Immobilization of spray dried *E. coli* containing *s*-aminotransferase using hydrous titanium oxide

Watson Neto

June 2009
Master thesis in Biological Engineering
Department of Biotechnology, Lund Institute of Technology, Lund University, Sweden

Abstract: Chiral amines are important building blocks for the pharmaceutical and agrochemical industry. They have many applications and are therefore of economical importance. Their production has mostly been achieved by enzymatic resolution. Aminotransferase, the enzyme most commonly used is known to be a pyridoxal-5-phosphate (PLP) dependent enzyme and it is commercialized in a spray dried *E. coli* formulation. Since there is a great market for this enzyme and all the products it can synthesize, it is essential to constantly improve its efficiency and make it even more economically attractive. Several studies have been conducted to achieve this purpose, one of these being the reutilization of the biocatalyst by immobilization. In this work, immobilization of the biocatalyst was performed using a different approach than what has been tried before. The approach is based on interaction between hydrous transition metal oxides, mainly the hydrous titanium oxide (TiOx), and biological entities such as cells, enzymes and similar. Hydrous titanium oxide has the ability to bind the cells and enzymes via hydroxyl groups forming partial covalent bonds. Using this method, the biocatalyst was successfully immobilized and its reutilization was demonstrated for several batches. Studies on enzyme kinetics, storage stability, temperature effect, and loading capacity were performed to validate the usefulness of the immobilization method for the potential use in a large scale process.

Keywords: Transition metal oxide, spray dried *E. coli* cells immobilization, Hydrous Titanium oxide, aminotransferase, chiral amines, acetophenone.

1. Introduction

Chiral amines, important building blocks for pharmaceutical and agrochemical industry, have been economically efficiently produced by enzymatic resolution. For this purpose, several biocatalytic methods have been studied, but recently great attention has been given to the use of transaminases or aminotranferases (ATs). These enzymes are known to be pyridoxal-5-phosphate (PLP)-dependent, and have received increased interest due to their great potential for production of natural and unnatural amino acids, amino alcohols, and amino sugars, as well as chiral amines which are valuable key intermediates or starting materials for chiral drugs [1], [2]. In the present study two main reactions using ω-transaminase were carried out: a) conversion of 7-methoxy-2-tetralone into (S)-7-methoxy-2-aminotetralin, which was the target reaction, and b) conversion of (S)-(−)-α-methylbenzylamine (*S*-MBA) into acetophenone, which was used as a model reaction. The first reaction is very slow and difficult to use in regular activity assays. On the other hand, the conversion of *S*-MBA into acetophenone is much faster. High conversion is achieved in a couple of minutes and it has been extensively studied, and for this reason it was chosen for the regular assays.

The application of this enzymatic synthesis at large scale is normally carried out using spray-dried cells having ATs activity. For a better economical large scale production of these chiral amines, continuous investigation must be carried out to improve the efficiency of this biocatalyst. Here, immobilization of *E. coli* spray-dried cells having S-aminotransferase activity was carried out as an approach to solve these problems. Immobilization of this biocatalyst has previously been reported using cell entrapment in calcium alginate beads. Immobilization by entrapment normally results in kinetic behaviour changes of the enzyme due to physical isolation from reaction medium and molecular association between the enzyme and the immobilization matrix that can lead to diffusion effects [3].

In this study, a different approach using surface adsorption immobilization technique was developed, using gelatinous hydrous transition metal oxides that associates with the cells' surface. The use of transition metal oxides for the immobilization of biological molecules is well established and documented. There are reports of immobilization of amino acids, peptides, enzymes, antibiotics, polysaccharides, and whole living cells using this method [4]. The immobilization is achieved due to chelation properties of transition metals, mainly titanium (IV) and zirconium (IV), which seem particularly attractive due to the non-toxicity properties of their oxides [5].

2. Background of the method:

2.1 Production of TiOx and cell immobilization

Investigations has shown that hydrous titanium(IV), zirconium(IV), iron(III), vanadium(III), and tin(IV) oxides can be used as matrices for immobilization of biocatalysts. They are able to associate with enzyme and cells, forming insoluble filterable complexes that are enzymatically active. Among them, titanium (IV) and zirconium (IV) proved to be the most satisfactory as they showed a little or no effect on the function of the biocatalyst [5]. In this work, full attention was given to hydrous titanium (IV). The TiOx is formed through addition of ammonium hydroxide to a solution of titanium (IV) tetrachloride until pH 7 is reached according to the following reactions:

$$TiCl_4 + H_2O \rightarrow TiOCl_2 + 2HCl \quad (1)$$

$$TiOCl_2 + 2HCl + 4NH_4OH \rightarrow 4NH_4Cl + 2H_2O \quad (2)$$

As the pH is increased until 7, white thin particles start to form in the solution consisting of titanium oxyhydrate, also called hydrous titanium oxide. This compound is the active part in the immobilization process. The mixture is then filtered and washed with physiological saline solution, revealing a white amorphous compound (TiOx). When mixed with a cell suspension it forms a complex that rapidly tends to aggregate and precipitate, being easily recovered by centrifugation or filtration (Figure 1).

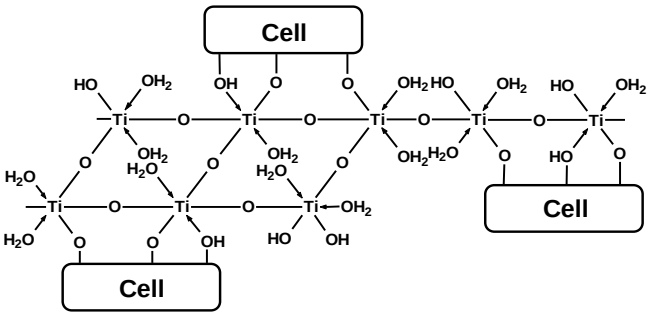


Figure 1: Titanium Oxyhydrate matrix chelating whole cell (Adapted from Kennedy *et al*, 1997 [4]).

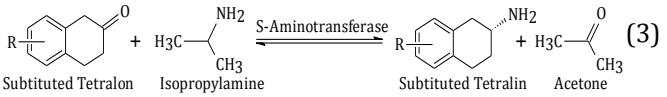
The immobilization process takes place due to a substitution reaction, in which hydroxyl groups on the surface of the hydrous metal oxide are replaced by suitable ligands from the cell, resulting in the formation of partial covalent bonds (Figure 1).

2.2 The transamination reaction

The ATs action is dependent of PLP as cofactor, which means that any reaction using this enzyme must have PLP in order for the reaction to take place.

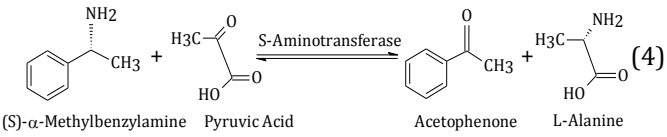
Depending on the substrate, different numbers of products can be obtained. The enzyme will transport an amine group from one substrate (the amine donor) to another (the amine acceptor).

The target reaction (3) consists of the transfer of the amine group from the isopropylamine to the substituted tetralone, converting it to a substituted tetralin and forming acetone as a secondary product.



(3)

However, this reaction occurs at very slow rate, achieving 85-90% of conversion in 12 hours [3]. On the other hand, the production of acetophenone (4) is catalyzed by the same enzyme but at faster rate, achieving 90-95% of conversion in less than 2 hours. This characteristic makes it more favourable to use this reaction as an activity assay to characterize the immobilized biocatalyst.



(4)

2.3 The activity assay

An absorbance spectrum of the substrate (MBA) and the product (acetophenone) showed that they have absorbance peaks at 213 and 245 nm respectively. According to this, a theoretical standard curve was developed, relating the absorbance measured in the spectrophotometer and the acetophenone concentration in the reactor.

The activity assays was performed at 55 ºC with pH set to 7 by adding 100 mM of Tris buffer at pH 7.2. In most of the reactions, the reaction mixture volume used was between 2.8 and 3 mL containing 15 mM of MBA, 100 mM of sodium pyruvate (SP) and 1 mM of PLP. The mixing was provided by the termoshaker (HLC Biotech) at 700 rpm. The reaction was stopped adding 0.1 M HCl and analysed by spectrophotometry (Shimadzu UV-1650PC) with a final dilution of 1:49

3. Material and Methods:

3.1 Production of hydrous TiOx and cell immobilization

To a solution containing 150 g/L of $TiCl_4$ (Fluka) and 150 g/L of HCl (Merck), 2 M of NH_4OH (VWR) was added slowly under gentle stirring until neutral pH was achieved. The pH was controlled by mean of a colour change marker (BTB from Fluka). The resulting solution was filtered under vacuum and washed with a 0,9% NaCl solution (Merck) to remove the remaining titanium tetrachloride and the pH indicator. The filtrate was stored in saline solution to keep the hydrous property with a concentration of 0,6 g/mL.

The immobilization takes place when a cell solution is mixed with the TiOx preparation and is given a few minutes (5-10 minutes) to settle down. The TiOx is heavier than the cells and will precipitate carrying them. The immobilization efficiency can early be confirmed by a quick visual analysis; a good immobilization will give a clear supernatant while a bad immobilization will produce a turbid supernatant.

3.2 Effect of immobilization on the biocatalyst activity

Immobilized cells (0.6 mg of cell with 1% of loading) was compared with free cells by following a 2 hours reaction, proceeding as described in 2.3 and taking samples each 10 minutes.

3.3 Storage stability

Free and immobilized cells (0.6 mg, 1% of cell loading in the immobilized preparation) was stored for 16 hours in the fridge, being one of each preparation kept in the presence of the cofactor (1mM PLP) and other without the cofactor. After that the preparation were tested for activity.

3.4 Effect of the temperature on the initial activity

Free and immobilized cells were incubated for 30 minutes at different temperatures (40, 50, 60 and 70 ºC) and tested for activity at 55 ºC. The cell mass used was 0.6 mg and the cell loading of the immobilized preparation was 1%.

3.5 Kinetics studies

The kinetics of immobilized and free cells was studied by running the activity assay for both preparations according to Table 1:

Table 1 – Composition of reaction mixture for kinetic studies of AT's activity in free and immobilized biocatalyst.

	MBA (mM)	SP (mM)	PLP (mM)	KH_2PO_4 (mM)
MBA Range	0 to 15	100	1	100
SP Range	15	0 to 15	1	100

3.6 Immobilization efficiency

Cell immobilizations with different loadings were prepared by adding 0.6 mg of cells to different amount of TiOx to achieve the desired loading. After preparation settled down, the entire supernatant was removed and tested for activity. This procedure was made for 4 assays to give statistical significance. The results were compared with the activity of the same amount of free cells.

3.7 Effect of preparation re-use on the initial activity

An immobilized preparation with 5 mg of cells (loading of 2%) was incubated for 70 minutes at 55ºC in a reaction mixture of 5 mL containing MBA (~16 mM), SP (100 mM), PLP (~1mM) and Tris buffer (100 mM). The reaction was carried out in a thermoshaker at 700 rpm rotation. Samples were taken at minute 5, 10, 15, 20, 30, 40, 50, 60, 70 for most of the batches, and centrifuged for 2 minutes. After centrifugation, 200 µL of the supernatant was collected in an *eppendorf* tube containing 800 µL of 0,1 M HCl. The remaining solution was returned to the reactor. After each batch the reactor was centrifuged for 10 minutes at 6000 rpm (WIFUG Lab Centrifuges), and the supernatant containing the old media was discarded and a fresh one was added starting the new batch.

The samples were analysed by spectrophotometry at 245 nm with a final dilution of 1:99.

3.8 Production of (s)-7-Methoxy-2aminotetralin

The immobilized biocatalyst with a loading of 1 % was tested in the target reaction (3) for 7 hours. The aim was to produce 7-methoxy-2-aminotetralin (7-MAT) from 7-methoxy-2-tetralone (7-MT) and isopropylamine (IPA).

The reaction took place in 50 mL reactor provided with a magnetic stirrer and water circulation jacket to maintain the temperature at 55 ºC. The cell mass used was 20 mg, in 20 mL reaction mixtures containing 68 mM of ketone (7-MT) diluted in 2% v/v ethanol, 0,5 mM PLP and 750 mM of IPA. Samples were taken at 0, 1, 2, 3, 4, 5 and 7 hours and then centrifuged for 2 minutes to remove the immobilized cells. 200 uL of supernatant was taken and diluted in 800 uL of 0,1 M HCl to stop the reaction. The remaining content after the centrifugation was returned to the reactor. The samples were analysed by HPLC with mobile phase composed two solutions; A: 100% methanol and B 0,01% trifluoroacetic acid in water. The results were compared to free cells (220 mg).

Between each samples, the reactor mixture was bubbled with nitrogen to remove the oxygen, since the ketone used is oxygen sensitive.

3.9 Calculation of the volume occupied by the preparation in the reactor

About 6 mg (dry weight) of spray dried *E. coli* was washed twice with 0.9% NaCl with 2 centrifugations at 6000 rpm (WIFUG Lab Centrifuge) in between and later immobilized with 594 mg (wet weight) of TiOx. The immobilized preparation with a final volume of 3 mL (filled with 0,9% NaCl) was put inside a graduated cylinder of 5 mL and its height during the sedimentation was observed for 2 hours.

4. Results and discussion

4.1 Effect of immobilization on the biocatalyst activity

The result shown in Table 2 suggests no great differences in behaviour between the immobilized and free biocatalyst. The maximum conversion achieved for free and immobilized cells was 69% and 65% respectively, and analysing the specific rate of both (0,900 U/mg *vs* 0,861 U/mg) we can assume that no great diffusion effects are produced upon the immobilization.

Table 2 – Maximun conversion and initial activity for free and immobilized cells.

	Free cells	Immobilized
Specific rate (U/mg)	0,900	0,861
Max Conversion (%)	69,43	64,76

4.2 Storage stability

It was found that storing both free cells and immobilized cells for 16 hours in the fridge was bad for the activity. Both preparations show a slight decrease in initial activity (data not shown) when compared to fresh free and immobilized cells. However, it is notable that the preparation that was kept in absence of the cofactor (PLP) showed a greater loss in activity. Comparing the preparations stored without PLP with the ones kept in PLP, both free and immobilized cells stored without the cofactor showed a decrease of about 18% and 26% respectively (Figure 2 A).

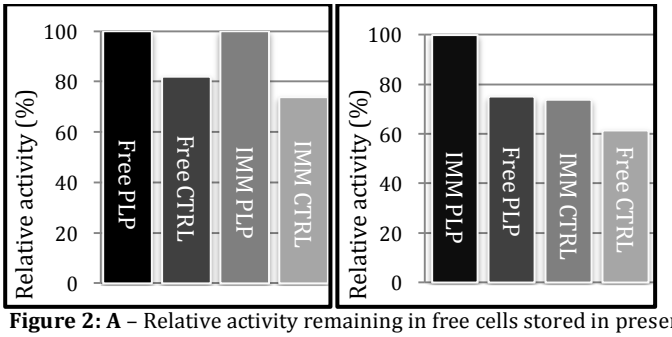


Figure 2: A – Relative activity remaining in free cells stored in presence of PLP (Free PLP) and absence of PLP (Free CTRL) and immobilized cells stored in presence of PLP (IMM PLP) and in absence of PLP (IMM CTRL). **B** –Remaining activity relative to the best activity result obtained (IMM PLP).

Among the 4 preparation, the immobilized cells stored in PLP had more retained activity than the similar preparation of free cells (Figure 2 B). Also, the immobilized cell preparation kept in absence of PLP (IMM CTRL) was slightly better (about 74% *vs* 61%) than the similar preparation of free cells (Free CTRL).

These results suggest strong protective behaviour of TiOx, but the experiment was not repeated and thus further studies are needed to show the significance of these results.

4.3 Effect of the temperature on the initial activity

The activity decreased as the incubation temperature was increased (Figure 3). As expected this phenomenon affects the immobilized cells as much as it affects the free cells. Both preparations showed a profound decrease in activity, especially at high temperatures.

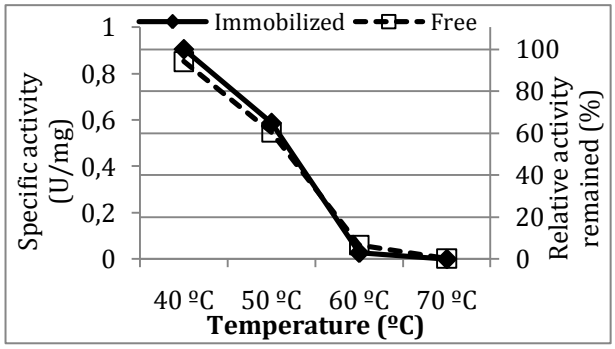


Figure 3 specific activities of free and immobilized cells after incubation for 30 minutes at 40. 50. 60 and 70 ºC

Comparing the activity of different incubation temperature with the one achieved at 40 ºC, it was observed that the incubation at 50 ºC resulted in 35% of activity loss relatively to incubation at 40 ºC. About 97% of activity was lost when the preparation was incubated at 60 ºC and at 70ºC no remaining activity was observed.

These results suggest that probably 55ºC is not the best temperature to run the reaction at with these cells. If a reaction of several hours is required with possible recycling between batches, studies are needed in order to find the best temperature that both allow cells to keep much of its activity without affecting the conversion rate too much. The economical impact must also be considered.

4.4 Kinetics studies

The resulting parameters shown in Table 3 were achieved by nonlinear regression (least squares) using the Michaelis-Menten model (Figure 4A,B) [3].

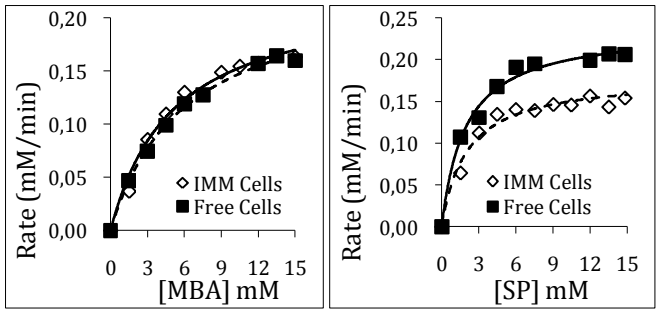


Figure 4: A and B Initial rate measurement when varying MBA and SP concentration for free (Free Cells) and immobilized cells (IMM cells)

These parameters confirm that there is no great difference in behaviour between immobilized and free biocatalyst. The affinity to the substrates (K_M) is similar for free and immobilized cells.

Table 3 – Kinetics parameters for free and immobilized cells

		MBA	SP
Free:	V_{max} (mM/min)	0,23	0,24
	K_M (mM)	5,99	1,94
IMM:	V_{max} (mM/min)	0,23	0,18
	K_M (mM)	5,11	2,03

4.5 Immobilization efficiency

Analyzing the data shown in Figure 5 we can assume that the window to get a good immobilization is located between 1% and 5% of cell loading; above 5% the losses increases to more than 50%.

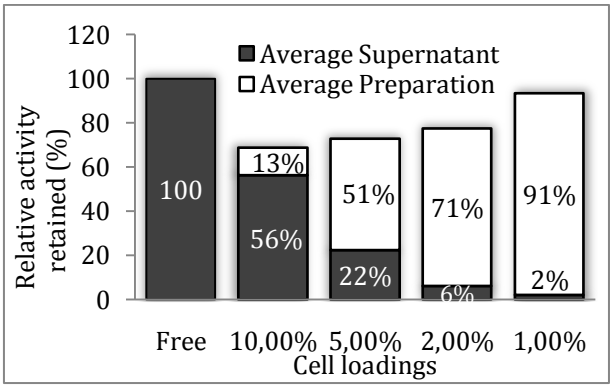


Figure 5 Compared relative activity in the supernatant of different cell loadings. **Average Supernatant:** average of 4 assays for activity present in the supernatant; **Average Preparation:** average of 4 assays for activity present on the immobilized preparation. **1%** to **10%** are cell loadings. **Free:** correspond to free cells

4.6 Effect of preparation re-use on the initial activity

Figure 6 shows the results, where can be seen that in the first 4 batches the maximum conversion was achieved after 40 minutes. As the preparation was recycled, the time for maximum conversion occurred later and later, but not longer than 70 minutes for any of the batches.

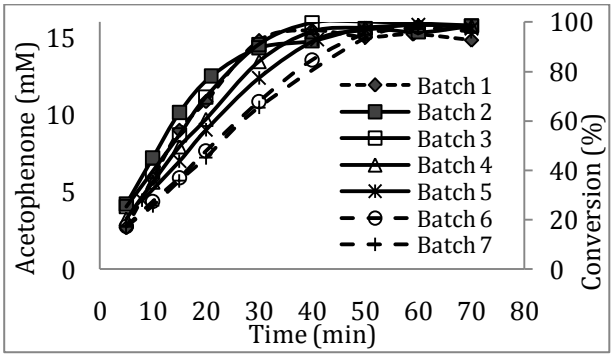


Figure 6 Acetophenone production for multi batches experiment (5 mg cells, 2% of loading).

Figure 7 show the relative activity based on the initial rate of each batch. Analysis of these data show that 50 %

of the initial activity is lost in 6-7 batches. This great loss of activity is mainly due to the temperature effect as which demonstrated before.

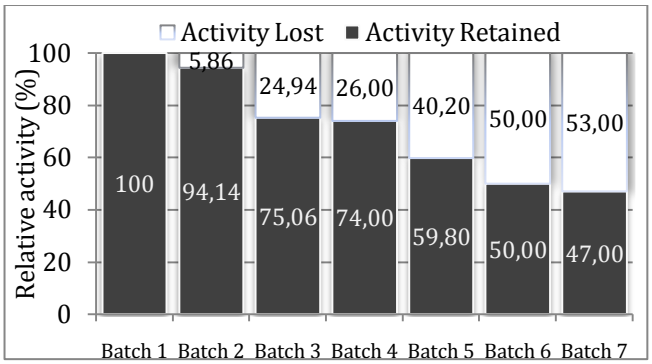


Figure 7 – Acetophenone production for multi batches experiment (5 mg cells, 2% of loading)

4.7 Effect Production of (s)-7-Methoxy-2aminotetralin

The results for production of 7-MAT are shown in Figure 8, where it can be seen that the maximum conversion achieved was only 20%. The reason for this was mainly the amount o cell used (20 mg). In later studies, 220 mg of free cells in the same volume of reaction produced about 95% of yield (data not shown).

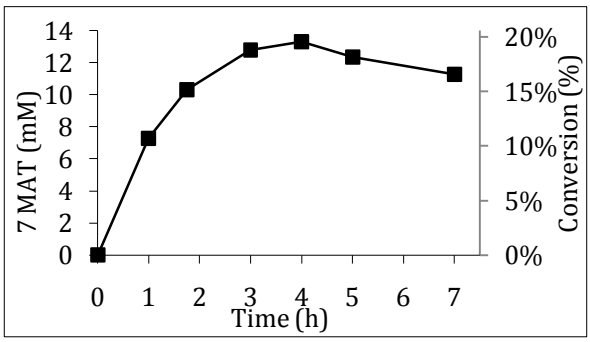


Figure 8 – Production of 7-MAT using immobilized cells (20 mg Cells – 1% Loading in 20 mL of reaction mixture)

The temperature used and the cell amount are the important keys in this reaction. The possibility of re-use the immobilized biocatalyst depends of these factors. After 5 hours both free and immobilized cells didn't shown any activity that may be related with the enzyme deactivation.

4.8 Calculation of the volume occupied by the preparation in the reactor

The volume the biocatalyst occupied in the reactor is very important and must be considered during the planning stage of a process. Most likely the immobilized biocatalyst will occupy a larger volume. After 120 min of sedimentation, the volume occupied by 600 mg of preparation was 1,6 mL. But, considering that if this immobilization method were to be applied in a larger scale, a centrifugation would promote a much faster sedimentation. The preparation was centrifuged for 5 minutes at 6000 rpm (WIFUG Lab Centrifuge). After that the volume occupied by the 600 mg of preparation (cell-TiOx) was decreased to 0,5 mL. With this relationship, we can predict the volume that the immobilization of 100 Kg of cells would occupy considering different cell loadings (Table 4).

Table 4 – Volume occupied by the preparation at different loadings when immobilizing 100 Kg of spray dried *E. coli* (dry weight)

Cell loading (%)	**1%**	**2%**	**3%**	**4%**	**5%**
Preparation (cell-TiOx) (Kg)	10000	5000	3333	2500	2000
Volume occupied (m^3)	8.33	4.17	2.78	2,08	1,67

The results shown in Table 4 allows one to predict the minum volume the preparation would occupied when immobilizing 100 Kg of spray dried *E. coli* cells. If one wishes to use a 6 m^3 reactor, according to table 4, the best loading would be 3% or 4%. As shown before, both these loadings have acceptable cell losses when immobilizing.

5. Conclusions:

The immobilization of *E. coli* spray dried cells was successful. The immobilization method allows reutilization of the preparation by centrifugation or filtration. The temperature used in the transamination reaction may not be the most appropriate. It was here demonstrated that great part of the activity was lost due to the temperature effect. It was observed that the cofactor has an important role in the enzyme stability. Further studies would allow a better compression of this phenomenon and it relation with the enzyme stability at different temperatures. The loading efficiency window achieved for spray dried *E. coli* immobilization was between 1 and 5%, which make the utilization of this method at larger scales possible but with some limitations. Its utilization may depend on the process, on the reactor size, the amount of biocatalyst used and the cell loading chosen. The production of 7-MAT was possible but with a poor conversion achieved (20%), mainly due to the low amount of cells used and possibly due to enzyme deactivation after 5 hours of reaction.

6. References

[1] Shin, J. S., Kim, B. G., Liese, A., Wandrey, C., "*Kinetic resolution of chiral amines with ω-transaminase using an enzyme-membrane reactor"*. Biotechnol Bioeng 2001, *73* (3), 179-87.

[2] Hwang, B. Y., Cho, B.K., Yun, H., Koteshwar, K., Kim, B. G., *"Revisit of aminotransferase in the genomic era and its application to biocatalysis".* Jornal of Molecular catalysis, 2005, (37) 47-55

[3] Martin, A. R., Shonnard, D., Pannuri, S., Kamat, S., *Characterization of free and immobilized (S)-aminotransferase for acetophenone production*. Appl Microbiol Biotechnol 2007, *76* (4), 843-51.

[4] Kennedy, J., Cabral, J., Kosseva, M., Paterson, M.; Methods in Biotechnology, Vol 1, Immobilization of Enzyme and cells, 1997, Chapter 39, pages 245-359

[5] Kennedy, J., Cabral, J., "*Use of titanium species for immobilization of cells".* Transition Met. Chem, 1990