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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 *Escherichia 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.

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

RESUMO ALARGADO

Aminas quirais consistem, nos dias de hoje, num importante intermediário para as indústrias farmacêuticas e agro-químicas com diversas aplicações, tendo por isso um grande atractivo comercial. A sua produção tem sido feita maioritariamente por resolução enzimática, sendo o enzima aminotransferase ou transaminase o responsável por essa resolução. Este enzima é conhecido por ser dependente de piridoxal-5-fosfato (PLP) que na reacção desempenha o papel de co-factor de activação. O enzima é normalmente comercializado na forma de células de *Escherichia coli* (*E. coli*) sujeitas a secagem por atomização.

Dependendo do substrato, um vasto leque de produtos finais pode ser obtido com este enzima. O enzima é responsável pela transferência de grupos amina de um substrato, o dador de amina, para outro substrato que será o receptor de amina (reacção de transaminação).

A reacção alvo neste projecto trata-se de uma transaminação que visa a produção de uma Tetralina substituída, mais concretamente a (S)-7-Metóxi-2-aminotetralina a partir da respectiva Tetralona substituída, neste caso a 7-Metóxi-2-tetralona. Nesta reacção há a transferência de um grupo amina a partir da Isopropilamina para a 7-Metóxi-2-tetralona que por sua vez será convertido em (S)-7-Metóxi-2-aminotetralina, formando-se Acetona como produto secundário.

Contudo, esta reacção é demasiado lenta e tem uma taxa de conversão muito baixa, atingindo-se 85 a 90% de conversão em cerca de 12 horas. Para um estudo eficaz que permita a avaliação de vários parâmetros inerentes a imobilização das células de *E. coli* contendo este enzima, tornou-se necessário a utilização de uma reacção que faça uso do mesmo enzima e que tenha uma taxa de conversão mais alta. A reacção de produção de Acetofenona a partir do Ácido Pirúvico e da Metilbenzilamina corresponde a este perfil. Embora esta reacção ocorra no sentido oposto ao da reacção alvo, isto é, em vez de se formar uma amina a partir de uma cetona, forma-se efectivamente uma cetona a partir de uma amina, nesta reacção obtém-se 90-95% de conversão em menos de 2 horas o que permite a elaboração de vários estudos num curto espaço de tempo.

Esta reacção é usada como modelo por diversos autores para caracterizar este enzima, e por esta razão existe na literatura um vasto conjunto de dados que permitem mais facilmente caracterizar o enzima em estudo.

Dada a existência de um grande mercado para este enzima e todos os produtos que pode formar, são de grande importância os estudos de melhoramento constantes que visem ao aumento da sua eficiência, tornando a sua utilização economicamente mais atractiva.

Até à data, vários estudos já foram efectuados nesse sentido, sendo um desses estudos baseados na reutilização do biocatalisador através de processos de imobilização.

Neste trabalho, estudos de imobilização deste biocatalisador foram efectuados, baseados num método diferente dos que já foram tentados até a data para este tipo de biocatalisador. Este método baseia-se na ligação aos óxidos hidratados de metais de transição, mais concretamente ao óxido de titânio (IV) hidratado ou titânio oxi-hidratado (TiOx) como também é conhecido. Este composto tem a capacidade de se ligar às células e enzimas através dos grupos hidroxilo presentes nestes, formando uma espécie de ligação parcial/semi covalente.

O processo de imobilização ocorre devido a uma reacção de substituição em que grupos hidroxilo presentes na superfície da matriz gelatinosa do óxido hidratado do metal de transição são substitutos por ligantes adequados na célula ou enzima. Devido ao peso do titânio, o complexo célula-TiOx irá precipitar em poucos minutos sem qualquer recurso a centrifugação, sendo que a sua utilização apressa o processo. O tamanho que o complexo apresenta torna-o também filtrável, podendo por isso ser utilizado um filtro para separar o biocatalizador imobilizado de uma mistura complexa de compostos.

O titânio oxi-hidratado é formado em dois passos principais, no primeiro passo uma mistura contento água, tetra cloreto de titânio e ácido clorídrico é preparada. Esta mistura de carácter ácido é no segundo passo neutralizada (até pH 7) por adição de hidróxido de amónio, formando-se o titânio oxi-hidratado como produto principal e cloreto de amónio como produto secundário. À medida que a base é adicionada, pequenas partículas de cor branca começam a formar-se no reactor, tendendo a precipitar caso a agitação seja interrompida. Estas partículas consistem no TiOx que a pH 7 tem uma consistência gelatinosa, sendo este depois filtrado e lavado com uma solução salina a fim de se descartar os compostos que não reagiram. O filtrado é depois misturado com uma suspensão celular e a imobilização ocorre. Este pode ainda ser

armazenado em solução salina para manter a sua propriedade hidratada, podendo ser utilizado até várias semanas depois.

Uma imobilização com sucesso pode ser numa primeira instância rapidamente observada sem recurso a qualquer equipamento, bastando-se observar a preparação de células imobilizadas, que quando visualmente comparada com uma solução de células livres produzem efeitos diferentes: o primeiro produz um sobrenadante translúcido, livre de qualquer célula após cerca de 10 minutos, ao contrário do segundo que precisará de alguma horas ou mesmo do recurso a centrifugação para promover o mesmo efeito.

Os primeiros ensaios feitos a este método de imobilização foram em células de levedura (*Saccharomyces cerevisiae*). Quando comparado com a imobilização das células de *E . coli* sujeitas a secagem por atomização, as leveduras revelaram uma maior afinidade para se agregarem à matriz de TiOx. Este facto poderá estar relacionado com as diferenças de tamanho entre os dois tipos de células.

Usando este método, as células de *E. coli* sujeitas a secagem por atomização foram imobilizadas com sucesso tendo sido possível a sua reutilização em vários *batches.*

Foram efectuados estudos de cinética enzimática onde se verificou que a imobilização por este método não produz grandes efeitos difusionais. O estudo de estabilidade de armazenamento das células imobilizadas e livres revelou a importância do co-factor PLP na estabilidade do enzima, observando-se também que as células imobilizadas apresentavam maior estabilidade após 16 horas quando comparadas com as células livres, ambas submetidas ao mesmo tratamento. Este fenómeno sugere alguma propriedade protectora por parte do TiOx. Observou-se também que a temperatura tem para este enzima um papel muito importante afectando ambas as células livres e imobilizadas de forma semelhante.

Foram ainda efectuados estudos de capacidade de carregamento celular, de eficiência de imobilização e cálculo do volume ocupado pela preparação no reactor, visando a validação deste método para uma possível aplicação industrial.

Palavras-chave: óxido de titânio hidratado, imobilização, *E. coli* sujeita a secagem por atomização, aminotransferase, transaminase, aminas quirais, acetofenona.

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ABREVIATIONS:

ATs – S-Aminotransferases

S-MBA - (S)-(−)-α-methylbenzylamine,

SP - Sodium pyruvate,

PLP -Pyridoxal-5-phosphate

TiOx - Titanium Oxyhydrate/ Hydrous Titanium Oxide

7-MAT - (S)-7-Methoxy-2-aminotetralin

7-MT - 7-Methoxy-2-tetralone

IPA – Isopropylamine

TFA – Trifluoroacetic acid

BTB - Bromothymol blue

DNS - 3,5-Dinitrosalicylic acid

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. When compared to other alternatives, these enzymes show many advantages such as high enantioselectivity and regioselectivity, broad substrate specificity and high reaction rate and stability [1], [2].

In the present study two main reactions using ω-transaminase were carried out: a) conversion of a substituted tetralone into a substituted (S)-aminotetralin—chiral amine, 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. The use of this enzyme at such scale generates two major challenges: a) loss of biocatalyst during product isolation and b) loss of product trapped in the cells or in cake formed during centrifugation/filtration to remove the spray-dried cells/enzymes [3].

For a better economical large scale production of these chiral amines, the problems listed above must be solved. 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 thesis, a different approach using surface adsorption immobilization technique was developed, using gelatinous hydrous transition metal oxides that associate 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 the 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 METHODS

2.1 – Production of hydrous titanium oxide 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 gelatinous matrix of hydrous titanium oxide is formed through addition of ammonium hydroxide to a solution of titanium(IV) tetrachloride until pH 7 is reached according to the following reactions:

$$TiCl_4 + H_2O \rightarrow TiOCl_2 + 2\,HCl \qquad \text{(Reaction 1)}$$

$$TiOCl_2 + 2\,HCl + 4\,NH_4OH \rightarrow \boldsymbol{TiO(OH)_2}(pp) + 4\,NH_4CI + 2\,H_2O \qquad \text{(Reaction 2)}$$

(Titanium Oxyhydrate)

As the pH is increased until neutral, white thin particles start to form in the solution consisting of titanium oxyhydrate, also called hydrous titanium oxide (TiOx). 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.

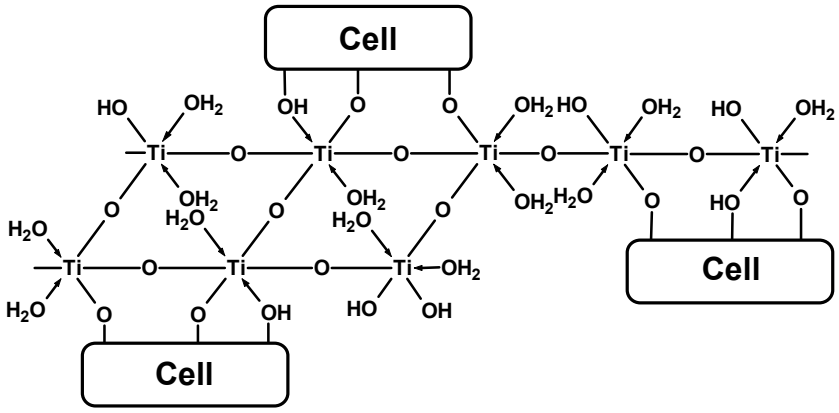


Figure 1 - Titanium Oxyhydrate matrix chelating whole cell (Adapted from Kennedy *et al*, 1997 [4]).

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).

Hydrous titanium oxide has a porous structure and several methods have been studied for the preparation of active gels with any desired pore radius in the 10-100 Å range.

Gelatinous hydrous titanium oxide, freshly precipitated from titanium(IV) chloride and ammonia, has pore radii in the 30 Å region; when dried, the pore size increases to 100 Å, and the amorphous structure crystallizes.

The pore size dimensions do alter on heating (50-350 ºC): temperatures above 200 ºC will cause removal of the molecular water by evaporation. Above 300 ºC, the hydrogen-bonded hydroxyl groups are removed progressively, and at 450 ºC only few isolated hydroxyl groups remain, causing the crystallization of the compound [6] which will no longer work as an immobilizer.

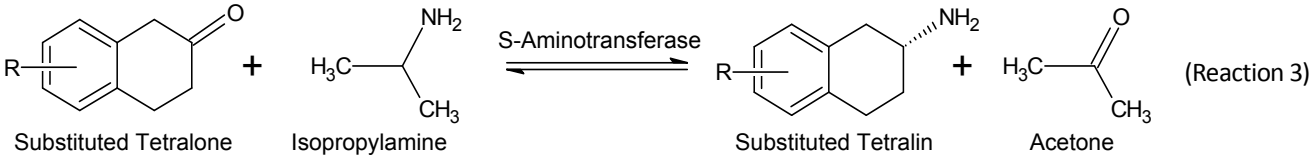
Since the *E. coli* cell is about 20.000 Å in length [7], it will not be able to diffuse into the TiOx matrix pores, thus the immobilization will occur on the surface of the carrier.

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.

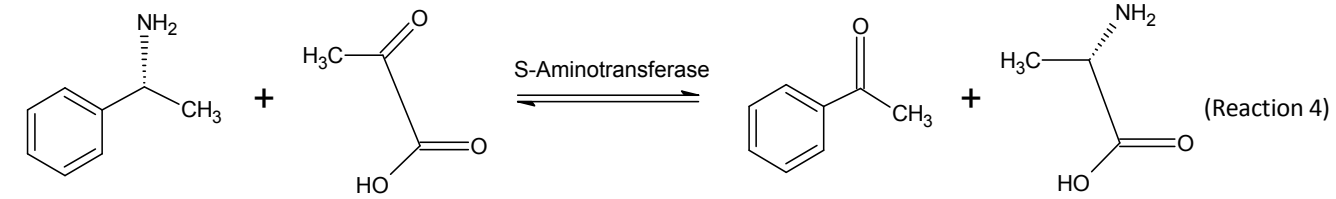
Depending on the substrate, different products can be obtained. The enzyme will transport an amine group from one substrate (the amine donor) to another (the amine acceptor).

The target reaction (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.



Substituted Tetralone + Isopropylamine ⇌ Substituted Tetralin + Acetone (Reaction 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 (Reaction 4) occurs in the opposite direction and it 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 enzyme.



(S)-α-Methylbenzylamine + Pyruvic Acid ⇌ Acetophenone + L-Alanine (Reaction 4)

The optimum temperature for ATs was reported to be around 55 ºC at pH values in the neutral range [3]. For this reason, the activity assays in this work were 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 SP and 1 mM of PLP.

2.3 – Development of an activity assay for the acetophenone reaction

In previous studies, the acetophenone reaction has been followed by HPLC [3]. However, the HPLC analysis requires a lot of preparations and takes time and is therefore not optimal if one wishes a quick analysis, able to process a lot of samples.

Instead a method to analyse the samples by spectrophotometry was developed in this project. The method is based on the difference between the absorbance of the product and the substrate in the ultra-violet region.

A spectrum scan of MBA and acetophenone showed that they have absorption peaks perfectly separated: approximately 213 nm for MBA and 245 nm for acetophenone (Figure 2). This allows the reaction to be followed either through the substrate consumption (213 nm) or through the product formation (245 nm).

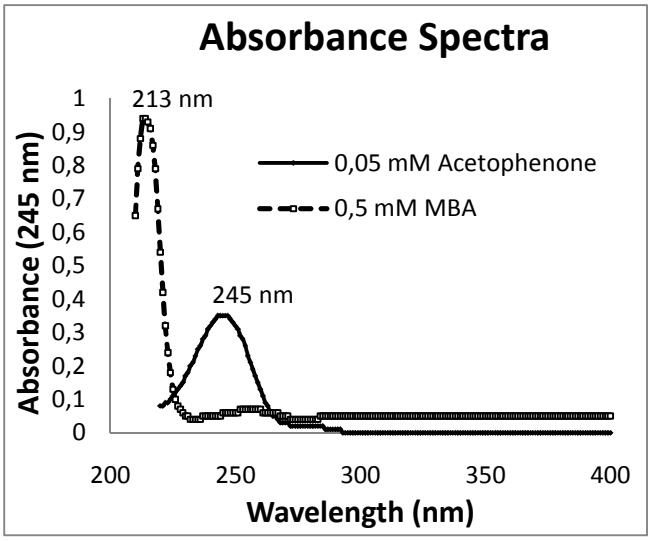


Figure 2 – Absorbance spectra for MBA and Acetophenone

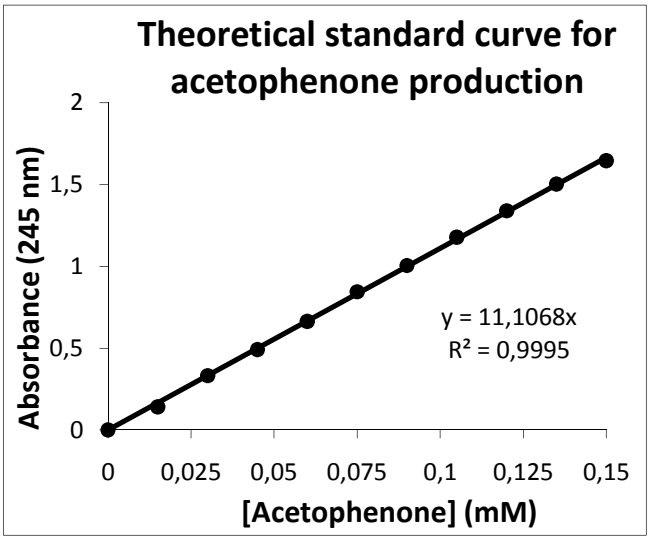


Figure 3 – Theoretical Standard curve for Acetophenone production

Considering the acetophenone wavelength of absorption at 245 nm, a theoretical standard curve was developed (Figure 3). The standard curve is said to be theoretical due to the fact that no enzyme was used. The reaction was simulated by mixing all the compounds needed (Appendix 1).

3 - MATERIAL AND METHODS

3.1 - Production and storage of hydrous titanium oxide

To a solution containing 150 g/L of titanium tetrachloride (Fluka) and 150 g/L of hydrochloric acid (Merck), 2 M of ammonium hydroxide (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 then 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 then stored in saline solution to keep the hydrous property with a concentration of 0,6 g/mL. (Appendix 2)

3.2 – Immobilization of yeast and E. coli *spray dried cells*

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 be confirmed early by a quick visual analysis; a good immobilization will give a clear supernatant while a bad immobilization will produce a turbid supernatant.

3.3 – Early experiments on yeast immobilization

The first immobilization experiments were conducted on yeast cells. The activity assays were based on invertase activity by the DNS Method (see Appendix 3).

3.3.1 – Yeast Immobilization efficiency

To test the immobilization efficiency for yeast, the supernatant of an immobilized preparation was compared with a second preparation in which the entire preparation was submitted to the activity test. The two preparations of immobilized yeast (A and B) of 50 mg each with approximately 1,45 % of cell loading was treated in the following way:

preparation A was tested for invertase activity without any pre-treatment; while preparation B, was gently centrifuged (*VWR, Galaxy MiniStar)* and the supernatant was recovered and tested for invertase activity (Appendix 4).

3.3.2 – Effect of immobilization on yeast cells activity

The activity of immobilized cells was compared with free cells by testing both preparations for invertase activity. The mass of free and immobilized cells were approximately the same (~1,45 mg) and the assay was made in duplicate.

Samples were taken at 0, 1, 10, 20 and 30 minutes and the reaction was stopped by putting the samples in an ice bath.

Additionally, a sample from each preparation was taken and analysed by an optic microscope.

3.3.3 – Loading capacity of yeast cells

To study how much cells can be bound by TiOx (loading capacity), the following experiment was carried out in parallel for free (Y) and immobilized cells (IMM):

1) Cell solutions containing 2, 4, 6, 8, 10, 12 and 20 mg of yeast (wet weight) diluted in 0,9% NaCl were prepared. 20 mg of TiOx (wet weight) was added to the cells to start the immobilization process and no TiOx was added to Y. Then, the preparations were mixed and given 10 min to settle down.

2) The 14 preparations (7 Y and 7 IMM) were centrifuged for 2 minutes (*VWR, Galaxy MiniStar*) and after that 100 µL was collected from the top of each one of the 14 preparations and added to 2 mL of 2% sucrose in 20 mM acetate buffer pH 4,5 in 3 mL vials.

3) The reaction mixture containing the cells was incubated for exactly 1 minute at 45 ºC in a termoshaker and the reaction was stopped by putting the vials in an ice bath.

4) To samples of 100 µL from each vial 100 µL of DNS reagent was added and then boiled for 5 minutes, cooled to room temperature and measured for absorbance at 540 nm.

3.4 – Experiments on spray dried **E. coli** *cells*

3.4.1 - Transamination activity assay

The activity assay used for *E. coli* spray dried cells is based on the production of acetophenone (Reaction 4) which was ran at 55 ºC with shaking (~700 rpm) in a termoshaker (HLC Biotech) using between 3-5 mL of reaction mixture. The reaction mixture was composed by 15 mM of MBA, 100 mM of Sodium Pyruvate, 1 mM of PLP and 100 mM of Tris Buffer pH 7,2. The mixture was kept closed, wrapped in aluminium foil and stored in the fridge.

The reaction was stopped by adding samples of 200 µL to 800 µl of 0.1 M HCl and then analyzed by spectrophotometry (Simadzu UV-1650PC) at 245 nm with a final dilution of 1:50.

3.4.2 – Immobilization efficiency of spray dried **E. coli** *cells*

The immobilization efficiency of spray dried *E. coli* cells expressing ATs was tested by comparing free and immobilized cells with the loading ranging from 0.5% to 10%.

The cell mass used was constant (0.6 mg – dry weight) and the TiOx was varied to achieve the desired loading. After immobilization the cells were given time to settle down (~10 min) and part of the supernatant (200 µL from 3 mL) was collected to perform the activity assay. Samples were taken at time 5, 10, 15 and 20 minutes.

The initial activities still present in the supernatant of different loadings were compared with the activity of free cells.

3.4.3 – Effect of immobilization on spray dried **E. coli** *activity*

Immobilized cells (0.6 mg of cells with 1% of loading) was compared with free cells by following a 2 hours reaction in parallel, taking samples every 10 minutes and proceeding as described above on the activity assay (3.4.1). In this way, the initial activity (mM/min) was determined as well as the maximum conversion.

3.4.4 – Storage stability

The storage stability was tested by storing the cells (free and immobilized) overnight in the fridge in presence or absence of PLP. Free and Immobilized cells were mixed with 1mM PLP solution (pH set to 7). And the control was kept without any PLP, in a saline solution (0,9% NaCl).

The cell mass used was 0.6 mg for both free and immobilized cells, and 1% of loading in the immobilized preparation.

The 4 vials were kept tightly closed and after 16 hours the cells were submitted to the activity assay.

3.4.5 – Effect of temperature on the initial activity of free and immobilized cells

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

3.4.6 – 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 for kinetic measurement of AT's activity in free and immobilized *E. coli* spray dried cells

Solution	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

3.4.7 – Effect of different salts on cell immobilization on TiOx.

Preparation with a cell loading of 2% was mixed with different salt solutions and after 10 minutes the results were observed.

When performing the immobilization, the cells used originally were diluted and mixed in phosphate buffer and then mixed with the TiOx preparation. Some results suggested that for any reason the phosphate buffer could affect the loading capacity of cells on TiOx.

To investigate this further, cells were mixed with 4 different buffers, phosphate buffer, Tris buffer, water and saline solution (0,9% NaCl).

3.4.8 - Effect of cell washing before the immobilization on loading capacity

The spray dried cells containing ATs is produced by growing *E. coli* MG1655 in a fermentor in the presence of a complex medium containing $MgSO_4.7H_2O$, antifoam, Fe (III) citrate hydrate, EDTA, $MnCl_2.2H_2O$, $Zn(CH_3COO)_2$, H_3BO_3, $Na_2MoO_4.2H_2O$, $CoCl_2.6H_2O$, $CuCl_2$ and phosphate Buffer (KH_2PO_4) [3].

After growth the cells were harvested by spray drying without removing the complex medium described above.

It was possible that some of the components in the medium could interfere with the immobilization as at least phosphate buffer was present in great amounts. For this reason, a washing step before the immobilization of the cells was included and it was found to be effective.

Cells were washed with saline solution (0,9% NaCl) twice with a centrifugation step in between (6000rpm for 15 min – WIFUG Lab Centrifuges). The immobilization process proceeded as previously and the produced preparations were tested for loading efficiency in parallel with preparations which did not include the washing step.

3.4.9 - Immobilization efficiency (multi assay)

Due to difficulties in estimating a constant value for the loading efficiency, that experiment was repeated, this time 4 assays in parallel were made to give a statistical significance and to predict the range of the efficiency for each immobilization loading.

Cell immobilization was performed as stated before in 3.4.2, but instead of removing only part of supernatant to perform the activity assay, it was totally removed to quantify all the cells present.

The immobilized preparation was also collected and the activity was measured. The cell mass used was 0.6 mg and the TiOx was once again varied to obtain the desired loading on the carrier.

3.4.10 - Effect of preparation re-use on 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 pH 7,2 (100 mM). The reaction was carried out in a thermoshaker at 700 rpm rotation.

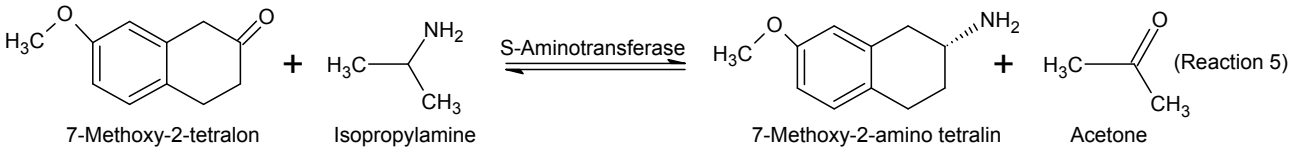
Samples were taken at 5, 10, 15, 20, 30, 40, 50, 60, 70 minutes for most of the batches, and centrifuged for 2 minutes. After centrifugation, 200 µL of the supernatant was collected in an *eppendorf* tube containing 800 µL of 0,1 M HCl. The remaining solution was returned to the reactor.

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 1:100.

3.4.11 Production of 7-MAT

The immobilized *E. coli* spray-dried cells with a loading of 1 % were tested in a 7 hours reaction that aimed to produce 7-methoxy-2-aminotetralin (7-MAT) from 7-methoxy-2-tetralone (7-MT) and isopropylamine (IPA) (Reaction 5). [8]



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 µL of supernatant was taken and diluted in 800 µL of 0,1 M HCl. The remaining content after the centrifugation was returned to the reactor.

The samples were analysed by HPLC with mobile phase composed of methanol, water and TFA in a gradient elution (see Appendix 14 for specification). The results were compared to free cells (220 mg) (Appendix 14).

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

3.4.12 – Calculation of the volume occupied by the preparation in the reactor

The volume occupied by the immobilized preparation in the reactor was measured by immobilizing 6 mg of the spray dried *E. coli* after washing the cells twice with 0.9% NaCl with 2 centrifugations at 6000 rpm (WIFUG Lab Centrifuge) in between.

The immobilized preparation with the characteristics showed in Table 2 was put inside a graduated cylinder of 5 mL and the height of TiOx during the sedimentation was observed for 2 hours.

Table 2 – Composition of immobilized preparation

Cell loading	1 %
Cell mass (dry weight)	6 mg
Cell sol. volume (6 mg/mL)	1 mL
TiOx mass (wet weight)	594 mg
TiOx sol. volume (0.6 g/ mL)	0,99 mL
Final volume (filled with 0.9% NaCl)	3 mL

4 – RESULTS

4.1 - Early experiments on yeast immobilization

4.1.1 – Yeast Immobilization efficiency

The results for the preliminary experiments on yeast immobilization revealed that the immobilization technique was applied correctly and that the TiOx has the potential to attach the yeast cells.

Figure 4 shows the compared activity between the preparation and the supernatant where it is notable that the supernatant does not show any activity at all, meaning that all (or almost all) cells were retained by the TiOx preparation.

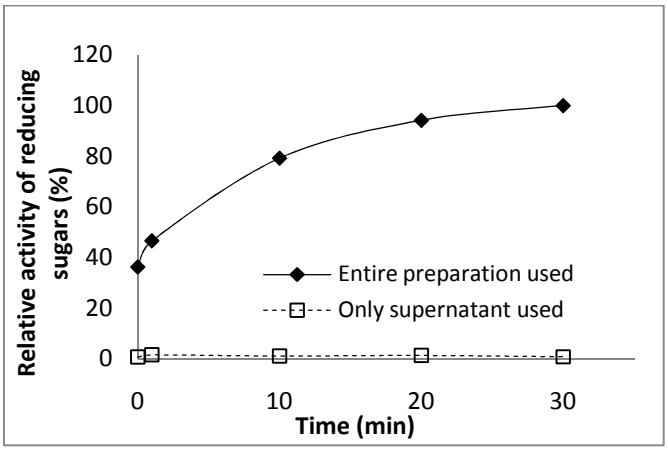


Figure 4 - Immobilization efficiency of yeast (50 mg of preparation, approximately 1,45 % of cell loading)

4.1.2 – Effect of immobilization on Yeast cells activity

After successful confirmation of the immobilization viability, the immobilized cells showed the same activity when compared to the free cells (Figure 5).

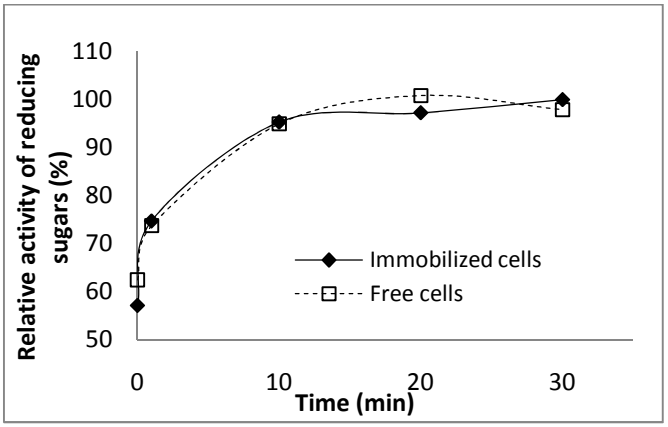


Figure 5 – Compared activity of free (1,45 mg cell) and immobilized yeast cells (1,45 mg cell, 1,45 % loading).

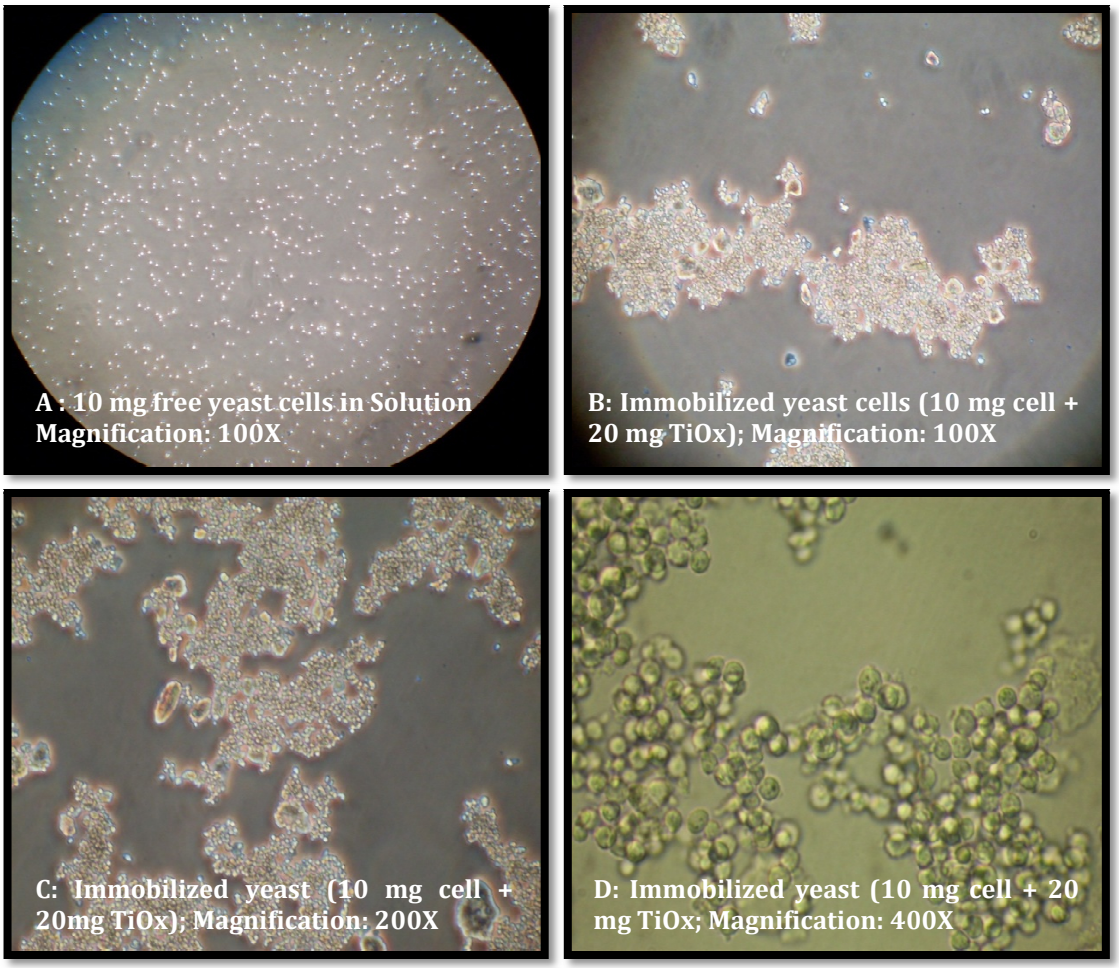


Figure 6 – Microcoscope pictures of immobilized (about 33% of cell loading) and free yeast cells.

From Figure 5 it is possible to confirm that no great diffusion effect is introduced when the cells are immobilized on TiOx. The immobilization itself does not seem to restrict the access of substrates to the enzyme.

Figure 6 shows how the immobilization looks like in an optical microscope. Fig 6.A shows the free cells in solution and it is notable that they are perfectly spread in the solution, on the other hand Fig 6.B shows the immobilized cells in solution where it can be seen that the cells are organized in clusters leaving the solution free of cells. The same organization can be seen in Fig 6.C and D at a higher magnification. The TiOx seems to work as glue that connects all the cells assembling in a cluster organization.

4.1.3 - Loading capacity of yeast cells

The loading capacity experiment for the yeast cells showed that it was possible to load at least 50% of cells on TiOx without great cell loss.

Figure 7 shows these results in which increasing mass of free yeast cells (2 to 20 mg) gave increasing activity in the supernatant. On the other hand, increasing mass of immobilized yeast did not show increasing activity in the supernatant. It was possible to see that all the way up to 50% of cell loading the activity in the supernatant remained low, meaning that there were few (or none) cells left in the supernatant after the immobilization. To confirm this results and exclude the hypothesis of TiOx being affecting the results by producing low activity for the immobilized yeast cells, a parallel preparation containing 12 mg of cells with a loading of 37,5% was tested without separate the supernatant from the solids (positive control). As expected, the use of the entire preparation produced an activity corresponding to the same amount of cells in free yeast cells preparation.

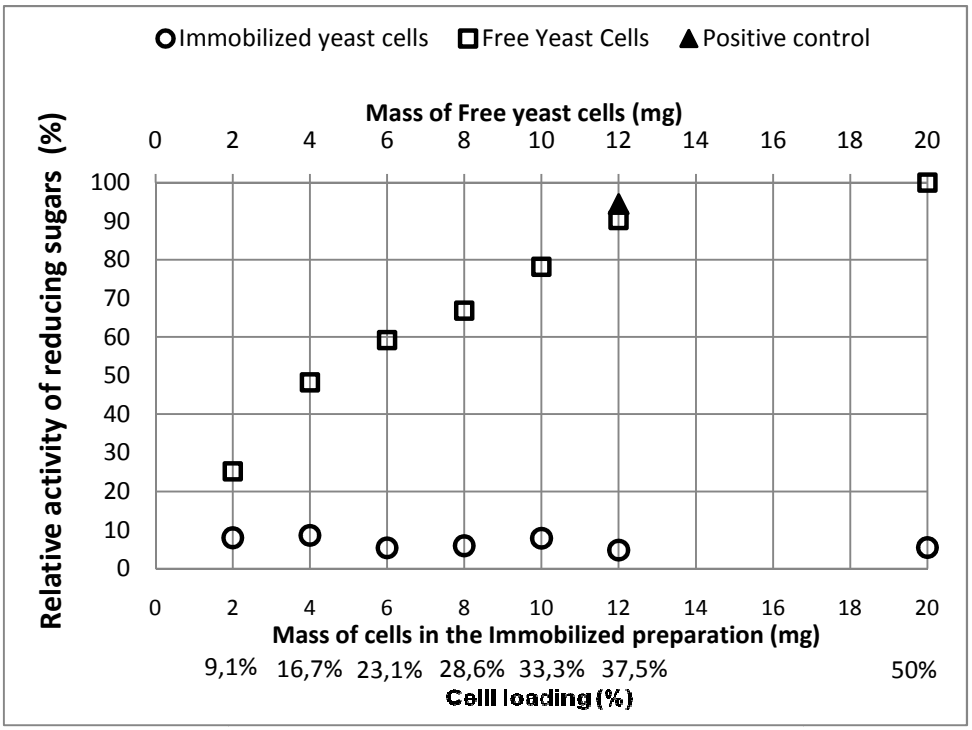


Figure 7 – Yeast loading capacity on TiOx compared with free cells.
(□) supernatant of free cells; (○) supernatant of yeast immobilized cells)
(▲) Positive control (12 mg of yeast cells immobilized with 37,5% of cell loading, the entire preparation was used.

4.2 – Experiments on spray dried E. coli *cells*

4.2.1 – Immobilization efficiency of spray dried E. coli *cells*

Compared to yeast cells, the TiOx seems to have less capacity to bind spray dried *E. coli* cells. The results in Figure 8 reveal that a loading of 2% produces an activity in the supernatant of about 28% relatively to the free cells, which means that almost 30% of the cells remained in the supernatant (not immobilized). At 5% and 10% it was even worse since more than 80% of the cells was not immobilized (Fig 8).

These results could also be seen directly in Figure 9 where it is obvious that the difference between loadings, 0.5% and 1% show a clear supernatant and higher than 2% the supernatant start to becomes turbid.

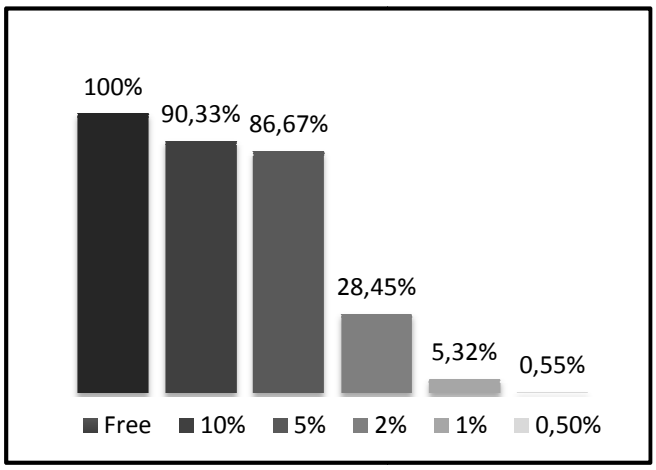


Figure 8 – Immobilization efficiency of *E. coli* spray dried cells (relative activity of the supernatant)

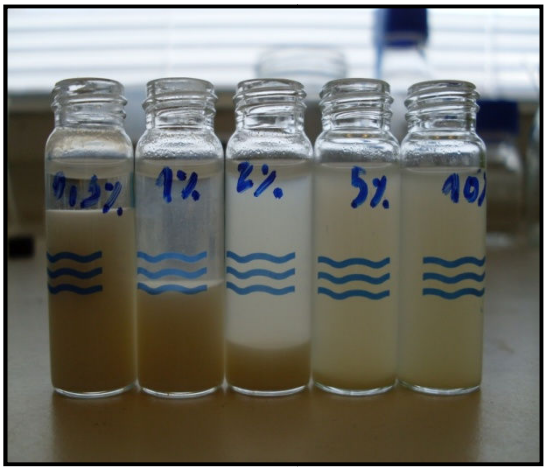


Figure 9 – Immobilization efficiency of E. coli spray dried cells (Picture of immobilized cells with different loadings (0,5; 1; 2; 5 and 10%)

The time for the preparation to settle down will influence the immobilization efficiency. More time means that more cells could be attached to TiOx. The results can be slightly improved if one waits longer (data not shown). However 5% and 10% loading will always be over the saturation limit.

This great difference between the loading capacity of yeast and spray dried *E. coli* cells may be related to the difference between the size of both microorganisms.

4.2.2 – Effect of immobilization on spray dried E. coli *activity*

Similarly to what happened with the yeast cells, the immobilization of *E. coli* spray dried cells does not seem to affect the cell's ability to catalyse reactions. Following a 2 hours reaction for both free and immobilized cells it became clear that the difference between them was very small. The maximum conversion achieved was about 70% and 65% for free and immobilized cells respectively and the specific rate 0,900 U/mg and 0,861 U/mg (Table 3).

The maximum conversion was far from 100% in this experiment, probably due to product evaporation since the assay was carried out in an open vessel. After a while it is possible that the evaporation rate becomes faster than the production rate, giving the negative activity shown in Figure 10.

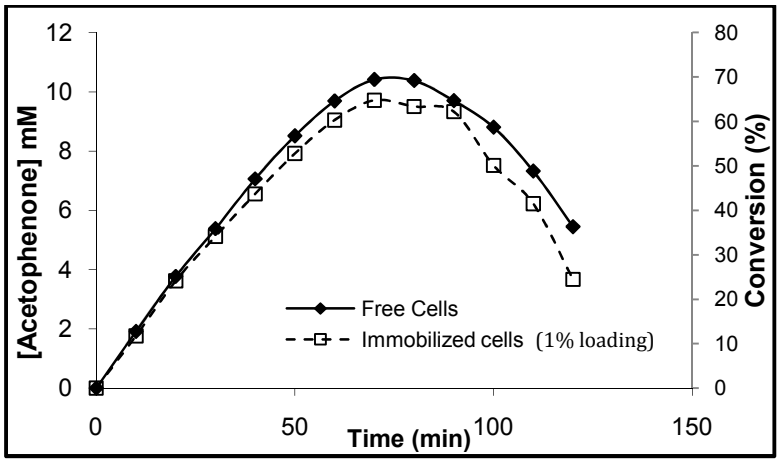


Figure 10 – Acetophenone production from MBA comparing Immobilized and free *E. coli* spray dried cells.

Table 3 – Maximun conversion and initial activity for free and immobilized cells.

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

4.2.3 - Storage stability

It was found that storing both free 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 amounting to about 18% and 26% respectively (Figure 11 A).

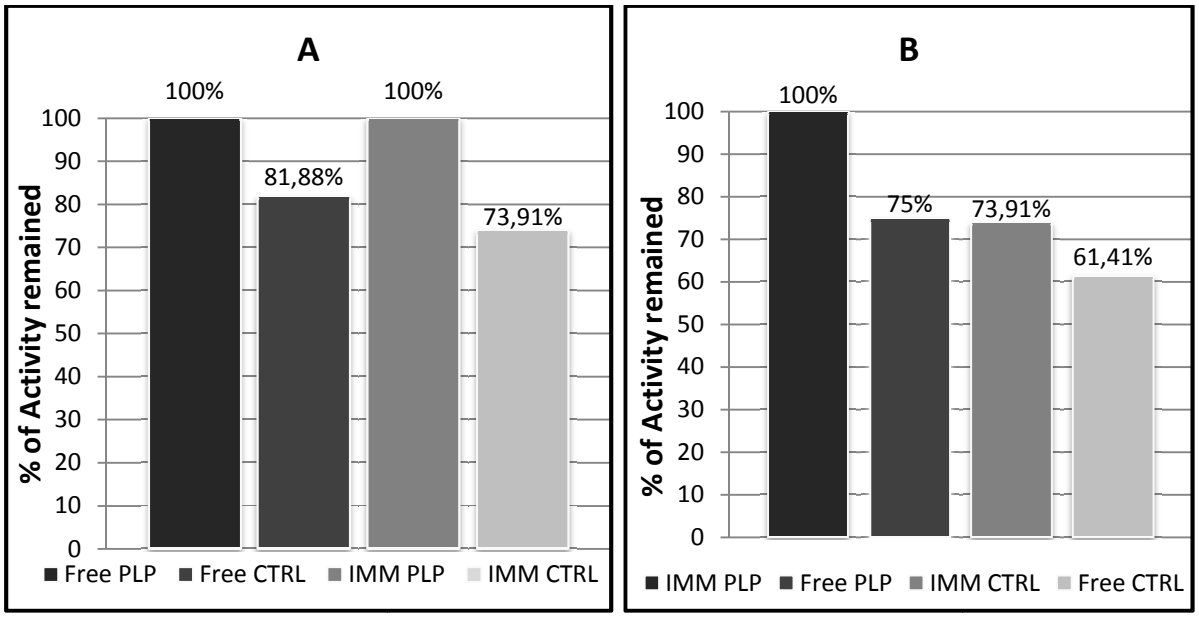


Figure 11: A – Relative activity remaining in free cells stored in presence of PLP (Free PLP) and absence of PLP (Free CTRL) and immobilized cells stored in presence of PLP (IMM PLP) and in absence of PLP (IMM CTRL), **B –**Remaining activity relative to the best activity result obtained (IMM PLP).

Among the four preparations, the immobilized cells stored in PLP had more retained activity than the similar preparation of free cells (Figure 11 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.

4.2.4 - Effect of temperature on initial activity of free and immobilized cells

Incubating the cells for 30 minutes at different temperatures seems to have a huge impact on cell activity. The activity decreased as the incubation temperature was increased (Figure 12). 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.

Assuming that the incubation at 40 ºC is the least harmful to the enzyme, 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 (Figure 13).

It is possible that incubation in the presence of cofactor (PLP) would reduce the loss of activity since the cofactor improved the stability during storage, demonstrated above.

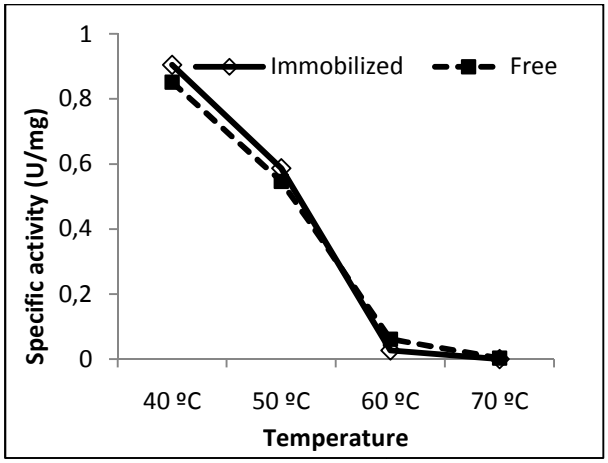


Figure 12 – specific activities of free and immobilized cells after incubation for 30 minutes at 40, 50, 60 and 70 ºC

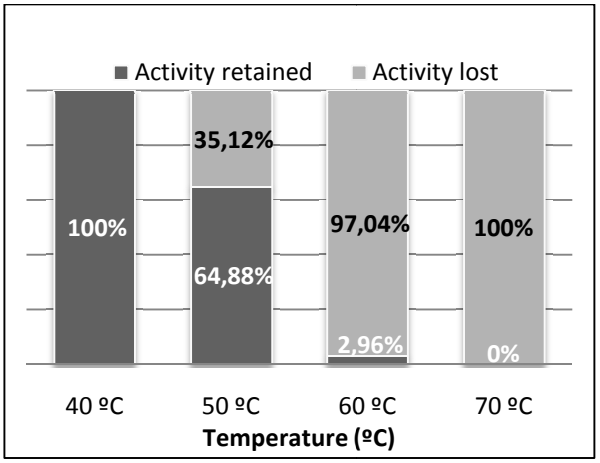


Figure 13 – Relative activity lost and retained for each temperature, for immobilized *E. coli* spray dried cells.

These results suggest that probably 55ºC is not the best temperature to run the reaction with these cells. It was shown to be the temperature at which the highest activity was achieved [3], but it was based on initial rate only.

If a reaction of several hours is required with possible recycling between batches, studies are needed in order to find the best temperature that allows cells to keep much of its activity without affecting the conversion rate too much. The economical impact must also be considered.

4.2.5 – Kinetics studies

Figures 14 and 15 show the effect of MBA and SP on the initial rate for free and immobilized *E. coli* spray dried cells.

The resulting parameters are shown in Table 4 and they were achieved by nonlinear regression (least squares) using the Michaelis-Menten model [3].

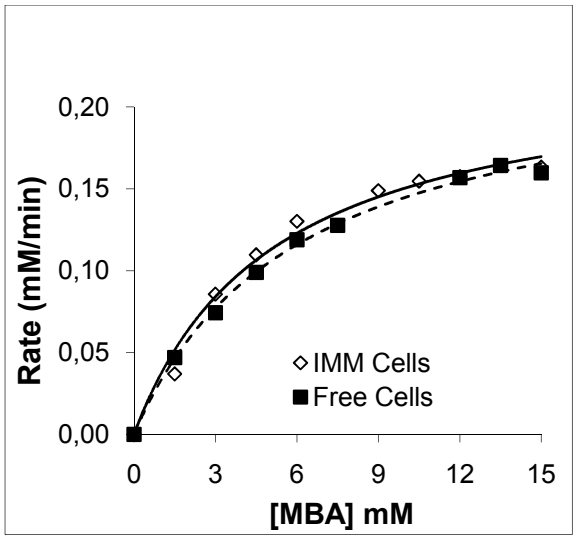


Figure 14 – Initial rate measurement when varying MBA concentration for free (Free Cells) and immobilized cells (IMM cells)

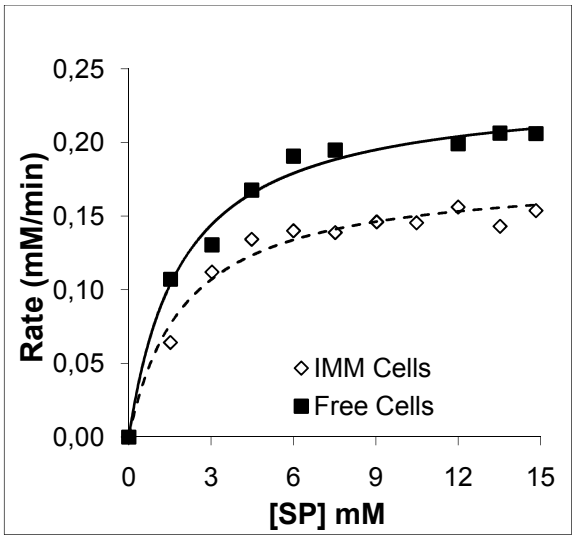


Figure 15 – Initial rate measurement when varying SP concentration for free (Free Cells) and immobilized cells (IMM cells)

Table 4 – 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

The kinetic parameters obtained here, do not show any similarity when compared to the ones estimated by other authors [3]. The parameters estimated here confirm that there is no great difference concerning enzyme behaviour between immobilized cells on TiOx and free cells containing ATs.

The parameters estimated by the referred author, suggested lower substrate affinity (higher K_M) for immobilized cells by entrapment on calcium alginate beads compared to free cells.

In this work, the V_{max} estimated for MBA does not vary much between free and immobilized cells, both were about 0.23 mM/min (Table 2).

The same phenomenon was observed for substrate affinity coefficient (Km) estimated for MBA: 5,99 mM for free cells and 5,11 mM for immobilized cells.

Similar results were obtained for SP, K_M is about 2 mM for both preparation but V_{max} was slightly lower for the immobilized preparation (~0,18 mM/min vs 0,24 mM/min).

In general these results confirm that no great diffusional effects affect the immobilized cells on TiOx as stated by Kennedy and Cabral, 1990 [4].

4.2.6 - Effect of different salts on cell immobilization on TiOx

During this study, phosphate buffer was used to dilute the cells as their activity is greatly affected by pH values out of the neutral range [3].

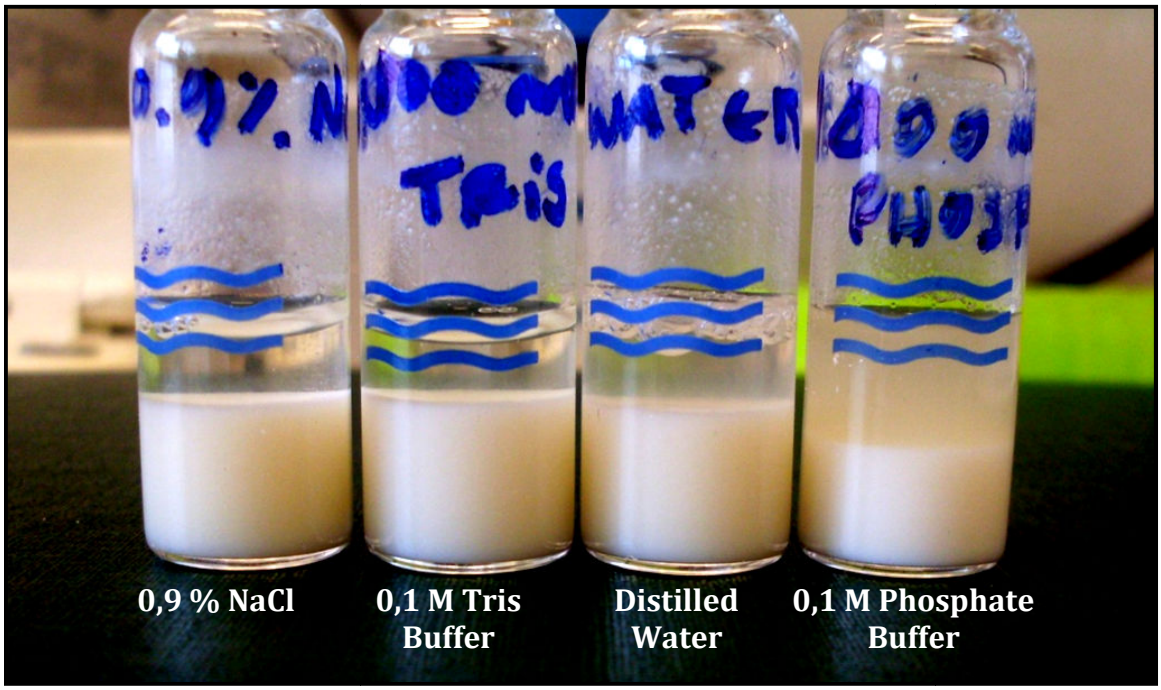


Figure 16 – effect of different buffer salts on the immobilization (2% of cell loading)

The results show that using phosphate buffer may not be the best option in this kind of immobilization. Comparing the 4 salt solutions, it is clear that the immobilization efficiency in phosphate buffer is rather inferior compared to the other solutions since the supernatant remained turbid for a loading of 2%. (Figure 16)

Among the other 3 solutions, distilled water seemed to be slightly worse than the saline solution and the Tris buffer.

No visual distinction was possible between Tris buffer and 0,9% NaCl.

These results suggest that there might be room for improvement of the immobilization if an optimal buffer and concentration can be found, and that phosphate buffer for any reason interfere with the TiOx immobilization.

4.2.7 - Effect of cell washing before the immobilization on loading capacity

Figure 17 shows the effect of washing the cells before the immobilization.

It's clear that removing the impurities present in fermentation broth improves the immobilization process. In all preparations the washed cells produced less activity in the supernatant, indicating a better immobilization.

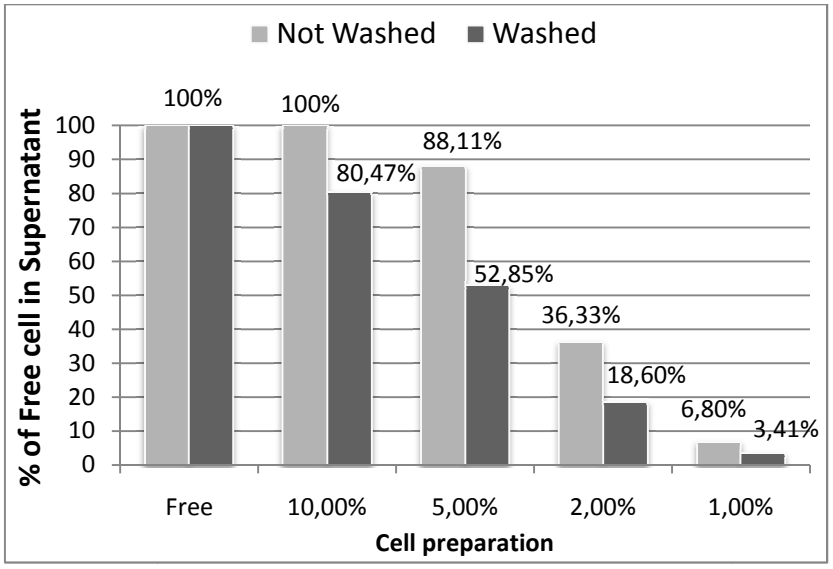


Figure 17 – Effect of washing the cells before the immobilization.
1% to **10%** are cell loadings. **Free:** correspond to free cells

4.2.8 –Immobilization efficiency (multi assay)

These results allow one to compare the activity retained on the immobilized preparation with the activity lost in the supernatant.

Figure 18 shows the activity retained on the immobilized preparation, where it is clear that a loading of 10% gives less than 20% of activity (or 20% of cells are retained on the preparation). Looking at Figure 19 at the same loading, it is possible to see that almost 60% of cells are present in the supernatant, explaining the low activity of this preparation.

Meanwhile looking at Figure 20 still at 10% loading, we see that the sum the activities of both supernatant and the preparation does not add up to 100%, about 30% of the activity is lost during the assay, most probably due to cell loss when harvesting the supernatant.

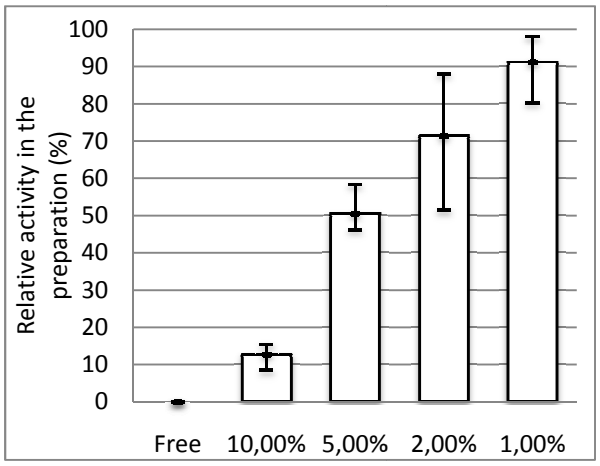


Figure 18 – Relative activity in preparation of different cell loadings compared to free cells. (The error bars show the highest and the lowest values obtained in different assays). **1%** to **10%** are cell loadings. **Free:** correspond to free cells

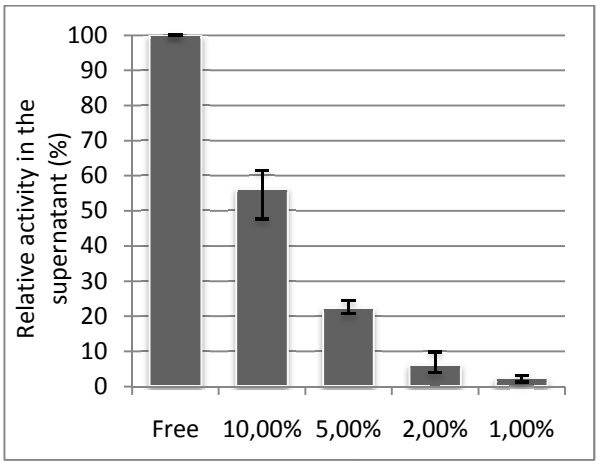


Figure 19 – Relative activity in supernatant of different cell loadings compared to free cells. (The error bars show the highest and the lowest values obtained in different assays). **1%** to **10%** are cell loadings. **Free:** correspond to free cells

Analyzing all the data generated by this multi assay, we can assume that the window to get a good immobilization is located between 1% and 5% of cell loading; above 5% the losses increase to more than 50%.

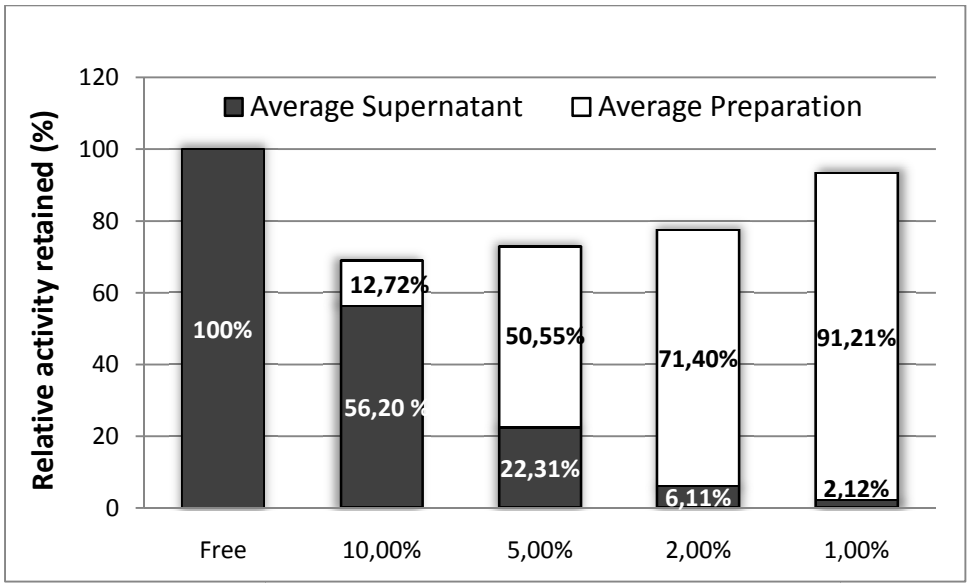


Figure 20 – Compared relative activity in the supernatant of different cell loadings. **Average Supernatant:** average of 4 assays for activity present in the supernatant; **Average Preparation:** average of 4 assays for activity present on the immobilized preparation. **1%** to **10%** are cell loadings. **Free:** correspond to free cells

4.2.9 – Effect of preparation re-use on initial activity

The results of re-use of immobilized cells in different batches are presented in Figures 21 and 22.

In the first 4 batches the maximum conversion was achieved after 40 minutes. As the preparation was recycled, the time for maximum conversion was achieved later, but not longer than 70 minutes for any of the batches.

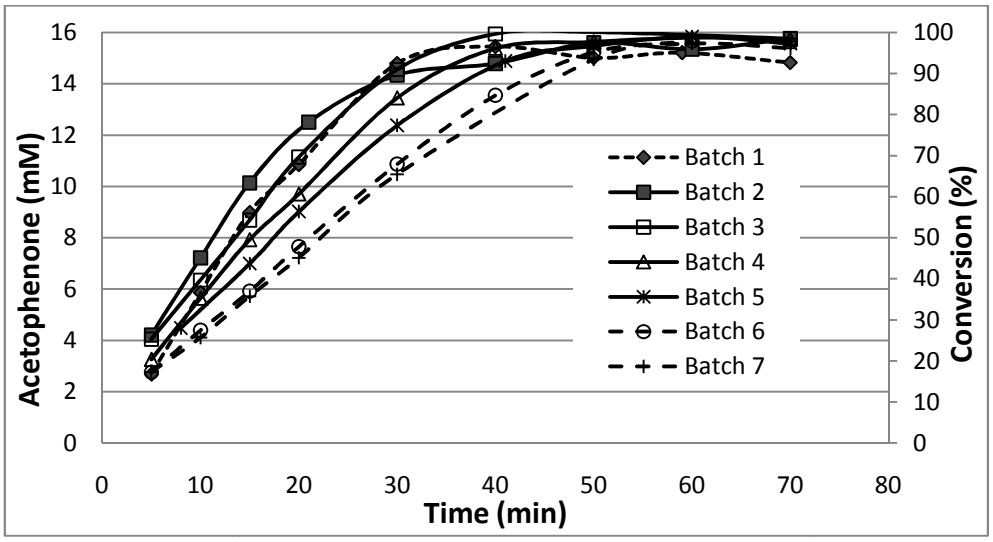


Figure 21 – Acetophenone production for multi batches experiment (5 mg cells, 2% of loading)

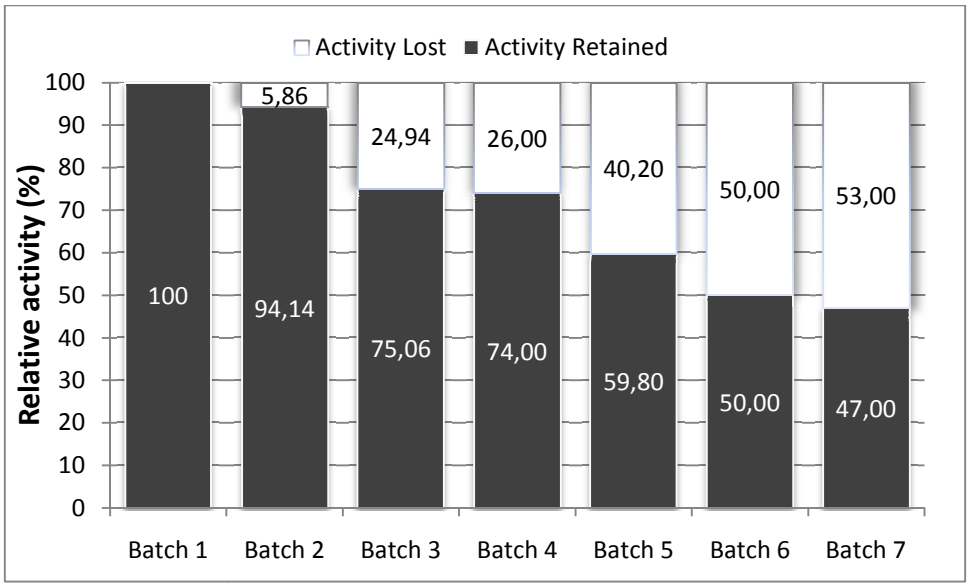


Figure 22 – Acetophenone production for multi batches experiment (5 mg cells, 2% of loading)

The data showed in Figure 22 was generated based on the initial activity of each batch. Analysis of these data show that 50 % of the initial activity is lost in 6-7 batches.

The recovery method selected was centrifugation, which seemed to be the easiest and simplest way to do it, but a filtration could also have been used.

In this experiment each batch had almost 1,5 hours of duration and after 7 recycles of the preparation, half of enzyme activity was lost. Considering that each batch of the 7-Metóxi-2-aminotetralin reaction needs at least 5 hours with a fresh cell preparation to reach its maximum conversion, we can predict that it would only be possible to perform 1 to 3 batches before the enzyme lost half of its activity.

This great loss of activity is mainly due to the temperature effect as was demonstrated before.

4.2.10 – Production of 7-MAT

Figure 23 shows the result for production of the substituted (S)-aminotetralin (7-methoxy-2-aminotetralin). In this reaction the 7-Methoxy-2-tetralone (7-MT) is converted into (S)-7-Methoxy-2-aminotetralin (7-MAT) by transfer of an amine group from the isopropylamine.

Similarly to the results achieved with free cells, the maximum conversion was after 5 hours of reaction. Comparing the conversion obtained for both TiOx and free cells, the first one gave only 20% of conversion while 95% was achieved with free cells. The great difference is due to the amount of cells used in both experiment (20 mg for TiOx and 220 mg for free cells experiment). The amount of biocatalyst used for the reaction using immobilized cells was too low and did not allow the conversion to reach maximum values. Both free and immobilized biocatalyst showed no activity after 4-5 hours of reaction.

Further studies with this reaction would allow one to calculate the suitable amount of biocatalyst for this reaction, since 20 mg in 20 mL seems to be too low and 220 mg probably is too much. The temperature has here an important role, it will dictate whether the preparation is able to be re-used or not.

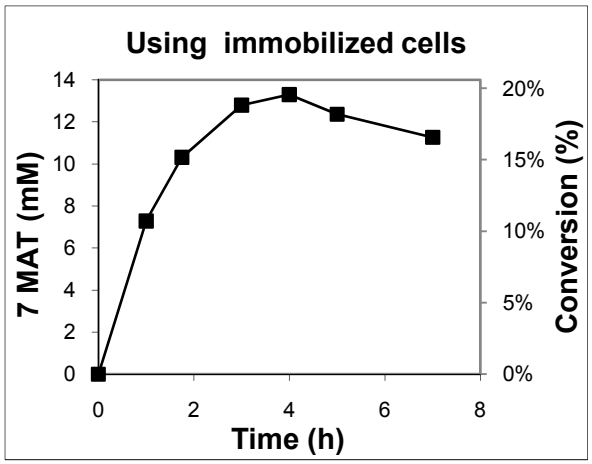


Figure 23 – Production of 7-MAT using immobilized cells (20 mg Cells – 1% Loading in 20 mL of reaction mixture)

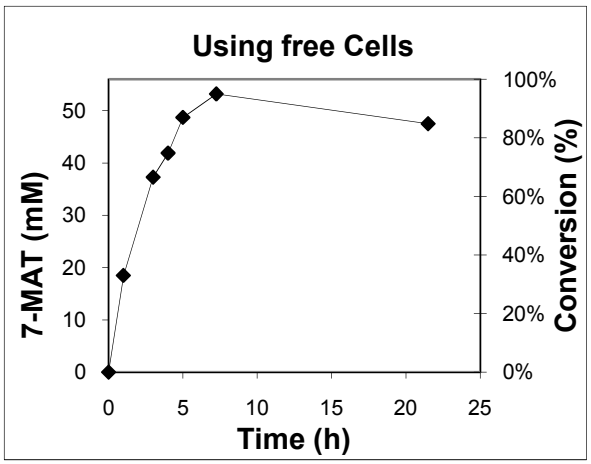


Figure 24 – Production of 7-MAT using free cells (220 mg in 20 mL of reaction mixture)

4.2.11 - 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. To investigate this, a simple experiment was carried and the results are shown in Table 5. It was observed that as the time passes the preparation (cell-TiOx) will precipitate and become compact in the bottom of the volumetric cylinder. After 120 min, the preparation occupied 1,6 mL in volume of compaction.

If this immobilization method is applied in a larger scale, probably it would be faster to centrifuge the preparation instead of waiting for it to settle down. Considering this, the preparation was centrifuged for 5 minutes at 6000 rpm (WIFUG Lab Centrifuge) and the volume occupied was 0,5 mL (Table 5). This means that 600 mg of preparation occupies 0.5 mL. In other terms, 1.2 ton of preparation occupies 1 m^3. Using this relationship, Table 6 was constructed to show the minimum volume the preparation would occupy at different cell loadings when immobilizing 100 Kg of spray dried *E. coli* (dry weight). If one wishes to use 6 m^3 reactor for example, 3 % or 4% may be the most suitable loading.

Table 5 – Volume occupied by the preparation with different sedimentation time.

Time (min)	Vol. occupied in the reactor (ml)
0	3.0
37	2.3
62	2.0
120	1.6
After centrifugation (6000 rpm - 5 min)	0.5

Table 6 – 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

5- CONCLUSION/DISCUSSION:

The immobilization of *E. coli* spray dried cells was successful. It was demonstrated that TiOx in solution arranges in clusters, attaching the cells. Due to its higher density, the titanium carries the cells rapidly to the vessel's bottom, which allows the preparation to be recovered either by centrifugation or filtration.

When comparing the *E. coli* spray dried cells to yeast cells, the loading capacity of the former in TiOx is lower. This difference may be associated with differences in the surface area of both microorganism or to the available ligands on their cell membrane.

The efficiency window achieved for spray dried *E. coli* immobilization (1 to 5%) makes 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.

It was demonstrated that the preparation can simply be recovered by a centrifugation for a few minutes. Even just stopping the stirring would also work.

Great attention must be given to the buffer used when performing this immobilization. Phosphate-based buffer appears to be unsuitable and disturbs the immobilization process. Tris buffer seems to be a good alternative, since it did not show any negative effect on the immobilization process.

Washing the cells before proceeding with the immobilization seems to be a good option as well, since the loading capacity was slightly increased after including this step in the process.

A lower reaction temperature should be considered, since it was shown that the biocatalyst stability was rather poor at 55ºC, especially without the cofactor PLP. A larger number of batches could probably be achieved if a lower temperature was used. It is important to find the best compromise in temperature between a good conversion rate, and biocatalyst stability. In future studies, the temperature stability should be tested in the presence of the cofactor, to see whether it helps or not to keep enzyme stability.

The TiOx storage appears to be simple and not affected with time, air exposure, light exposure or mechanical force. The preparation was kept stored in saline solution for months and continually used, without showing any decrease in efficiency.

During the titration to form hydrous titanium oxide, the stirring speed, the titration speed and the final pH may play a role on the particle structure and size, which may influence the loading capacity [6].

It would be industrially interesting if one could create TiOx directly from commercial titanium oxide powder, avoiding the titration step.

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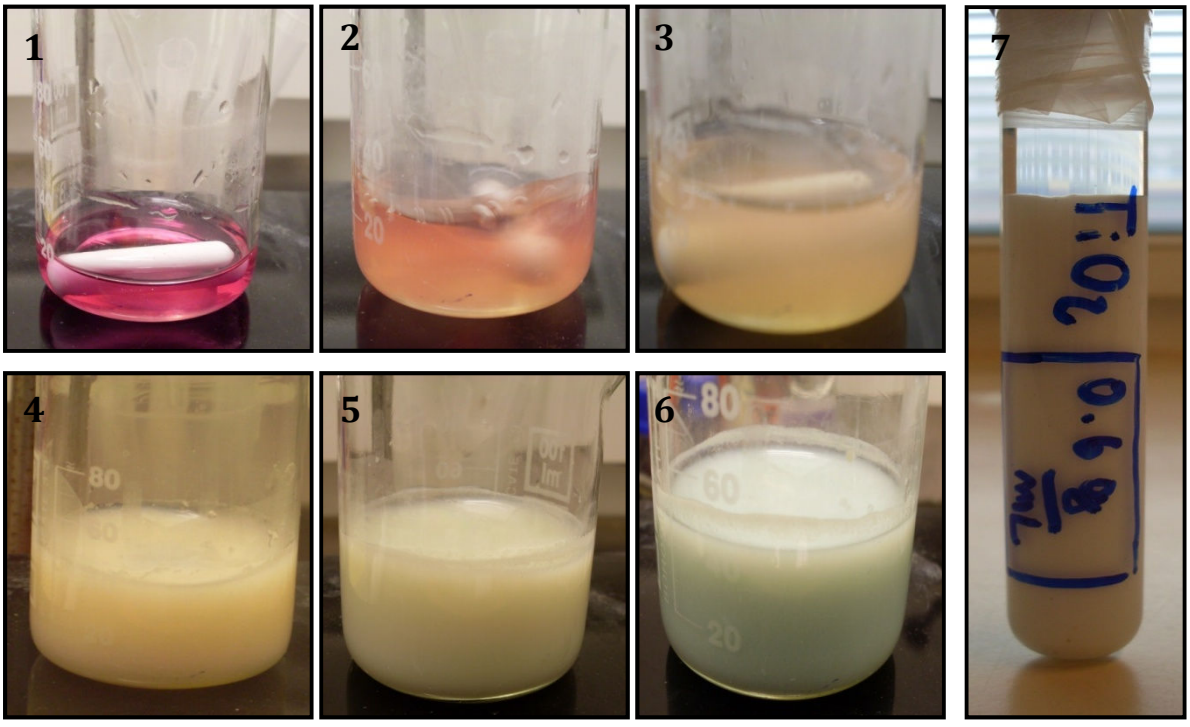
APPENDIX

1) Standard Curve

Theoretical Conversion	MBA	Sodium Pyruvate	Acetophenone	Alanine	PLP	Phosphate buffer	Absorbance (1:100)
(%)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(245 nm)
0,00	15,00	100,00	0,00	0,00			0,113
10,00	13,50	98,50	1,50	1,50			0,254
20,00	12,00	97,00	3,00	3,00			0,445
30,00	10,50	95,50	4,50	4,50			0,604
40,00	9,00	94,00	6,00	6,00			0,777
50,00	7,50	92,50	7,50	7,50	1	100,00	0,958
60,00	6,00	91,00	9,00	9,00			1,118
70,00	4,50	89,50	10,50	10,50			1,291
80,00	3,00	88,00	12,00	12,00			1,453
90,00	1,50	86,50	13,50	13,50			1,616
100,00	0,00	85,00	15,00	15,00			1,759

2) Production and storage of hydrous titanium oxide



3) DNS Method originally described:

Incubate the yeast cells with a 2,0% (w/v) solution of sucrose in 20 mM sodium acetate buffer, pH 4,5, at 45 ºC. At zero time and at various time intervals along an incubation period of 30 min at 45ºC, take 100 µL samples and place them in an ice bath to stop the reaction.

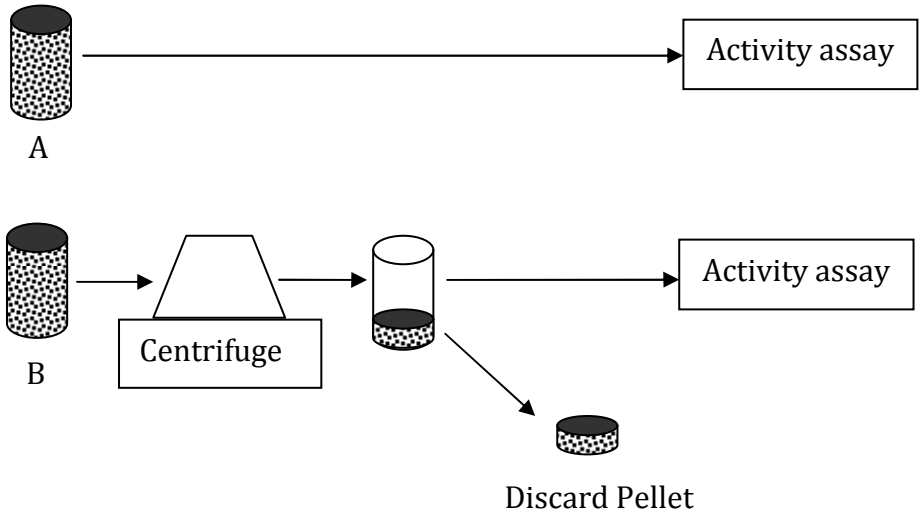
Thaw the samples to room temperature, and add 100 µL of DNS reagent. Mix thoroughly and heat in a boiling water bath for 5 min. Cool to room temperature add 1 mL of distilled water and measure the absorbance of the solutions at 540 nm.

Composition of the reagent:

DNS solution:

3,5-dinitrosalicylic acid (DNS)	10 g
Phenol	2.0 g
Na_2SO_3	0.5 g
Sodium Potassium Tartare	200 g
NaOH solution (10 g/L)	1000 mL (dissolve the reagents in 500 mL and then make up to 1 L

4) Immobilization efficiency:



Results from absorbance measurement at 540 nm

	50 mg IMM Yeast Cells	
Time	**(A) Solution**	**(B) Supernatant**
0	0,456	0,008
1	0,587	0,019
10	1,000	0,013
20	1,189	0,016
30	1,263	0,009

5) Effect of immobilization on yeast cells activity

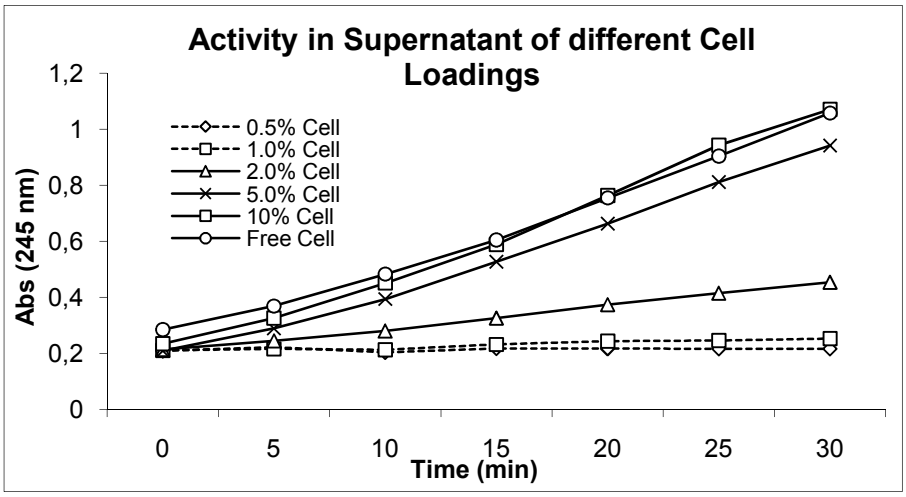
Results from absorbance measurements at 540 nm

	100 mg IMM Yeast Cells		1 mg Yeast Free Cells		Average	
Time (min)	Abs(245 nm) 1st Assay	Abs(245 nm) 2nd Assay	Abs(245 nm) 1st Assay	Abs(245 nm) 2nd Assay	**IMM**	**Y**
0	0,685	0,656	0,802	0,665	0,6705	0,7335
1	0,865	0,890	0,800	0,932	0,8775	0,866
10	1,15	1,088	1,152	1,077	1,119	1,1145
20	1,157	1,126	1,17	1,197	1,1415	1,1835
30	1,175	1,173	1,181	1,117	1,174	1,149

IMM = 100 mg of Immobilized Cells (~1,45 mg of Yeast)
Y = 1 mg Free Yeast Cells

6) **E. coli** *spray dried cells immobilization efficiency*

Time	Abs (245 nm)					
(min)	0.5% Cell	1.0% Cell	2.0% Cell	5.0% Cell	10% Cell	Free Cell
0	0,209	0,211	0,214	0,209	0,235	0,285
5	0,222	0,216	0,245	0,289	0,326	0,369
10	0,204	0,213	0,28	0,394	0,451	0,483
15	0,218	0,232	0,326	0,527	0,589	0,605
20	0,217	0,244	0,374	0,663	0,764	0,755
25	0,216	0,246	0,415	0,812	0,943	0,904
30	0,216	0,253	0,454	0,942	1,071	1,058



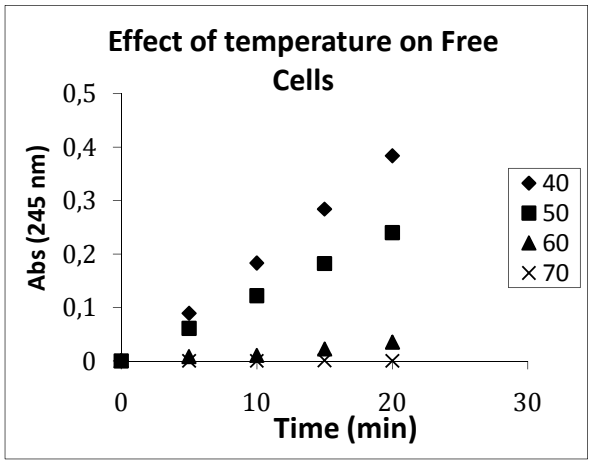
7) Effect of immobilization on activity of E. coli *spray dried cells*

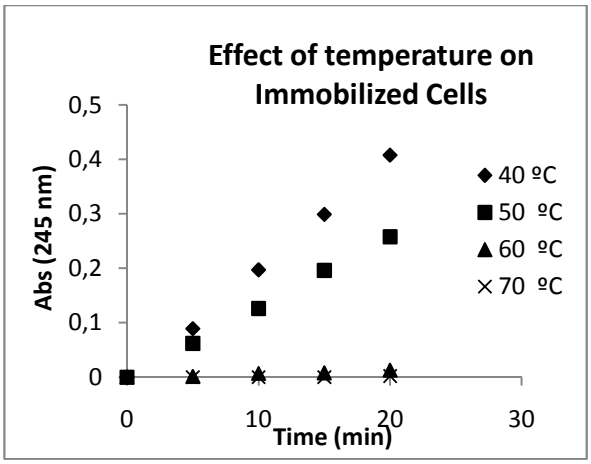
Time	Abs(245 nm) 1:49		mM Acetophe		% Conv	
(min)	Free Cells	Imm cells	Free Cells	Imm cells	Free Cells	Imm cells
0	0	0	0,000	0,000	0,00	0,00
10	0,344	0,318	1,911	1,766	12,74	11,77
20	0,679	0,652	3,771	3,621	25,14	24,14
30	0,968	0,922	5,376	5,121	35,84	34,14
40	1,271	1,18	7,059	6,554	47,06	43,69
50	1,533	1,426	8,515	7,920	56,76	52,80
60	1,745	1,629	9,692	9,048	64,61	60,32
70	**1,875**	**1,749**	**10,414**	**9,714**	**69,43**	**64,76**
80	1,868	1,711	10,375	9,503	69,17	63,35
90	1,7463	1,68	9,699	9,331	64,66	62,21
100	1,585	1,353	8,803	7,515	58,69	50,10
110	1,319	1,121	7,326	6,226	48,84	41,51
120	0,981	0,661	5,449	3,671	36,32	24,48

Blanc Free (nm) **0,26**
Blanc IMM (nm) **0,284**

8) Effect of temperature on initial activity of free and immobilized cells

Free Cells					**Immobilized cells**				
Time (min)	**40 ºC**	**50 ºC**	**60 ºC**	**70 ºC**	**Time (min)**	**40 ºC**	**50 ºC**	**60 ºC**	**70 ºC**
0	0,000	0,000	0,000	0,000	**0**	0,000	0,000	0,000	0,000
5	0,089	0,061	0,008	0,000	**5**	0,089	0,062	0,001	0,000
10	0,183	0,122	0,010	0,000	**10**	0,197	0,126	0,007	0,000
15	0,284	0,182	0,022	0,001	**15**	0,299	0,196	0,008	0,000
20	0,384	0,240	0,035	0,000	**20**	0,408	0,258	0,013	0,002
Vo (mM/min)	0,019	0,012	0,001	0,000	Vo (mM/min)	0,020	0,013	0,001	0,000
Relative activity (%)	100,00	64,16	7,19	0,32	Relative activity (%)	100,00	64,88	2,96	0
Correlation	0,9996	1,0000	0,9655	0,7746	Correlation	0,9993	0,9996	0,9559	-





9) Storage stability

Time (min)	Free CTRL	IMM CTRL	Free PLP	IMM PLP
5	0,160	0,173	0,180	0,189
10	0,217	0,241	0,249	0,279
15	0,273	0,314	0,318	0,373
20	0,330	0,383	0,381	0,460
Slope	0,0113	0,0136	0,0138	0,0184
R	1,0000	0,9998	1,0000	0,9999
Rel. activ	81,88406	73,91304	100	100

10) Kinetics studies

Free cells					IMM cells				
[MBA] mM	**Rate (mM/min)**	**Rate* (mM/min)**	**Regression Residual**	**(Regression Residual)^2**	**[MBA] mM**	**Rate (mM/min)**	**Rate* (mM/min)**	**Regression Residual**	**(Regression Residual)^2**
0	0	0	0	0	**0**	0	0	0	0
1,5	0,0470	0,0464	0,0006	3,619E-07	**1,5**	0,0369	0,0516	-0,0147	2,15E-04
3	0,0743	0,0773	-0,0029	8,583E-06	**3**	0,0857	0,0841	0,0016	2,51E-06
4,5	0,0989	0,0993	-0,0004	1,634E-07	**4,5**	0,1097	0,1065	0,0032	1,01E-05
6	0,1188	0,1159	0,0030	8,758E-06	**6**	0,1302	0,1228	0,0073	5,36E-05
7,5	0,1277	0,1288	-0,0010	1,091E-06	**9**	0,1489	0,1451	0,0038	1,48E-05
12	0,1569	0,1545	0,0024	5,799E-06	**10,5**	0,1547	0,1530	0,0016	2,67E-06
13,5	0,1642	0,1604	0,0038	1,466E-05	**12**	0,1574	0,1595	-0,0022	4,71E-06
15	0,1597	0,1655	-0,0058	3,337E-05	**15**	0,1633	0,1697	-0,0063	4,03E-05
			sum	7,279E-05				sum	3,44E-04

11) Effect of cell washing before the immobilization on loading capacity

Results from absorbance measurements

Washed cells					
Supernatant:	Dilution: 1st 1000/400, 2nd 1000/50, Final 50 X				
Time (min)	1%	2%	5%	10%	Free
0					
5	0,267	0,331	0,436	0,532	0,601
10	0,274	0,394	0,621	0,818	0,972
15	0,29	0,466	0,807	1,126	1,319
20	0,302	0,527	0,999	1,381	1,668

Not Washed cells					
Supernatant:	Dilution: 1st 1000/400, 2nd 1000/50, Final 50 X				
Time (min)	1%	2%	5%	10%	Free
0					
5	0,283	0,381	0,553	0,58	0,577
10	0,307	0,51	0,866	0,926	0,909
15	0,329	0,628	1,147	1,268	1,238
20	0,35	0,739	1,423	1,591	1,561

Calculations for relative activity

Washed cells				Not Washed cells			
Loading	Slope	%	Correl	Loading	Slope	%	Correl
Free	0,0710	100,00	1,000	Free	0,0656	100,00	1,000
10%	0,0571	80,47	0,999	10%	0,0675	100,00	1,000
5%	0,0375	52,85	1,000	5%	0,0578	88,11	1,000
2%	0,0132	18,60	1,000	2%	0,0238	36,33	0,999
1%	0,0024	3,41	0,990	1%	0,0045	6,80	1,000

12) Immobilization efficiency (multi assay)

Calculations of relative activity for different assays

	Relative activity (%)		**Relative activity (%)**		**Relative activity (%)**		**Average**	
Cell loading	**A Prep**	**A Supe**	**B Prep**	**B Supe**	**C Prep**	**C Supe**	**Prep**	**Super**
Free	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
10,00%	**15,50**	**59,36**	**14,12**	**47,71**	**8,55**	**61,52**	**12,72**	**56,20**
5,00%	**58,41**	**20,79**	**46,92**	**21,67**	**46,32**	**24,47**	**50,55**	**22,31**
2,00%	**88,09**	**9,83**	**74,55**	**3,98**	**51,54**	**4,51**	**71,40**	**6,11**
1,00%	**98,11**	**1,89**	**95,23**	**1,39**	**80,29**	**3,09**	**91,21**	**2,12**

13) Effect of preparation re-use on initial activity

Batch 1				Batch 2				Batch 3				Batch 4			
Time (min)	Abs (245 nm)	Aceto (mM)	Conv (%)	Time (min)	Abs (245 nm)	Aceto (mM)	Conv (%)	Time (min)	Abs (245 nm)	Aceto (mM)	Conv (%)	Time (min)	Abs (245 nm)	Aceto (mM)	Conv (%)
5	0,298	2,68	17,4	5	0,47	4,2	27,3	5	0,449	4,043	26,3	5	0,362	3,259	21,2
10	0,651	5,86	38,1	10	0,8	7,21	46,8	10	0,705	6,347	41,2	10	0,626	5,636	36,6
15	0,998	8,99	58,3	15	1,13	10,1	65,8	15	0,965	8,688	56,4	15	0,88	7,923	51,4
20	1,205	10,85	70,4	21	1,39	12,5	81,2	20	1,238	11,15	72,4	20	1,078	9,706	63,0
30	1,643	14,79	96,1	30	1,59	14,3	93,1	30	1,617	14,56	94,5	30	1,494	13,45	87,3
40	1,716	15,45	100	40	1,64	14,8	96,1	40	1,771	15,95	103,5	40	1,709	15,39	99,9
50	1,667	15,01	97,5	50	1,73	15,6	101,3	50	1,779	16,02	104,0	50	1,737	15,64	101,6
59	1,689	15,21	98,7	60	1,71	15,4	99,7	70	1,751	15,77	102,4	60	1,755	15,8	102,6
70	1,646	14,82	96,2	70	1,75	15,8	102,4					70	1,748	15,74	102,2

	Batch 1	Batch 2	Batch 3	Batch 4
Vo(mM/min)	0,630	0,593	0,473	0,466
Correlation	1,000	1,000	1,000	1,000
Relative Activity	100	94,1	75,06	74

Batch 5				Batch 6				Batch 7			
Time (min)	Abs (245 nm)	Aceto (mM)	Conv (%)	Time (min)	Abs (245 nm)	Aceto (mM)	Conv (%)	Time (min)	Abs (245 nm)	Aceto (mM)	Conv (%)
8	0,498	4,48	29,1	5	0,31	2,76	17,9	5	0,306	2,755	17,9
15	0,777	7	45,4	10	0,49	4,39	28,5	10	0,456	4,106	26,7
20	1,002	9,02	58,6	15	0,66	5,92	38,4	15	0,635	5,717	37,1
30	1,375	12,4	80,4	20	0,85	7,65	49,7	20	0,802	7,221	46,9
41	1,654	14,9	96,7	30	1,21	10,9	70,6	30	1,163	10,47	68,0
50	1,719	15,5	100,5	40	1,51	13,6	88,0	50	1,661	14,95	97,1
60	1,759	15,8	102,8	50	1,69	15,2	98,9	60	1,729	15,57	101,1
70	1,729	15,6	101,1	60	1,74	15,7	101,7	70	1,709	15,39	99,9

	Batch 5	Batch 6	Batch 7
Vo(mM/min)	0,377	0,315	0,296
Correlation	0,999	1,000	1,000
Relative Activity	59,8	50	47

14) Production of 7-MAT

Mobile phase HPLC

- 2 Solutions: A(100% Methanol), B(0,01% TFA in water)
- Gradient:

  Time 0 – 25% A + 75% B:

  Time 4.5 min change to 50% A + 50% B

  Time 5 min change to 35% A + 65% B

Composition of reaction mixture

	Free cells	Immobilized cells
7-MT (mM)	56	68
IPA (mM)	750	750
PLP (mM)	0,5	0,5
EtOH (% v/v)	2	2
Cell mass (mg)	220	20
Reaction mix (mL)	20	20

Free cells

Time (h)	Area 1	Area 2	av. Area	Conc vial (mM)	Dilution	Conc react (mM)	Conversion (%)
0	0	0	0	0	100	**0,0**	0,0%
1	894027	956503	925265	0,185053	100	**18,5**	33,0%
3	1884567	1842913	1863740	0,372748	100	**37,3**	66,6%
4	2099574	2090353	2094964	0,418993	100	**41,9**	74,8%
5	2427049	2443118	2435084	0,487017	100	**48,7**	87,0%
7,25	2679229	2641373	2660301	0,53206	100	**53,2**	95,0%
21,5	2335636	2414675	2375156	0,475031	100	**47,5**	84,8%

Immobilized cells

Time (h)	Area 1	Area 2	av. Area	Conc vial (mM)	Dilution	Conc react (mM)	Conversion (%)
0	**0**	**0**	0	0	20	**0,0**	0,0%
1	1824363	1815953	1820158	0,364032	20	**7,3**	10,7%
1,75	2585579	2569794	2577687	0,515537	20	**10,3**	15,2%
3	3193871	3205300	3199586	0,639917	20	**12,8**	18,8%
4	3330288	3320386	3325337	0,665067	20	**13,3**	19,6%
5	3127074	3054400	3090737	0,618147	20	**12,4**	18,2%
7	2815309	2816607	2815958	0,563192	20	**11,3**	16,6%