0	150	0
Figure 3.26	20	130	40
Figure 3.27	45	105	90
Figure 3.28	60	90	120
Figure 3.29	95	55	190

Table 3.30

- Second experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL (mg/ml)
16	0,1	40	0-200

Table 3.31

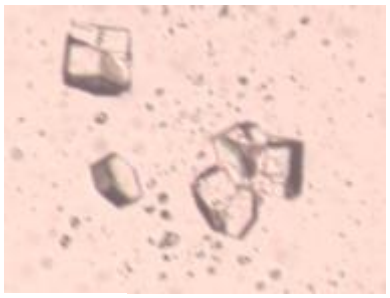


Figure 3.32

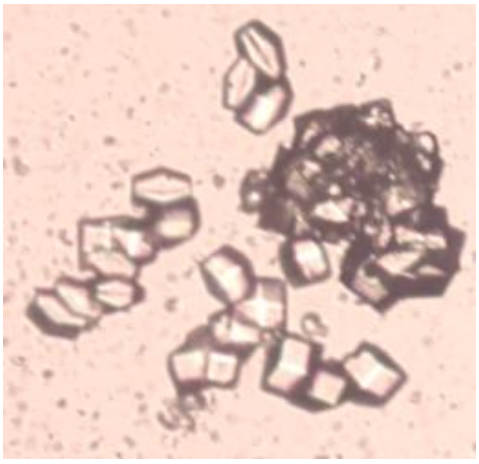


Figure 3.33

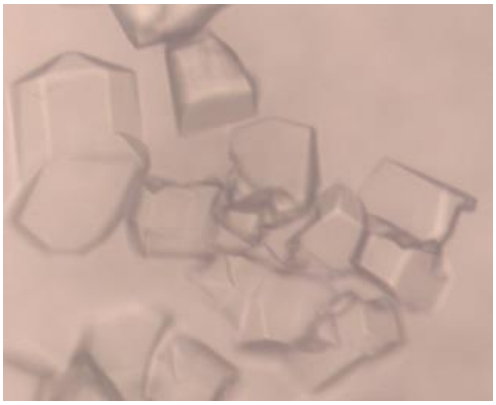


Figure 3.34

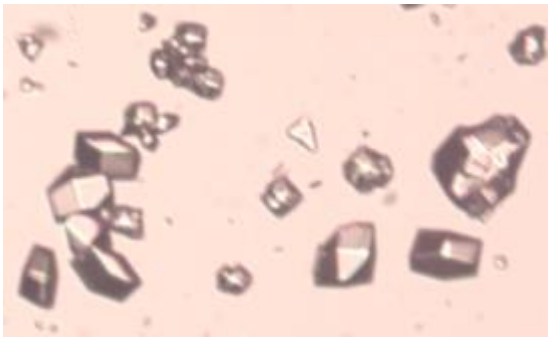


Figure 3.35

Figure 3.36

	IL (µl)	water (µl)	IL (mg/ml)
Figure 3.32	0	150	0
Figure 3.33	20	130	40
Figure 3.34	45	105	90
Figure 3.35	70	80	140
Figure 3.26	100	50	200

Table 3.37

- Third experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL (mg/ml)
16	0,1	60	0-200

Table 3.38

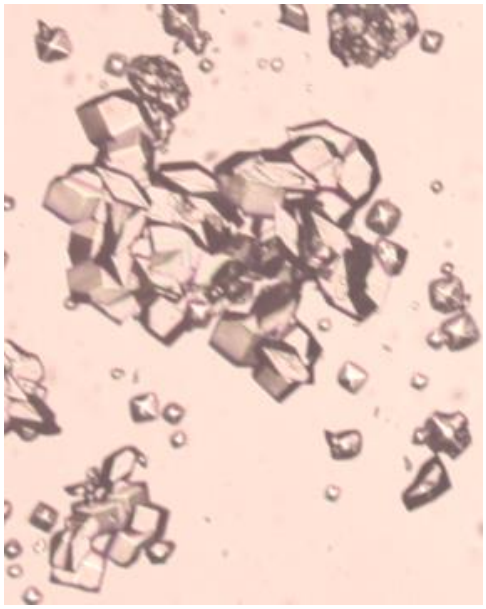


Figure 3.39

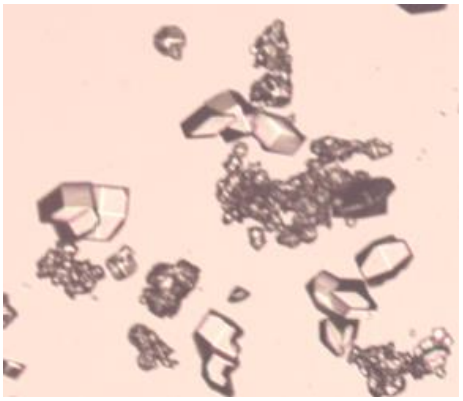


Figure 3.40

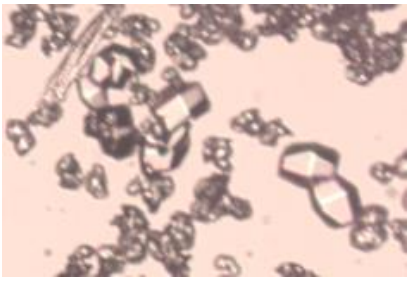


Figure 3.41

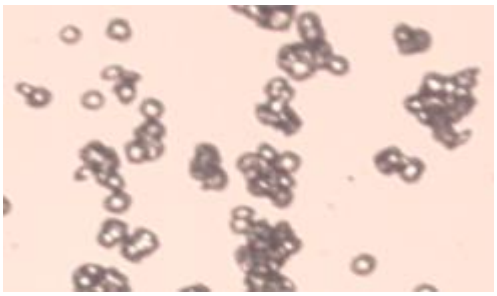


Figure 3.42

Figure 3.43

Figure 3.44

	IL (µl)	water (µl)	IL (mg/ml)
Figure 3.39	0	150	0
Figure 3.40	20	130	40
Figure 3.41	40	110	80
Figure 3.42	60	90	120
Figure 3.43	80	70	160
Figure 3.44	100	50	200

Table 3.45

- Fourth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL (mg/ml)
10	0,1	40	0-200

Table 3.46

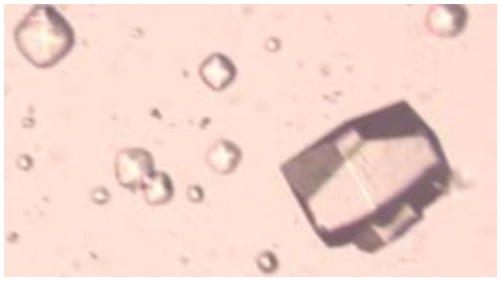


Figure 3.47

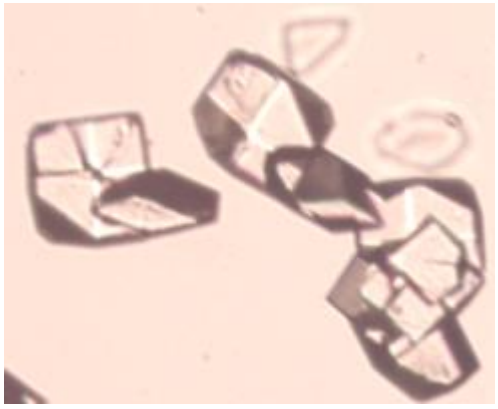


Figure 3.48

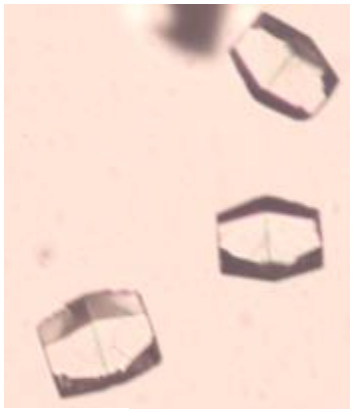


Figure 3.49

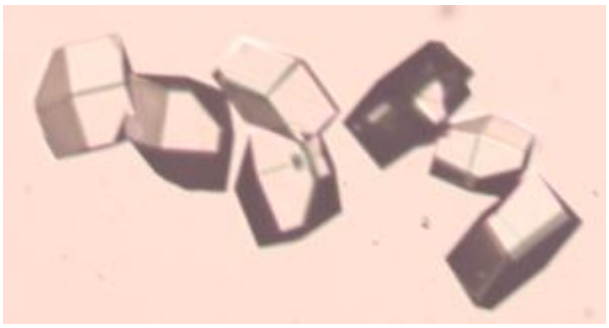


Figure 3.51

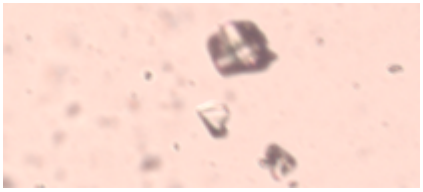


Figure 3.50

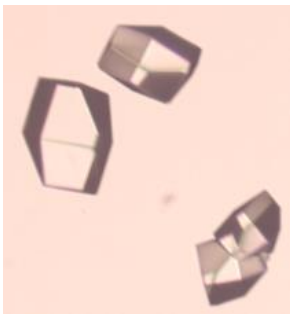


Figure 3.52

	IL (µl)	water (µl)	IL (mg/ml)
Figure 3.47	0	150	0
Figure 3.48	20	130	40
Figure 3.49	40	110	80
Figure 3.50	60	90	120
Figure 3.51	80	70	160
Figure 3.52	100	50	200

Table 3.53

- Fifth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL (mg/ml)
10	0,1	60	0-200

Table 3.54

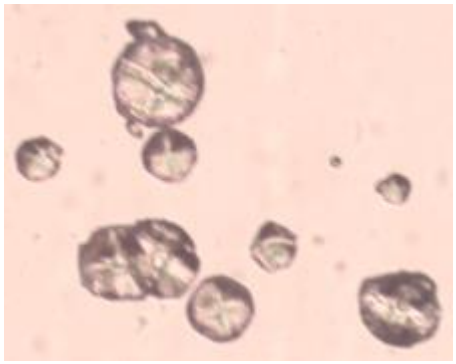


Figure 3.55

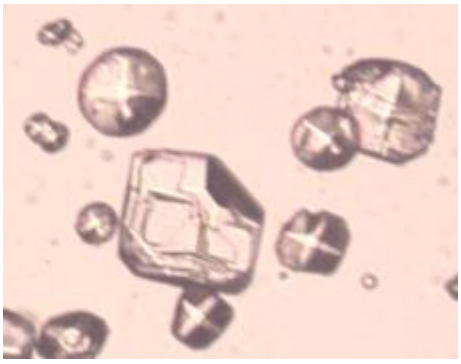


Figure 3.56

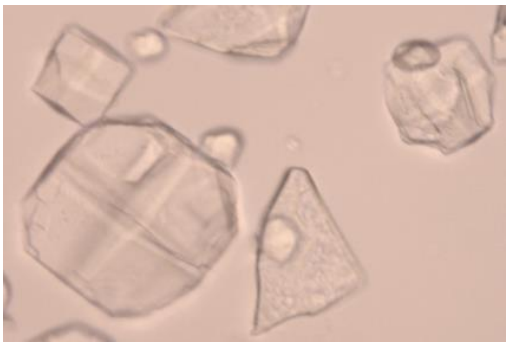


Figure 3.57

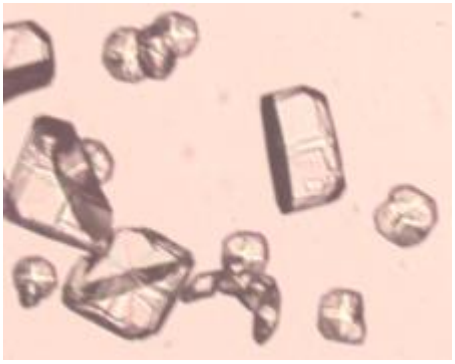


Figure 3.58

Figure 3.59

Figure 3.60

	IL (µl)	water (µl)	IL (mg/ml)
Figure 3.55	0	150	0
Figure 3.56	20	130	40
Figure 3.57	40	110	80
Figure 3.58	60	90	120
Figure 3.59	80	70	160
Figure 3.60	100	50	200

Table 3.61

➢ **Continuous Process**

- First experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
15	0,1	40	0	0,4

Table 3.62

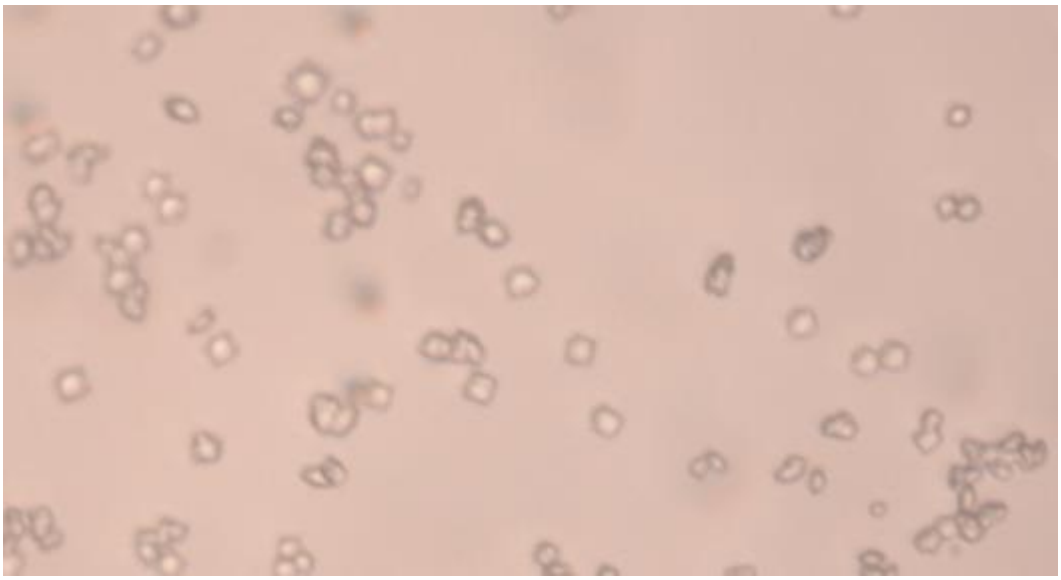


Figure 3.63

- Second experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
10	0,1	40	0	0,4

Table 3.64

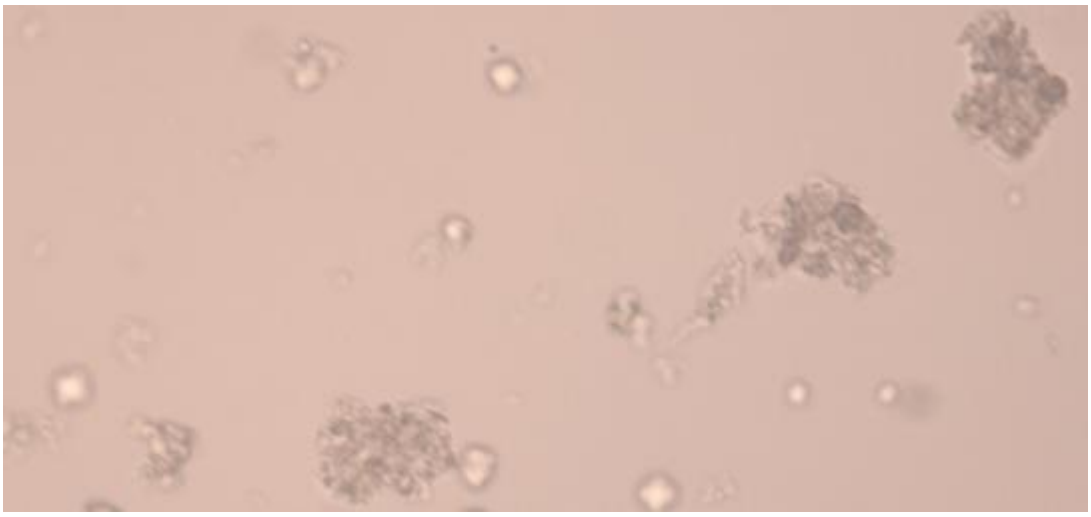


Figure 3.65

- Third experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
10	0,1	40	0	0,8

Table 3.66

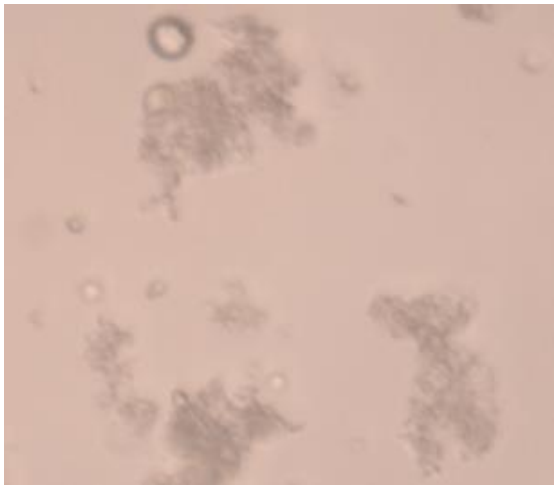


Figure 3.67

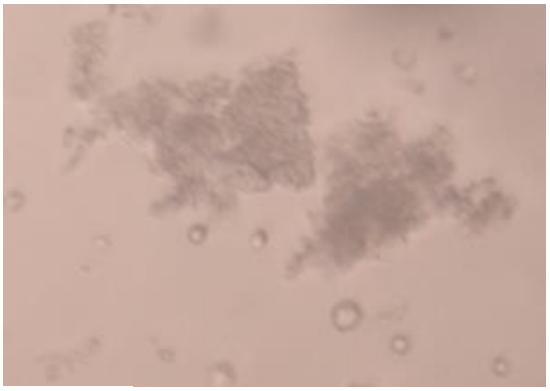


Figure 3.68

- Fourth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
14	0,1	40	0	0,8

Table 3.69

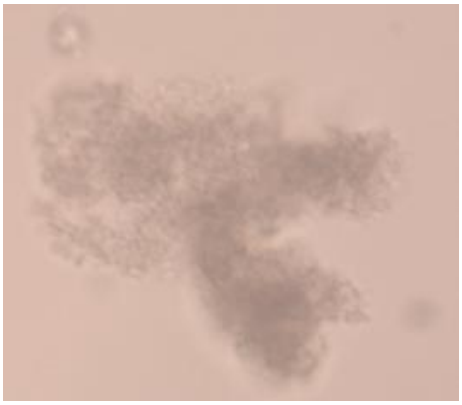


Figure 3.70

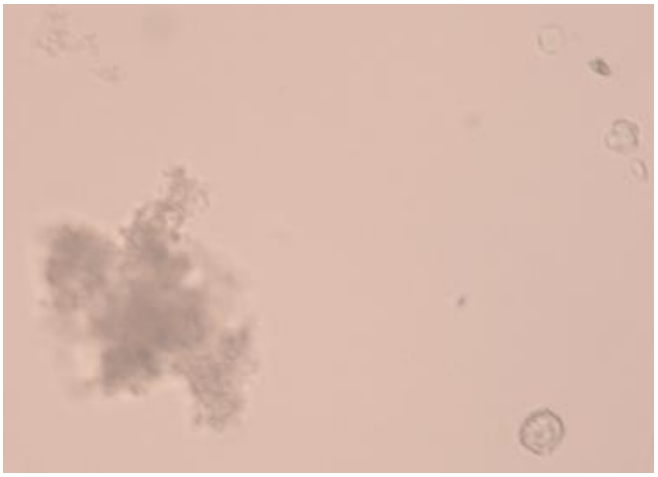


Figure 3.71

- Fifth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
16	0,1	40	0	0,8

Table 3.72

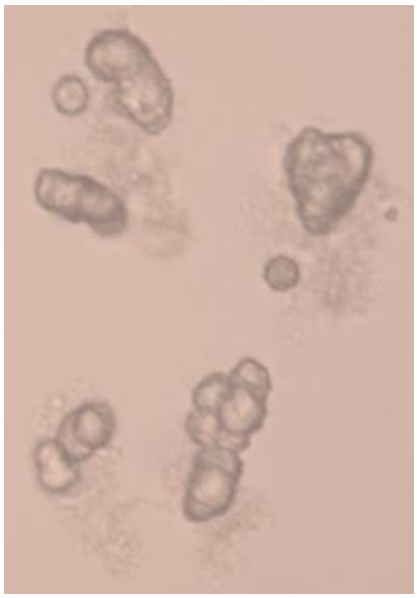


Figure 3.73

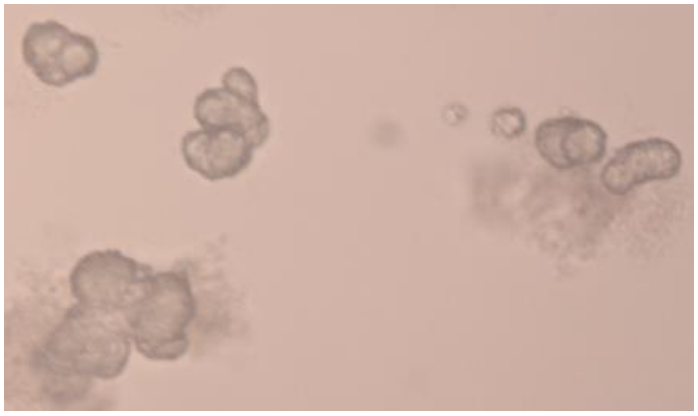


Figure 3.74

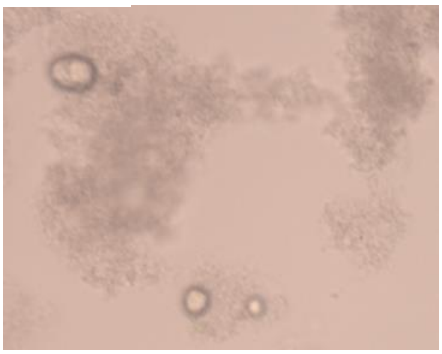


Figure 3.75

- Sixth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
20	0,1	40	0	0,8

Table 3.76

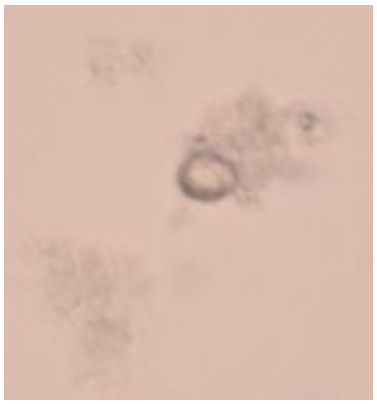


Figure 3.77

Figure 3.78

- Seventh experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
20	0,1	40	20	0,8

Table 3.79

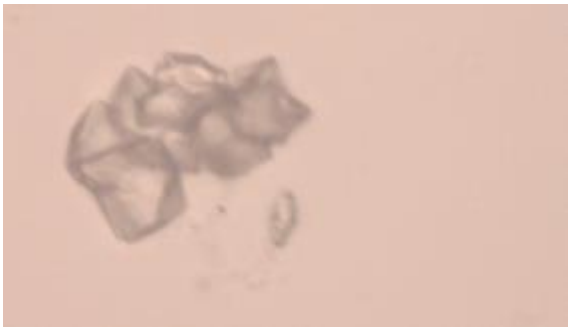


Figure 3.80

- Eighth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
16	0,1	40	20	0,8

Table 3.81

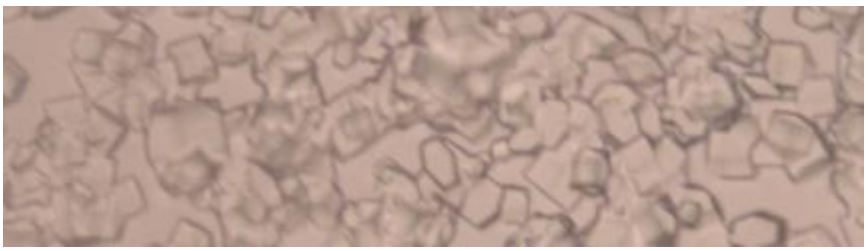


Figure 3.82

- Ninth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
14	0,1	40	20	0,8

Table 3.83

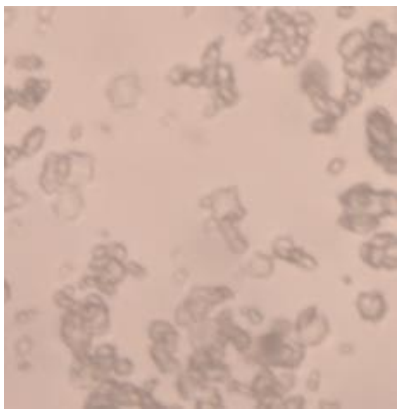


Figure 3.84

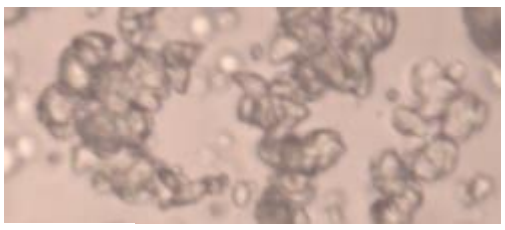


Figure 3.85

- Tenth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
10	0,1	40	20	0,8

Table 3.86

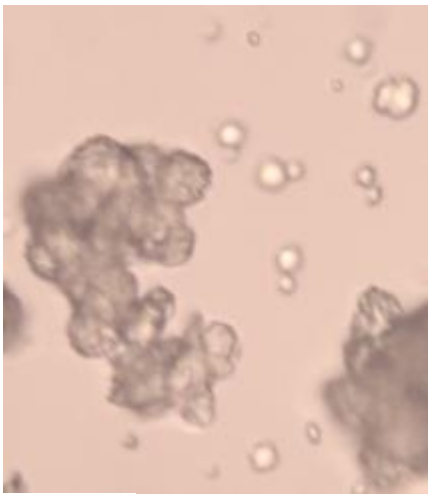


Figure 3.87

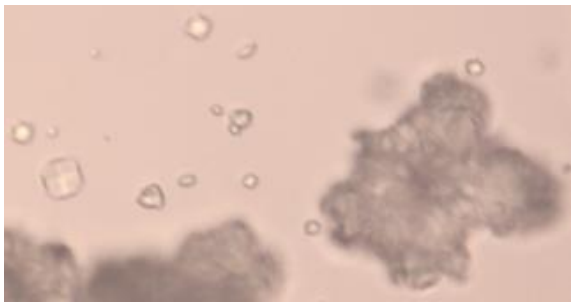


Figure 3.88

- Eleventh experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
20	0,1	40	40	0,8

Table 3.89

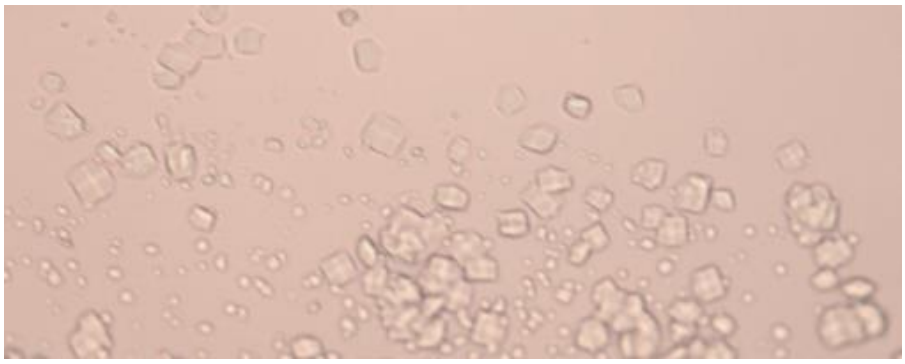


Figure 3.90

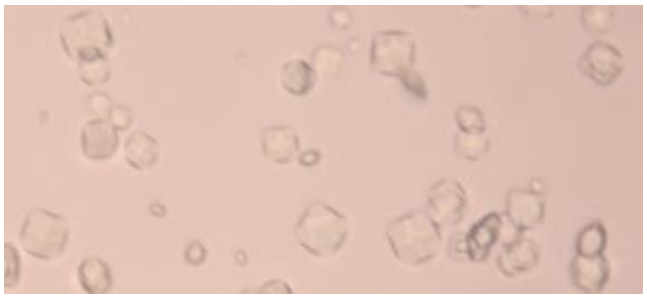


Figure 3.91

- Twelfth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
16	0,1	40	40	0,8

Table 3.92

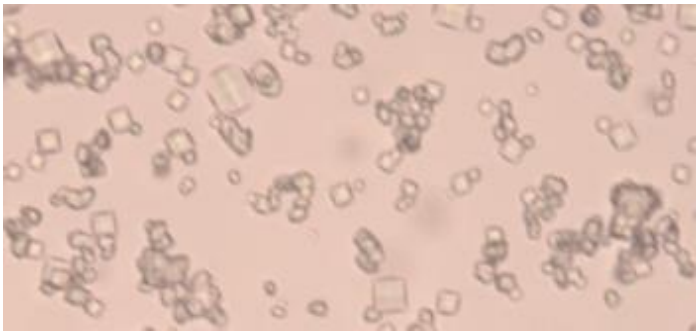


Figure 3.93

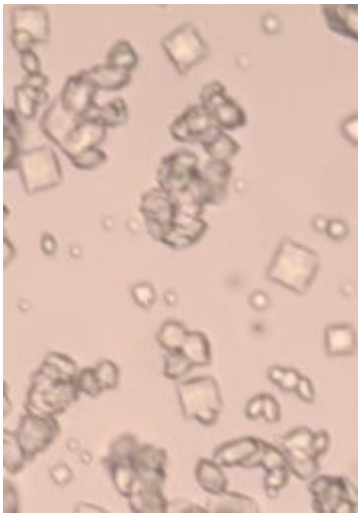


Figure 3.94

- Thirteenth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
14	0,1	40	40	0,8

Table 3.95

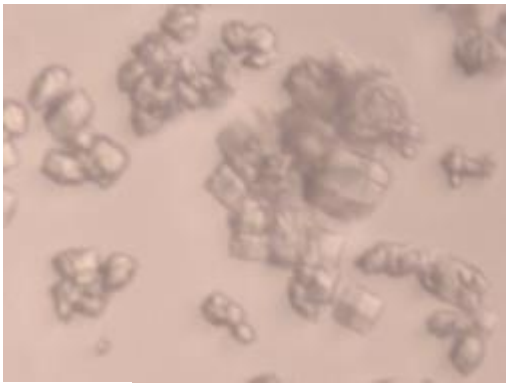


Figure 3.96

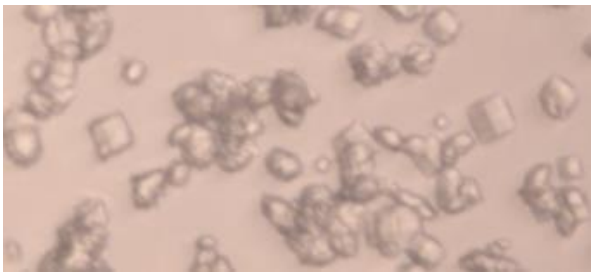


Figure 3.97

- Fourteenth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
10	0,1	40	40	0,8

Table 3.98

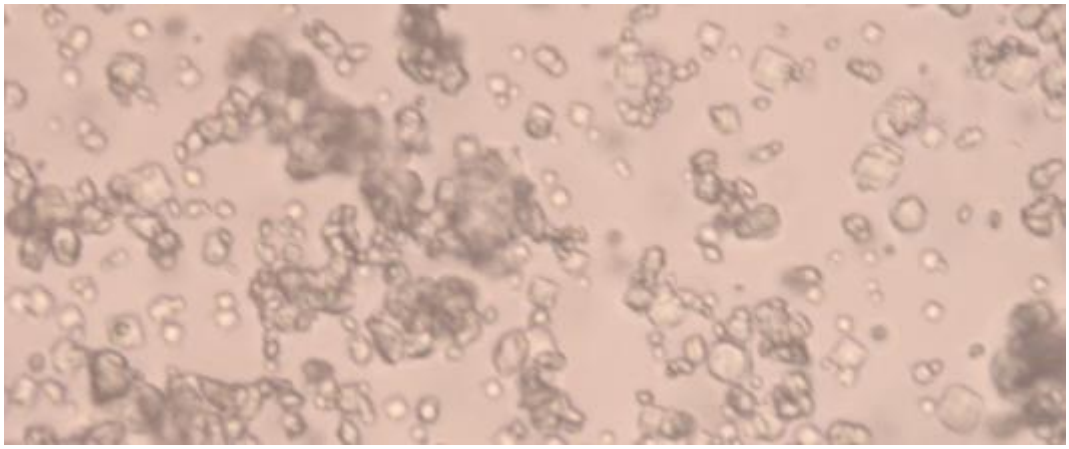


Figure 3.99

- Fifteenth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
20	0,1	40	100	0,8

Table 3.100

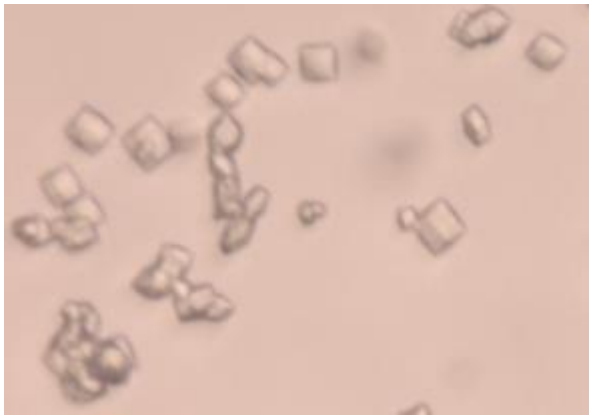


Figure 3.101

Figure 3.102

- Sixteenth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
16	0,1	40	100	0,8

Table 3.103

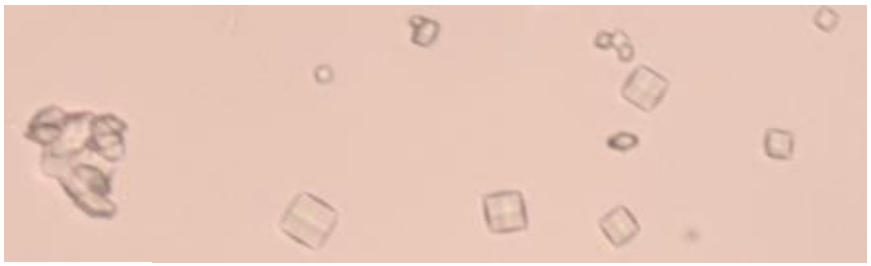


Figure 3.104

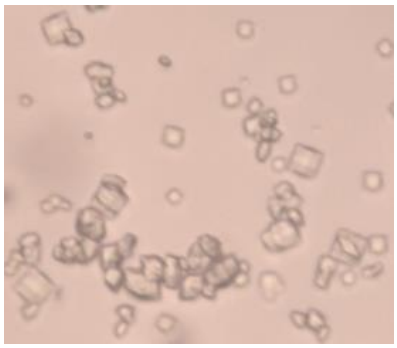


Figure 3.105

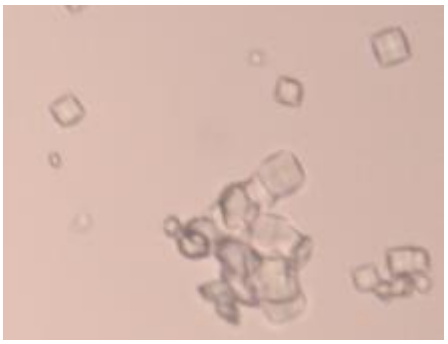


Figure 3.106

- Seventeenth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
14	0,1	40	100	0,8

Table 3.107

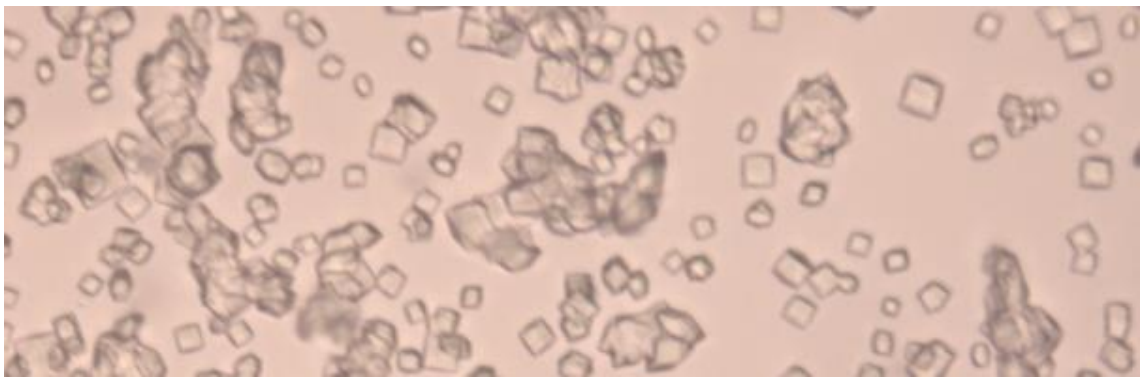


Figure 3.108

- Eighteenth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
10	0,1	40	100	0,8

Table 3.109

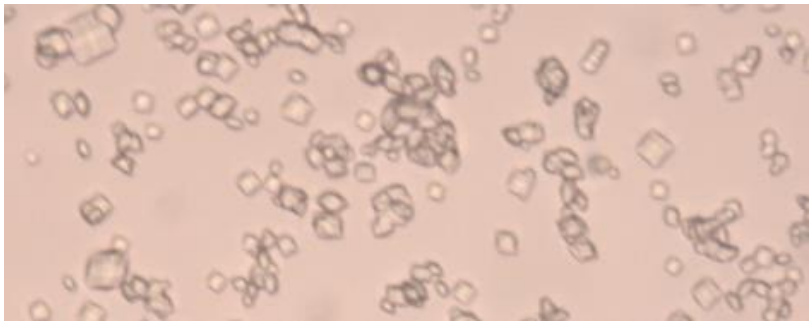


Figure 3.110

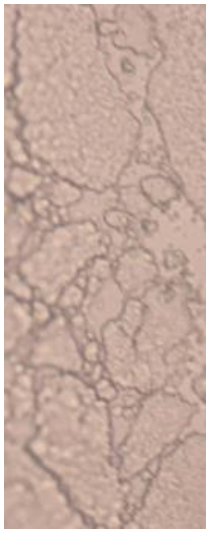


Figure 3.101

- Nineteenth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
7	0,1	40	0	0,8

Table 3.112

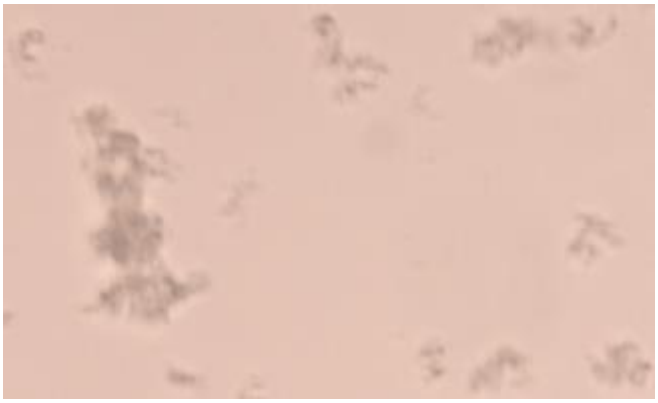


Figure 3.113

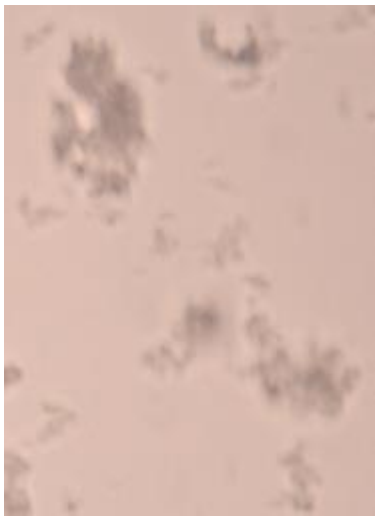


Figure 3.114

- Twentieth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
7	0,1	40	20	0,8

Table 3.115

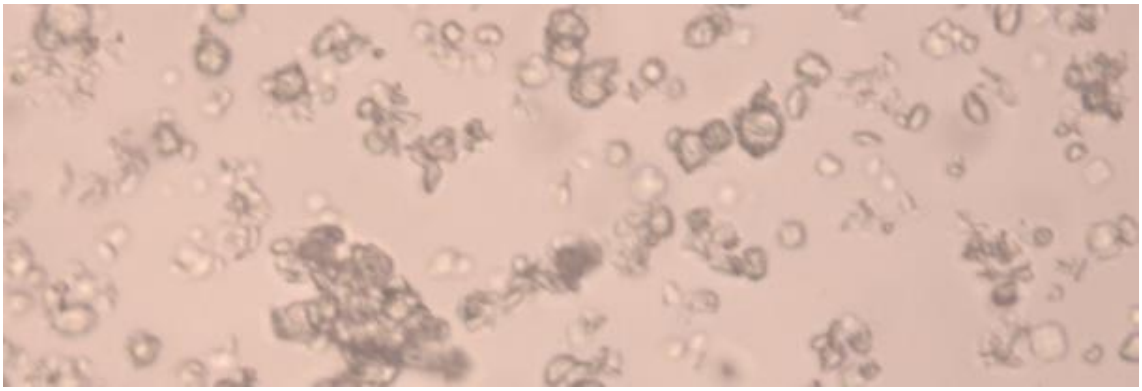


Figure 3.116

- Twenty-first experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
7	0,1	40	100	0,8

Table 3.117

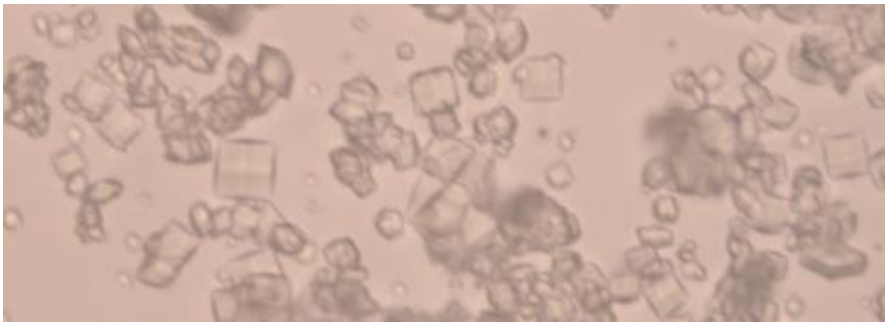


Figure 3.118

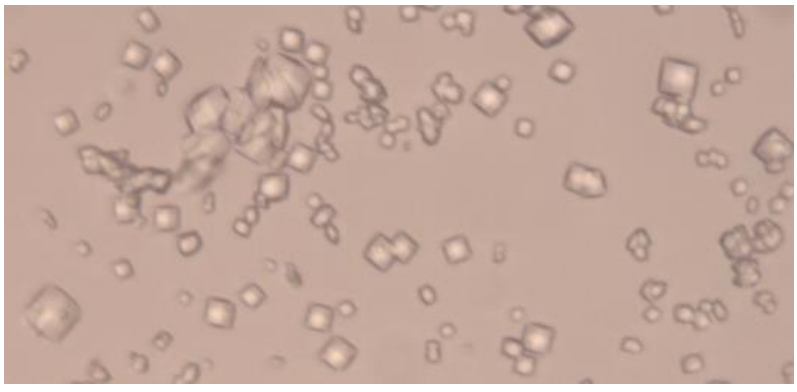


Figure 3.119

- Twenty-second experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
7	0,1	40	40	0,8

Table 3.120

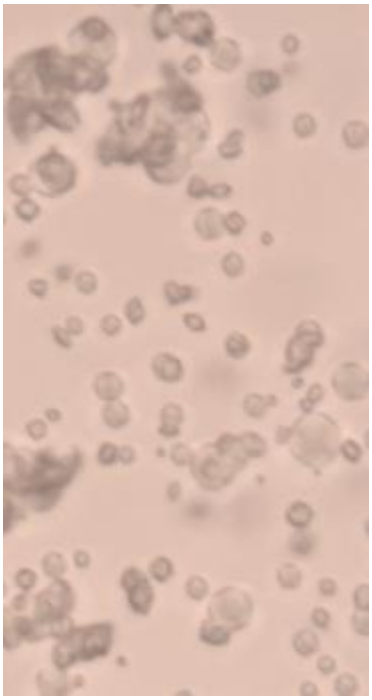


Figure 3.121

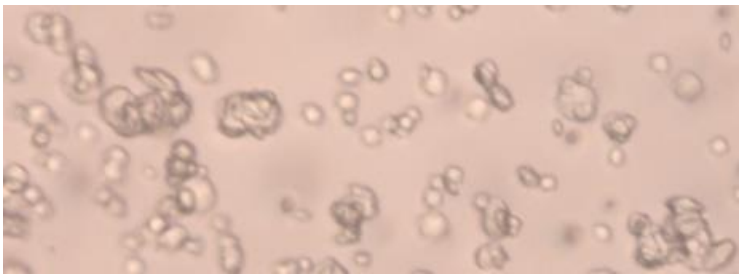


Figure 3.122

- Twenty-third experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
20	0,1	40	10	0,8

Table 3.123

Figure 3.124

- Twenty-fourth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
16	0,1	40	10	0,8

Table 3.125

Figure 3.126

Figure 3.127

- Twenty-fifth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
14	0,1	40	10	0,8

Table 3.128

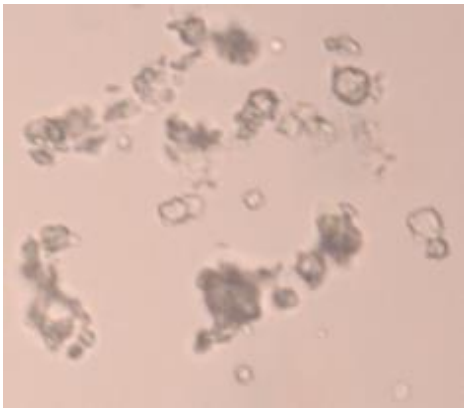


Figure 3.129

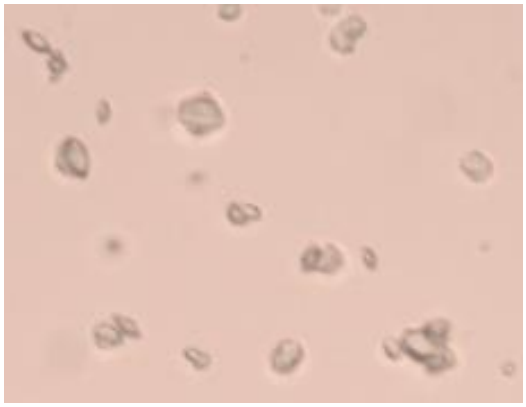


Figure 3.130

- Twenty-sixth experiment

Temperature (ºC)	Protein (g/ml)	NaCl (mg/ml)	IL(mg/ml)	Flow rate (ml/min)
10	0,1	40	10	0,8

Table 3.131

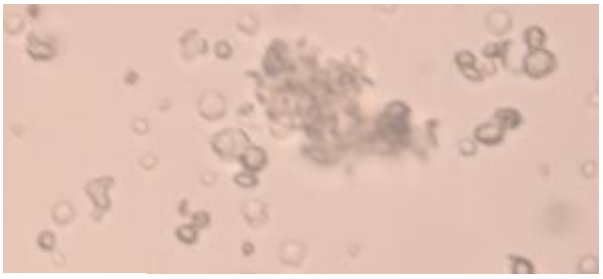


Figure 3.132

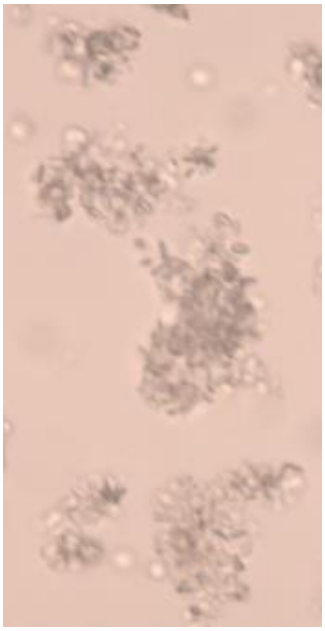


Figure 3.133

- Naked eye observations of crystallization through the first water bath in continuous experiments.

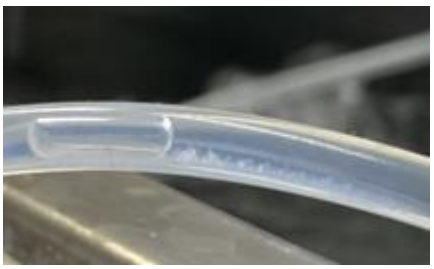


Figure 3.134. Air bubble pushed the crystal forward.

All over the solubility experiments, we have focused as well on the visible influence (Figure 3.134) of several parameters in the protein crystallization, like Ionic Liquid concentration or temperature, along the first water bath in continuous processes. The Ionic Liquid has a strong influence in the crystallization but as the following tables show, the temperature plays also an important role during the first tank. The following tables (from Table 3.135 to Table 3.139) show the evolution of the nucleation in the first water bath (amount of formed crystals in front of the bubbles), working with different temperatures and different Ionic Liquid concentrations.

Ionic Liquid concentration = 0 mg/ml	
Temperature = 20ºC	No crystals
Temperature = 16ºC	No crystals
Temperature = 14ºC	No crystals
Temperature = 10ºC	No crystals
Temperature = 7ºC	Crystals appear even earlier than the first 10 cm and in a great amount

Table 3.135

Ionic Liquid concentration = 10 mg/ml	
Temperature = 20ºC	Not crystals
Temperature = 16ºC	Not crystals
Temperature = 14ºC	Little amount of crystals after the first 20-30 cm
Temperature = 10ºC	Little amount of crystals after the first 10-20 cm

Table 3.136

Ionic Liquid concentration = 20 mg/ml	
Temperature = 20ºC	No crystals
Temperature = 16ºC	~2 cm of crystals in front of the bubble after the first bath
Temperature = 14ºC	~2 cm of crystals in front of the bubble after the first bath
Temperature = 10ºC	Little amount of crystals after the first ~20 cm
Temperature = 7ºC	Crystals appear even earlier than the first 10 cm

Table 3.137

Ionic Liquid concentration = 40 mg/ml	
Temperature = 20ºC	Little amount of crystals in front of the bubble at the end
Temperature = 16ºC	~1-1.5 cm of crystals in front of the bubbles
Temperature = 14ºC	~2 cm of crystals in front of the bubbles
Temperature = 10ºC	First crystals appear after the first ~20-30 cm
Temperature = 7ºC	Crystals start appearing between the first 15-20 cm

Table 3.138

Ionic Liquid concentration = 100 mg/ml	
Temperature = 20ºC	First crystals start appearing after the first ~30-40 cm. At the end, ~2-3 cm in front the bubbles
Temperature = 16ºC	First crystals start appearing after the first ~30-40 cm. ~4 cm of crystals in front of the bubble at the end of the pipe
Temperature = 14ºC	First crystals start appearing after the first ~30-40 cm. ~4 cm of crystals in front of the bubble at the end of the pipe
Temperature = 10ºC	At the very beginning, there is not a big amount of crystals in front of the bubble, but they start appearing after the ~20-30 cm At the end of the pipe, a large amount of crystals are appreciable in front of the bubble, between 5-7 cm
Temperature = 7ºC	Crystals do not appear until the first ~50-60 cm

Table 3.139

**The data from tables (3.135 to 3.139) were taken to the naked eye, so the results are approximate values*

4 DISCUSSION and CONCLUSION

All over the experiments, many parameters have been varied to achieve the different goals which were proposed in the project. Obviously, the variation of some parameters have a greater influence on the crystallization, and higher yield levels can be reached. However, there are some others which hardly modify the final yield but they have a strong influence on the crystallization time. As we know, this two factors, yield and time, are between the most important in any industry, so knowing how to obtain the optimal value of each one at the same time becomes essential.

One of the first questions someone could ask himself/herself could be the difference between working continuously or discontinuously in crystallization. Well, based on the results of our experiments, the fact that working with batch reactor means that the maximum reached yield is higher than the yield we can get with the continuous reactor as the table 4.1 shows. However, if we focus on time, the time difference between each reactor to get their respective yield is huge. For instance, a continuous reactor with 3 water baths which works with a flow rate of 0.4 ml/min and with Ionic Liquid, needs 2 hours to achieve a yield of 70%, while a batch reactor which works with Ionic Liquid, needs 24 hours to achieve 87%.

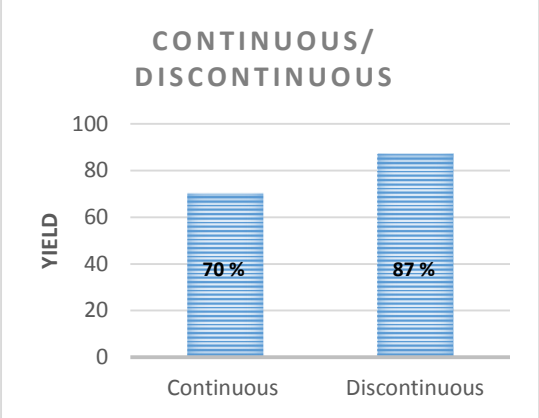


Table 4.1. Yield comparison between continuous and discontinuous reactor

One of the parameters which has a greater influence on the yield is the Ionic Liquid. However, depending on which type of reactor we work with, the yield progression will be modified in one or other way. On one hand, the effect of the Ionic Liquid in the batch experiments has the greater impact in the first hours, as the Figure 4.2 shows. This means that the IL allows the formation of the first crystals earlier than if we do not work with Ionic Liquid, where the crystals take much more time to start appearing. However, the use or not of Ionic Liquid doesn´t have a big influence in the final yield. Both of them achieve a yield around 87%.

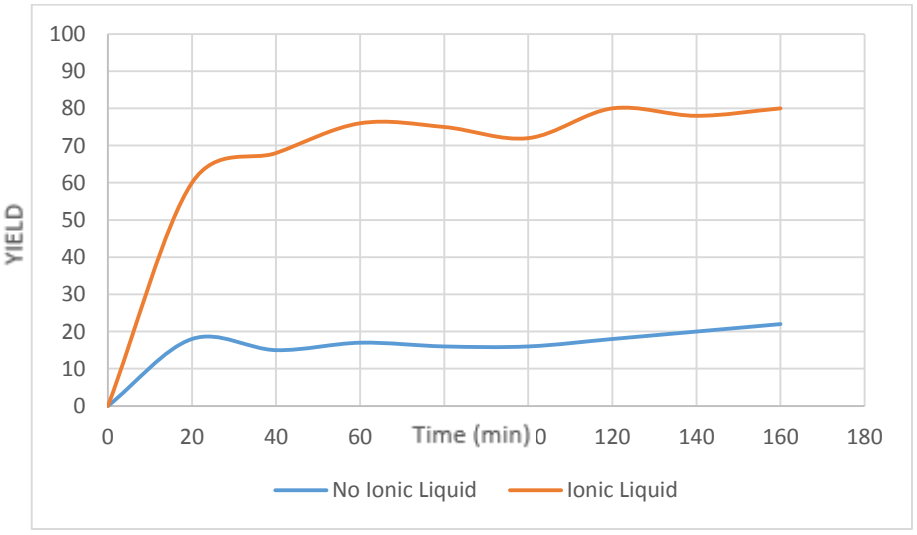


Figure 4.2. Yield Evolution in batch reactor with and without IL in the first 3 hours

On the other hand, working continuously, both the Figure 4.3 and the Figure 4.4 show clearly the influence of the Ionic Liquid takes place both in the initial crystallization steps and in the final yield, where the yield is almost 30% higher working with Ionic Liquid. At the end of the first water bath (after the first 26 minutes) the influence of the Ionic Liquid is appreciable as well. If we don´t work with IL, the process doesn´t reach even 20%, meanwhile, if we work with IL, the process already achieve 50% in the first water bath.

This Figure 4.3, 4.4 and 4.5 illustrate the yield after each water baths by four or five samples taken at the end of each one.

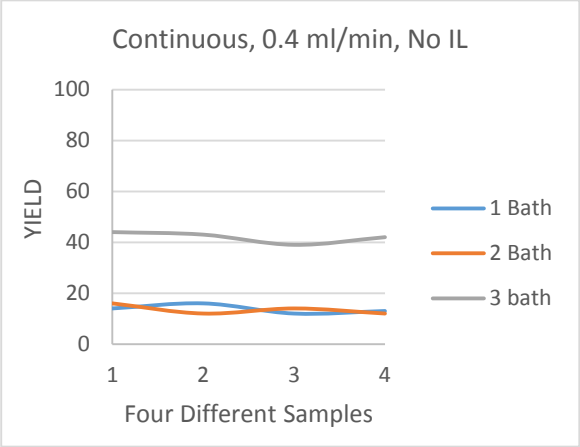


Figure 4.3. Yield comparison after the first, second and third water bath, in an experiment without IL and a flow rate of 0.4 ml/min

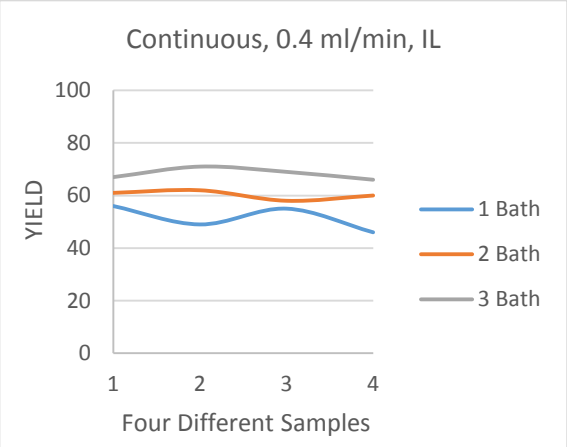


Figure 4.4. Yield comparison after the first, second and third water bath, in an experiment with IL and a flow rate of 0.4 ml/min

Furthermore, we have carried out some experiments doubling the flow rate. In other words, working with 0.8 ml/min as the Figure 4.5 shows instead of working with 0.4 ml/min. However, apparently its influence on the yield is not really noticeable. Both the Figure 4.4 and 4.5 show a yield of around 50% after the first water bath, 60% after the second one and quite close to 70% in the final one. But it is important to realize that if we work with double flow rate, the whole residence time will be half. So, we get the same amount of crystallization in half time.

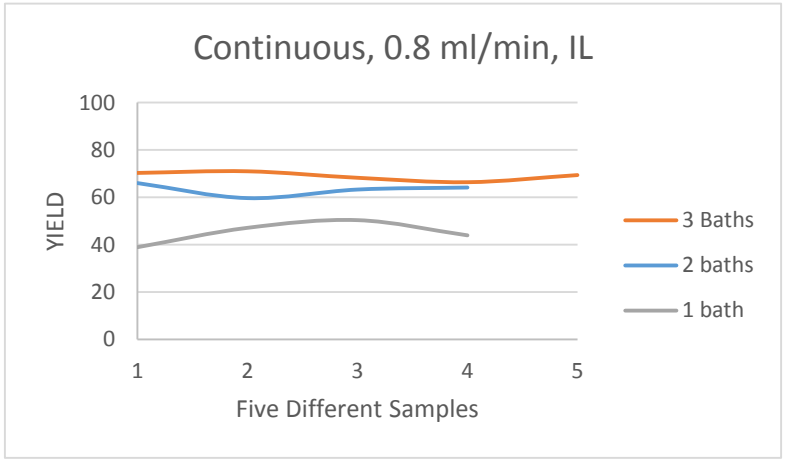


Figure 4.5. Yield comparison after the first, second and third water bath, in an experiment with IL and a flow rate of 0.8 ml/min

The Figures 4.4 and 4.5 show that crystallization takes place mainly during the first water bath, where the yield achieves values around 50%. Once the solution leave the first 3 meters where nucleation takes place, it continues through the second and third water bath, where supersaturation is chosen to be below the critical supersaturation level in order to promote growth of nuclei, instead of their formation. Therefore, the yield increases just around 20% along the last 10 meters (the last 2 water baths). In the other case, without Ionic Liquid, it is appreciable that occurs exactly the opposite. The main yield increase takes place during the last water bath.

As the results chapter shows, many continuous experiments with just one water bath and a couple of discontinuous experiments have been made in order to focus on the crystal shape and on the position of the precipitation area and the crystallization area for the solubility diagram of lysozyme. The position in the diagram depends on, as the Figure 4.6 shows, the IL concentration and the temperature. So, depending on the working conditions, the protein could either crystallize or precipitate.

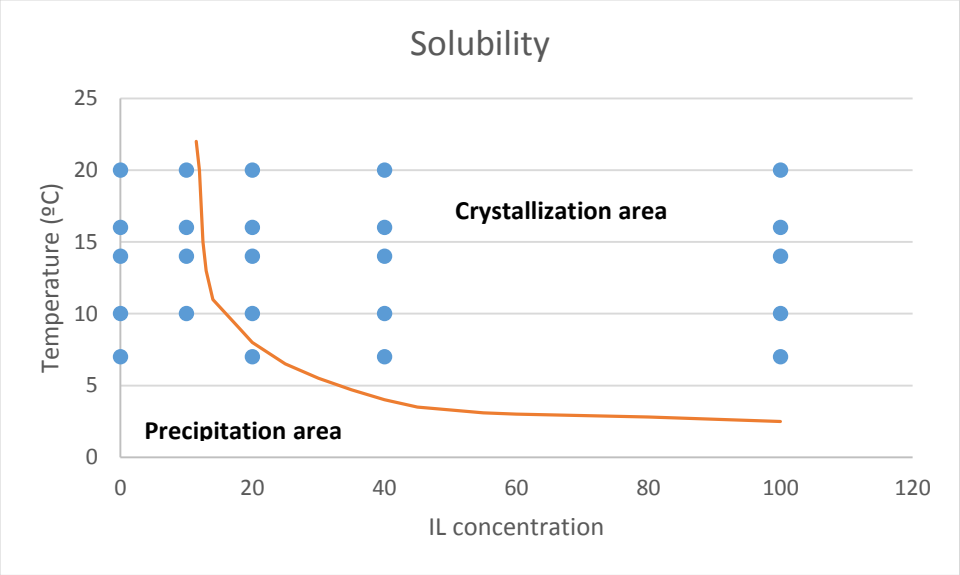


Figure 4.6. Ionic Liquid solubility in continuous crystallization. Each point belongs to a single experiment

The points are dispersed all over the Figure 4.6, where there are two remarkable areas. The one over the precipitation line (orange line) and the on below. Thus, this line splits up two physical phenomena, crystallization and precipitation. On one hand, it is important to take into account that a dramatic physical change between both sides of the precipitation line never happen, which means that points close to the orange line contain both crystallization and precipitation as we can see in the Figure 4.7 (the photo belongs to an experiment without Ionic Liquid at 16ºC). On the other hand, points which are located further from the solubility line contain just precipitation or crystallization as the Figure 4.8 (just crystallization) and Figure 4.9 (just precipitation) show.

Figure 4.7. Precipitation and crystallization. Experiment without Ionic Liquid at 16ºC

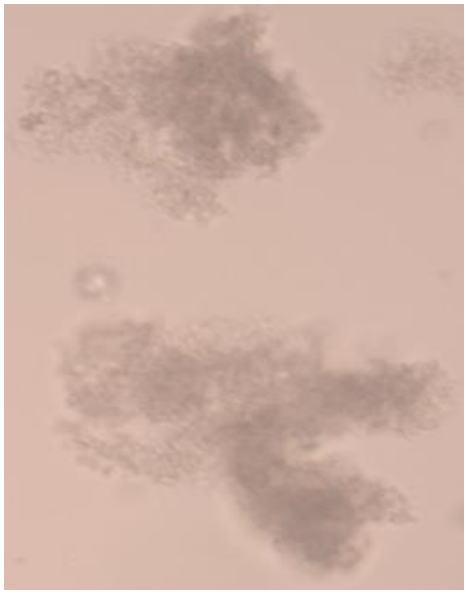


Figure 4.9. Just Precipitation. Experiment without Ionic Liquid at 7ºC

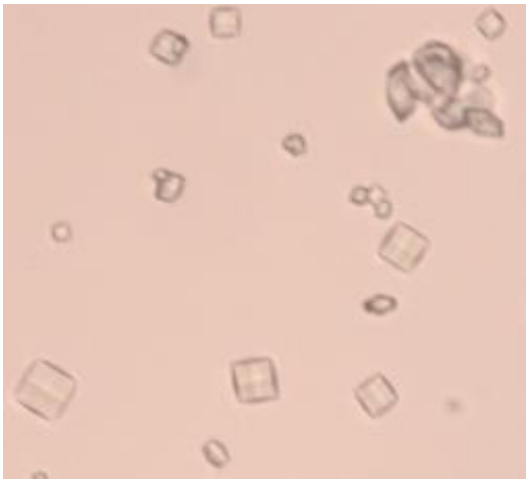


Figure 4.8. Just Crystallization. Experiment with Ionic (100 mg/ml) at 16ºC

Once the solubility diagram (Figure 4.6) had been analysed, we could clearly conclude that the addition of Ionic Liquid to the crystallization process helped in formation of crystals instead of precipitation. Besides, as the temperature decrease, we got closer to reach the precipitation phenomena but it depended again on the concentration of IL. When working with high concentration of Ionic Liquid the precipitation area was reached only at very low temperatures or not at all, thereby prohibiting us from characterizing the precipitation line.

For instance, when we carried out the experiment with a constant Ionic Liquid concentration (10 mg/ml) and we decreased the temperature from 20ºC to 10ºC, we moved vertically through the solubility diagram (Figure 4.6). At 20ºC (twenty-third experiment, Figure 3.67) we obtained just precipitation. As we went down through this vertical line, precipitation remained happening, but crystals started to appear at the same time at the end of each experiment (twenty-fourth, twenty-fifth and twenty-sixth experiment). This is because we work so close to the precipitation line, and both phenomena could occur.

However, we were able to study in depth, on the other hand, the crystallization area. Superficially, all crystals seemed really similar. Nevertheless, the crystallization conditions hat a strong influence on the crystal shape, leading in some cases to geometrical crystals (with remarkable edges) and in other cases more rounded and smooth crystals. Then, if we performed the same experiment as in the last paragraph but with a constant Ionic Liquid concentration of 100 mg/ml, several differences in the crystals were found as we decreased the temperature. At 20ºC, the crystals had a cubic geometrical form and they are pretty separated, no agglomeration, as the Figure 4.10 shows. At a lower temperature, both at 16ºC, 14ºC and 10ºC the final crystals present a cubic geometric form as well, but some agglomeration between crystals was detected. Finally, at the lowest covered temperature, Figure 4.11, at 7ºC, the crystals shape remained the same as the previous ones, but a higher level of agglomeration was found. The same steps occurred when working with a constant Ionic Liquid concentration of 40 mg/ml, differing from the lowest temperature experiment (twenty-second experiment, 7ºC) where the crystals lose their geometrical shape.

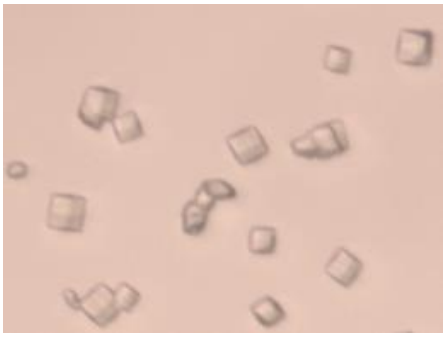


Figure 4.10. Continuous crystallization, [IL] = 100 mg/ml, T=20ºC, cubic geometrical crystals, No agglomeration

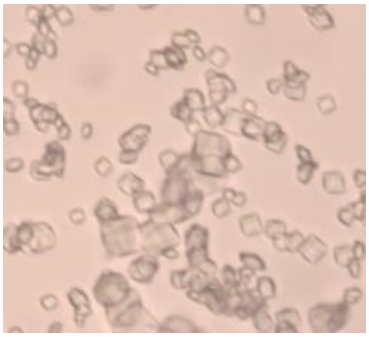


Figure 4.11. Continuous crystallization, [IL] = 100 mg/ml, T=7ºC, cubic geometrical crystals, Agglomeration

Moving horizontally through the solubility diagram, we can compare the crystallization between experiments which differ in the Ionic Liquid concentration at the same temperature. For instance, if we fixed a temperature of 14ºC, we obtained the following crystallization features (Table 4.12):

T = 14ºC				
[IL] = 0 mg/ml	[IL] = 10 mg/ml	[IL] = 20 mg/ml	[IL] = 40 mg/ml	[IL] = 100 mg/ml
Precipitation. Crystallization. Crystals with a certain shape, mainly rounded, but without and specific form. No agglomeration	Crystallization. Crystals without a specific geometrical form. Agglomeration.	Crystallization. Crystals present certain geometrical shape but really variable. Agglomeration.	Crystallization. Crystals present certain geometrical form, mainly cubic. Agglomeration.	Crystallization. Crystals with a very specific geometric shape, mainly cubic. Agglomeration

Table 4.12. Crystal features at a fixed temperature for different Ionic Liquid concentration

The influence of the temperature and the Ionic Liquid concentration along the first water bath can be easily analysed from the Tables (3.135 to 3.139). We could conclude that decreasing the process temperature, the amount of crystals in front of the bubble increase and the formation of crystals occurs even earlier. Besides, the influence of the Ionic Liquid, as we have been discussing all over the project, is clearly remarkable. The higher concentration of IL allows the bigger amount of formed crystals. When we carried out experiments ay 7ºC at first glance crystals seemed to appear very early when doing experiments without IL. Though, under the microscope it was visible that in this case only precipitation occured. But, if we work with a higher concentration (100 mg/ml for example) the crystals do not appear before the ~50-60 cm (Table 3.139). So, studying this results, and knowing that 0 mg/ml of IL and 100 mg/ml of IL at any temperature means precipitation and crystallization respectively, we can conclude that the precipitation occurs almost instantaneously and the crystallization takes a little more time.

Until now, we have just focused on the comparison between crystals which were produced continuously at different conditions. However, the first five experiments are related with discontinuous crystallization, where the produced crystals carry a really specific geometry, from cubic geometries to rounded, or even diamond shape when the temperature is really low (10ºC). In this five experiments, we worked with two different NaCl concentration (40 mg/ml or 60 mg/ml), varying both the IL concentration and the temperature (see point 3.2, Crystal´s shape after crystallization, batch processes)

To end up the work, I would like to add a comparison between one of the first graphics (Figure 1.7) and one of the last ones (Figure 4.6) which keep a strong relation between each other. Both graphics show a crystallization area and a precipitation area. The first figure shows with arrows, a possible way to achieve crystallization, which is represented in the second figure with a point in the crystallization area (depending on the working conditions, mainly IL concentration and temperature, the point will be placed further or closer from the precipitation line). However, if the experiment conditions lead the horizontal line of the Figure 1.7 until the precipitation zone, the point will be placed below the precipitation line in the Figure 4.6 instead of above. So to sum up, we must maintain the reaction in the nucleation zone to produce crystals, avoiding then the precipitation zone.

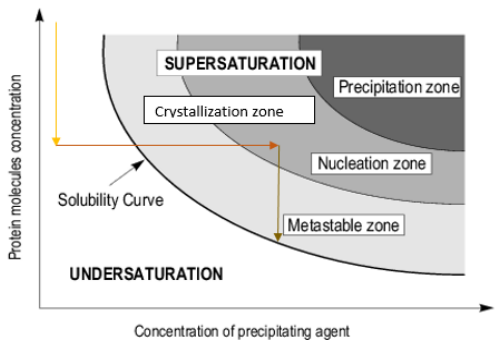


Figure 1.7. Solubility curve

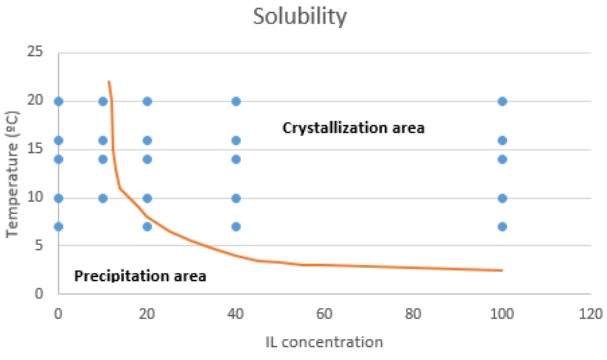


Figure 4.6. Ionic Liquid solubility in continuous crystallization. Each point concerns to a single experiment

5 REFERENCES

(UniProt), U. P. (2015). *Lysozyme C*. Retrieved from http://www.uniprot.org/uniprot/P00698

Asherie, N. (2004). Protein Crystallization and Phase Diagrams. *Methods, 34(3)*, 266-272.

Bicak, N. (2005). A New Ionic Liquid: 2-hydroxy ethylammonium formate. *Journal of Molecular Liquid, 116(1)*, 15-18.

De Yoreo, J. J., & Vekilov, P. G. (2003). Principles of Crystal Nucleation and Growth. *Reviews in mineralogy and geochemistry, 54(1)*, 57-93.

Hekmat, D., Hebel, D., Schmid, H., & Weuster-Botz, D. (2007). Crystallization of Lysozyme: From vapor diffusion experiments to bath crystallization in agitated ml-scale vessels. *Process Biochemistry, 42(12)*, 1649-1654.

Huettmann, H., Zich, S., Berkemeyer, M., Buchinger, W., & Jungbauer, A. (2015). Design of Industrial crystallization of Interferon Gamma: Phase Diagrams and Solubility Curves. *Chemical Engineering Science, 126*, 341-348.

Introduction to Protein Crystallization. (2011, July 04). Retrieved from http://www.xray.bioc.cam.ac.uk/xray_resources/whitepapers/xtal-in-action/node3.html

Judge, R. A., Takahashi, S., Longenecker, K. L., Fry, E. H., Abad-Zapatero, C., & Chiu, M. L. (2009). The Effect of Ionic Liquid on Protein Crystallization and X-ray Diffraction Resolution. *Crystal Growth and Design, 9(8)*, 3463-3469.

Leunissen, M. (2001). Protein crystallization. *An essay on several aspects of protein*.

Lipp, E. (2006, September 1). *Protein Crystallization in Pharma Research*. Retrieved from Refinements of the Technology Are Taking place very quickly: http://www.genengnews.com/gen-articles/protein-crystallization-in-pharma-research/1868/

Mesters, J. R. (2007). *Nucleation and Crystal Growth.*

Neugebauer, P., & Khinast, J. G. (2015). Continuous Crystallization of Proteins in a Tubular Plug-Flow Crystallizer. *Crystal growth & design, 15(3)*, 1089-1095.

Pusey, M. L., Paley, M. S., Turner, M. B., & Rogers, R. D. (2007). Protein Crystallization Using Room Temperature Ionic Liquids. *Crystal growth & design, 7(4)*, 787-793.

Ratnaparkhi, M. P., Chaudhari, S. P., & Pandya, V. A. (2011). Peptides and Proteins in Pharmaceuticals. *Int J. Curr. Pharm. Res, 3*, 1-9.

Samant, K. D., & O´Young, L. (2006). Understanding Crystallization and Crystallizers. *Chemical engineering process, 102(10)*, 28-37.

Schmid, F. X. (2001). Biological Macromolecules: UV-visible Spectrophotometry. *eLS*.

Sigma-Aldrich. (n.d.). *Product Information, Lysozyme from Chicken egg white*. Retrieved from http://www.sigmaaldrich.com/catalog/product/sigma/l6876?lang=de&region=AT

Wang, Z., Dang, L., Han, Y., Jiang, P., & Wei, H. (2010). Crystallization control of thermal stability and morphology of hen egg white lysozyme crystals by ionic liquids. *Journal of agricultural and food chemistry, 58(9)*, 5444-5448.