High Throughput in Biocatalysis

Steroid Bioconversions

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Abstract

Microreactors have been widely used in biocatalytic processes due to their intrinsic characteristics, namely, easier handling, lower mass transfer limitations and smaller volume requirements. Additionally, it benefits overall process economics, lowering cost by reducing quantity of reagents and manpower. The aim of this work was to obtain a micro-industrial platform for the continuous production of steroid molecules. The performed reaction was the oxidation of cholesterol performed by cholesterol oxidase, yielding 4-cholesten-3-one. This system forms hydrogen peroxide as a by-product, which hinders industrial application. To overcome this limitation, catalase was coupled to reduce this product. The overall process was tentatively optimized concerning both operational conditions and biocatalytics and the final process was assembled according to all the experimental data gathered. The optimization process demonstrated that the enzymes should work in separate reactors (Packed-bed reactor for cholesterol oxidase and Fluidized-bed reactor for catalase) immobilized in a PVA-PEG support, which provided the reuse of the enzymes. The maximum cholesterol conversion obtained was near 50% but hydrogen peroxide degradation was effective. These results show that, despite the required further process optimization, indications were given concerning the use of both enzymes within the same system, the by-product elimination and the reuse of the enzymes, which provided a suitable base line strategy to facilitate industrial implementation of this multienzyme process.

Key-words: cholesterol oxidase, catalase, microreactors, continuous-mode systems, multienzyme processes.

Introduction

Biocatalysis is the use of natural occurring compounds (e.g. proteins) as catalysts. The use of enzymes as biocatalysts is nowadays widely spread in industrial scale and many studies have been done with the goal to improve the role of enzymes in industrial processes, such as the application of enzymes in organic synthesis and the development of green industrial products and procedures (Illanes, 2008, Woodley, 2008, Wohlgemuth, 2010). Some examples of green practices concern the minimization of wastes, the minimization of hazardous reagents and intermediates, the minimization of energy expense and, when using hazardous compounds, a recovery or reutilization process must be envisaged (EPA, August 2002).

Studies concerning biocatalysis have been carried out using isolated enzymes with different purification grades or whole cells with given catalytic activities. In both cases and, in order to maximize the efficiency of the processes, a combination of biocatalytic transformations either separated in time and space or in one-pot or cascade, is gaining scientific relevance (Burton et al., 2008). One example of the application of these processes is steroid biotransformations. In order to achieve a better

description of the behavior of a given biocatalytic process, the representation of the reaction mechanism through mathematical models can be a key step during the process development since it leads to a better exploitation of the material and human resources (Santacoloma et al., 2011).

To overcome the relatively slow implementation of biocatalysis on industrial scale, wide research efforts have been made towards the development of miniaturized devices, termed microreactors (Fernandes, 2010a). Those provide a faster transfer of results, the high level of parallelization required to the several stages of the process, lower costs and lower manpower. Some examples used are microwell plates (useful for screening) and microfluidic devices, like microchannel reactors and minireactors (vessels with similar behavior to bench scale fermentation systems but with reduced volumes, e.g. 10 ml) (Marques et al., 2010a).

Enzyme immobilization enhances the stability and hence applicability of biomolecules as reusable and robust biocatalysts. Some relevant characteristics of immobilization that make this process economically feasible are: i) the allowance for high enzyme content and therefore high volumetric productivity, ii) the increased control of the reaction, iii) easier recuperation of the used enzyme, iv) reduced contamination of the final product, v) minimization of substrate inhibition, vi) prevention from denaturation and vii) possibility of reuse. On the other hand the major drawbacks are the mass transfer limitations and the possibility of activity loss during the immobilization procedure (Purich, 2010, Fernandes, 2010b). Nevertheless, with a rational design of an immobilization process, a high performance biocatalyst may be obtained.

Steroids are lipidic molecules that are characterized by the presence of a four-ring carbon atom core, called cyclopentanephenanthrene (Fahy et al., 2005). The most commonly used raw materials in steroid industry are cholesterol and phytosterols and, nowadays, the steroid industry has a well established position in the pharmaceutical market, in which biological conversions are widely used, both with whole cells and isolated enzymes.

The present work concerns the oxidation of cholesterol using the enzyme, cholesterol oxidase. Cholesterol oxidases (3β-hydroxysterol oxidases) are flavin adenine dinucleotide (FAD) dependent enzymes that catalyse two reactions in one single active site: the initial oxidation of a Δ^5-ene-3β-hydroxysterol to Δ^5-ene-3β-ketosteroid with the reduction of molecular oxygen to hydrogen peroxide, followed by isomerization of Δ^5 bond yielding a Δ^4-ene-3β-ketosteroid as the final product (Aparício et al., 2008). This enzyme has been used to determine the amount of serum cholesterol in biological samples (Mendes et al., 2007), in the search and study of lipid rafts (London, 2002), to determine cholesterol and other sterols in foods and in steroid-production industry (Fernandes et al., 2010). The aim of this work is to obtain a micro-industrial production platform using the oxidation of cholesterol by cholesterol oxidase yielding cholestenone, as a model reaction. This reaction produces hydrogen peroxide along the way and, although it can be useful in some clinical applications, it is also a constraint in the downstream process. Thus, the main objective is to incorporate two enzymes working properly together, using cholesterol oxidase to convert cholesterol and catalase to dissociate the hydrogen peroxide into oxygen and water. Besides the verification of the enzymes behavior by itself, immobilization techniques were studied and activity profiles were determined. Since the substrate is hydrophobic it will be incorporated in an organic phase, therefore, a screening system for the suitable organic solvent was delivered.

The reactors configuration to test will be operating in batch or continuous systems

Materials and Methods

Cholesterol oxidase (EC 1.1 .3.6) was purchased from BBI Enzymes Ltd. (Gwent, UK) and catalase (EC 1.11.1.6) from Sigma (St. Louis, MO, USA). 4-cholesten-3-one was purchased from Acros (Geel, Belgium), cholesterol and progesterone were obtained from Sigma (St. Louis, MO, USA) and hydrogen peroxide from Merck (Germany). To PVA (poly(vinyl alcohol))-based material (LentiKat® Liquid), was purchased from GeniaLab (Braunschweig, Germany). The needles used to form the PVA beads were obtained by Terumo (Tokyo, Japan) with an internal diameter of 0.8 mm and 50 mm of length. The syringes were also purchased from Terumo and had a 2 ml capacity. Polyethylene glycol (PEG) 600 was purchased from Fluka (Deisenhofen, Germany). All other chemicals used during this work were of analytical or HPLC grade and purchased from assorted suppliers.

Solvent screening

Solvent screening was performed either in microtiter plates or in 10 ml microreactors where 2.5 ml of the organic solvents were placed in 10 ml screw caped vessels together with 2.5 ml of 100 mM phosphate buffer pH 7 containing 0.1 $g.l^{-1}$ of the cholesterol oxidase. Assays were conducted during 2 hours at 30ºC.

Bioconversion trials and continuous mode operation

In all bioconversion trials, either for stability evaluation, different aqueous-organic system ratio, co-immobilization trials, oxygen supply trials and hydrogen peroxide toxicity the defined conditions were temperature of 30ºC, using 10 ml capacity microreactors under 600 rpm magnetic stirring. In continuous mode, both enzyme concentrations were 0.1 $g.l^{-1}$. Figure 1 and 2 represent the two different setups proposed and assembled in the continuous operation mode. Different temperatures were applied to the system: whilst the reactions occurred at 30ºC, the main reservoir was maintained at 4ºC by a refrigeration device (Thermomix), provided by Braun (Germany) to avoid solvent evaporation. An HPLC pump was used (L-6000 from Merck Hitachi) and also a peristaltic pump (LKB from Pharmacia) was incorporated into one of the continuous setups (Figure 3). The packed-bed reactor used for cholesterol oxidase had a 2 ml capacity with dimensions of 2.5 cm length and 1 cm external diameter. The fluidized-bed reactor where catalase was incorporated had a 3 ml capacity with dimensions of 7.7 cm length and 0.7 cm of external diameter. The main reservoir contained a two phase aqueous-organic system composed of 100 mM phosphate buffer solution and *n*-heptane, respectively. The main reservoir was covered with a PTFE lid and additionally with a parafilm layer. In these, six inlets/outlets were inserted: 1) to enable the withdrawal of the organic phase to begin the reactional circuit (out), 2) to enable the withdrawal of the aqueous phase continuous monitoring (out), 3) air inlet, 4) to release the built-up pressure from the system, 5) to enable the organic phase to return to the main reservoir after completing the circuit (in) and 6) to enable the aqueous phase to return to the main reservoir (in) (Figure 3).

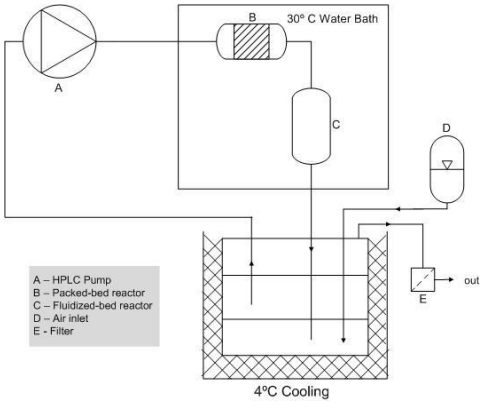

Figure 1 – Schematic representation of the continuous mode setup without the continuous hydrogen peroxide monitoring

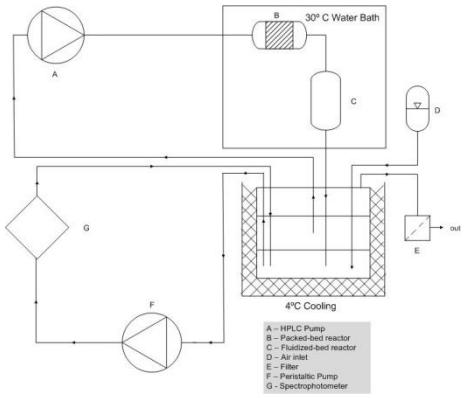

Figure 2 – Schematic representation of the continuous mode setup with the hydrogen peroxide monitoring incorporated

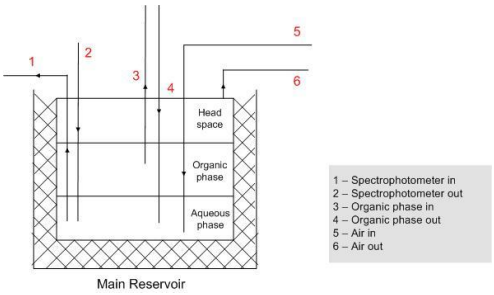

Figure 3 – Schematic representation of each inlet/outlet inserted in the main reservoir and the respective function

Hydrogen peroxide analysis

The hydrogen peroxide degradation was monitored according to Li and Schellhorn (2007) in 96-well UV-transparent microtiter plates (Greiner, Bio-One GmbH, Germany) at 240 nm in a microtiter plate spectrophotometer (SpectraMax Plus 384, Molecular Devices, Inc., CA, USA). The continuous mode setup had an on line monitoring device composed by an UV cuvette, connected to the aqueous phase of the main reservoir, which was placed inside the spectrophotometer. A peristaltic pump allowed the recirculation of the aqueous phase from the reservoir to the cuvette and back to the reservoir.

Steroid analysis

Samples from the steroid biotransformation media were extracted with a solution of progesterone (0.12 $g.l^{-1}$) in *n*-heptane in a 1:1 proportion. This solution was used as an internal standard. After mixing on a vortex (Reax 2000 by Heidolph Instruments, Schwabach, Germany) for 1 minute the samples were centrifuged at 4500 rpm during 5 minutes (Sigma 201). The organic phase was collected and analyzed with HPLC analysis that was performed by using a Licrospher Si-60 column (5µm particle size, Merck, Germany) with 1 $ml.min^{-1}$ isocratic elution and UV detection at 254 nm. The mobile phase was composed of *n*-heptane and absolute ethanol (90:10 v/v). Conversion yields were calculated on the basis of product formation since, besides 4-cholesten-3-one, no other product was formed, as assumed from the chromatograms, given the absence of unidentified peaks.

Dissolved oxygen analysis

To determine the dissolved oxygen concentration, Oxodish® 24-well microtiter plates (Presens, Germany) containing oxygen probes were used. The assays were carried out both under orbital agitation (200 rpm) and non-agitated conditions in two phase aqueous-organic systems composed of either ethanol and 100 mM phosphate buffer pH 7 solution or *n*-heptane and 100 mM phosphate buffer pH 7 solution.

Results and Discussion

Immobilization

Three different immobilization methods were performed, namely PVA-PEG, the sol-gel encapsulation and the immobilization on magnetite.

PVA-PEG immobilization method was already been proved effective to relevant biotransformation processes (Fernandes et al., 2009, Marques et al., 2011), sol-gel bioencapsulation has been applied in a large number of biological materials (Catana et al., 2005, Gill et al., 2000) and, to enhance simplicity in downstream process, the immobilization of the enzyme onto magnetite was tentatively used.

The activities of both enzymes (cholesterol oxidase and catalase) either isolated or combined in the three different immobilization supports were studied (Figure 4). As it can be observed, in sol-gel and magnetite immobilization the enzymes presented no activity. In sol-gel procedure the activity loss may be related to the release of methanol by TMOS or related to the drying process. In the case of magnetite, the usage of glutaraldehyde during the immobilization procedure may have inhibited the enzymes proper activity. These immobilization procedures must be optimized and a dedicated work should be necessary, which was out of scope for the present.

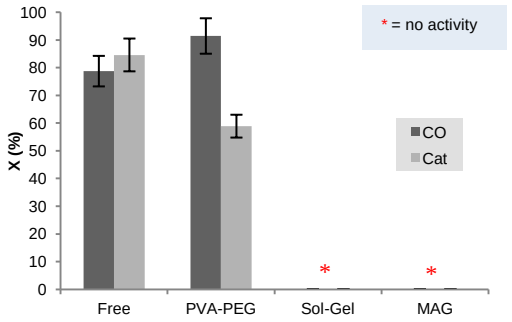

Figure 4 - Activity trials for both enzymes in the soluble form and in the three immobilization procedures (CO: cholesterol oxidase, Cat: catalase)

Unlike the results obtained for cholesterol oxidase and catalase in the sol-gel and magnetite supports, when the PVA-PEG supports were used, promising conversion yields were obtained with both immobilized enzymes. Although in catalase the PVA-PEG support lowered the conversion yield when compared to the free enzymes, in the case of cholesterol oxidase that parameter was not affected by the immobilization procedure, since similar conversion yields were observed either in free or immobilized forms of the biocatalyst.

Kinetic parameters

Table 1 - Kinetic parameters of free and immobilized enzymes

	Catalase		Cholesterol oxidase	
	K_m (M)	v_{max} ($M.s^{-1}$)	K_m (M)	v_{max} ($M.s^{-1}$)
Free	2.85×10^{-4}	2.38×10^{-6}	1.57×10^{-6}	2.74×10^{-6}
PVA-PEG	8.72×10^{-3}	2.48×10^{-3}	6.54×10^{-7}	1.67×10^{-6}

When comparing the free and immobilized enzyme (Table 1), a higher K_m is observed for the immobilized catalase. This reduction of the enzyme's affinity to its substrate has two reasons: a possible conformational change in the enzyme catalytic centre caused by the immobilization procedure or, mass transfer limitations. In cholesterol oxidase the immobilization procedure did not affect the enzyme's affinity towards the substrate. This can be explained by the protective characteristics of the immobilization support, which may have protected the enzyme function when in direct contact with the organic phase, despite the mass transfer limitations inherent to the immobilization support.

A comparison between the kinetic parameters obtained for this work (both free and immobilized in assorted supports) with previous data was performed, where few favorable results were found concerning different enzyme sources and immobilization supports. (Cirpan et al., 2003, Akkaya et al., 2009, Srisawasdi et al., 2006, Doukyu et al., 1998, Çetinus et al., 2000 and Marques et al., 2011).

Activity profiles

By assessing BRENDA[1] database it was verified that both cholesterol oxidase (derived from *Brevibacterium sp.*) and catalase (derived from bovine liver) were referenced with an optimum temperature of 30º C. Moreover, in MacLachlan et al. (2000), the same temperature was estimated to be the optimum. Knowing this

[1] http://www.brenda-enzymes.org, assessed on 30 September, 2011

parameter, the optimum pH and ionic strength of the buffer was determined (data not shown). It was stated that both enzymes are able to work under the same conditions, namely in phosphate buffer 100 mM, pH 7 (optimum molarity range from 50 mM to 200 mM and optimum pH range from 6 to 8).

Solvent Screening

During the preliminary assays, the organic phase was composed by ethanol. Nonetheless, it was necessary to determine which organic solvent had the suitable characteristics to develop a continuous mode platform with the two enzymes working simultaneously. This screening assay was supported not only by experimental data, obtained by conversion reactions performed in 96-well microtiter plates and in magnetically stirred microreactors but also crossing these data with some physicochemical properties of the solvents, in order to obtain correlation patterns between them.

A set of 53 different organic solvents were tested and preliminary testes had reduced the initial set to 24. With the 24 solvents, a correlation between the conversions obtained and the physic-chemical properties (namely, molecular weight, specific gravity, logP, vapor pressure, solubility, dielectric constant and Henry's law constant) was attempted and the construction of a multi-parametric analysis was suggested.

Methanol, ethanol, 2-propanol and *n*-heptane presented the higher conversion rates, 28.41%, 52.00%, 35.42% and 47.81%, respectively and the stability of cholesterol oxidase was tested in these four solvents (in the scope of a continuous mode operation). The enzyme had a higher stability when *n*-heptane was used as organic phase. The different stability patterns may be related with the fact that the solvents impose different biocompatibility problems (e.g. phase toxicity vs. molecular toxicity).

The oxygen concentration was estimated in a biphasic environment composed by either ethanol or *n*-heptane. Two conditions were used to mimic efficient aeration and non-ideal aeration (200 rpm and 0 rpm, respectively). Knowing the solubilities of oxygen in both solvents and the oxygen transfer coefficients it was possible to calculate the amount of oxygen available to be used in the reaction of cholesterol oxidation. The expression that follows those assumptions is:

$$\frac{dC}{dt} = k_L a(C - C_L) - q_{O_2} x \qquad (1)$$

Where $K_L a$ is the oxygen transfer coefficient (s^{-1}), C is the oxygen concentration in the media (mM), C_L the oxygen concentration in the liquid (mM), q_{O_2} is the consumed oxygen during the reaction period (mM) and x the enzyme concentration (mM).

It was verified that the oxygen availability was not a limiting step to this reaction since both in ethanol and *n*-heptane $k_L a(C - C_L) > q_{O_2} x$ and a favorable OTR was achieved when agitation was imposed to the system. *N*-heptane presented higher oxygen avaliability due to the higher oxygen concentrations present in this solvent. In ethanol, the concentration is lower since it is soluble in water, thus forming a monophasic media. The *n*-heptane insolubility in water does also provides an easier withdraw of the samples along the reactions, which makes the analytical process faster and simpler. It also provides a higher capacity to carry larger amounts of cholesterol, in comparison with ethanol.

Bioconversion trials

In order to analyze the stability results concerning both enzymes, additional information was gathered with the usage of two different activity models, bi-exponential adjustment model and exponential deactivation model: $Act = Ae^{\beta t} + (1 - A)e^{\alpha t}$ and $Act = e^{-kt}$ respectively (Aymard et al., 2000). Following the observation of the results obtained, the most accurate model was the bi-exponential adjustment, which was chosen to predict the behavior of the enzymes.

Stability trials were performed using catalase and cholesterol oxidase in the free and immobilized form for 100 hours and the enzymes behavior was predicted up to 300 hours. The aim was to choose suitable conditions to perform a continuous mode operation using both enzymes, specifically to decide if both enzymes should work separately or together in the same reactor system and if they should be co-immobilized or catalase should be used in the free or immobilized form (data not shown).

The behavior of the enzymes in a non-aqueous system was tested. A trial was performed to determinate the effect of the reduction of the water content in the two-phase aqueous-organic system and the results revealed that, even when the enzyme was immobilized, it was not advisable to remove the aqueous phase from the reactor. The decrease of the aqueous phase fraction significantly lowered the conversion yield, which only achieved promising values for an aqueous phase fraction in excess of 30%. The differences observed may have occurred due to alterations in the interfacial area arising from the variation of the volume of both phases, as reported in (Cruz et al., 2001).

The next trial was mainly to evaluate the differences between the usage of both enzymes simultaneously in the same support or separately (either in the free form or immobilized). Inside each batch reactor different combinations of the enzymes were placed and the results obtained are showed in the next figure. The results represent the behavior of the enzymes after 5 hours of reaction and reveal a highest substrate conversion when cholesterol oxidase and catalase were separately immobilized. This may be due to a hydrogen peroxide free environment given by the action of catalase over the reaction media and, therefore, over the activity of cholesterol oxidase.

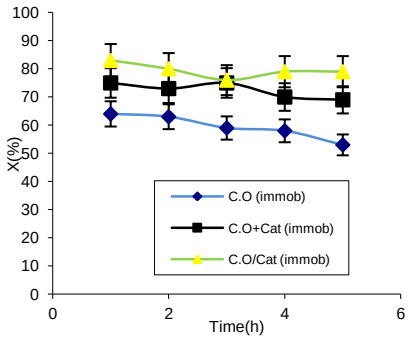

Figure 5 – Immobilized cholesterol oxidase (CO) and catalase (Cat) activity curves after 5 hours of reaction.

The oxygen supply trial consisted on the evaluation of the enzyme behavior when different concentrations of oxygen were present inside the reactor. This trial aimed to establish whether or not an oxygen supply device should be integrated in the continuous mode setup and to verify if a higher amount of oxygen supply would inhibit the bioconversion, as it had been reported by Marques et al. (2010). Four assays were performed: a control reactor with no oxygen content (the oxygen was removed by the use of a nitrogen inflow), a reactor opened to atmosphere with no forced aeration; two reactors, submitted to 5 and 20 minutes of forced aeration, providing higher concentrations of oxygen.

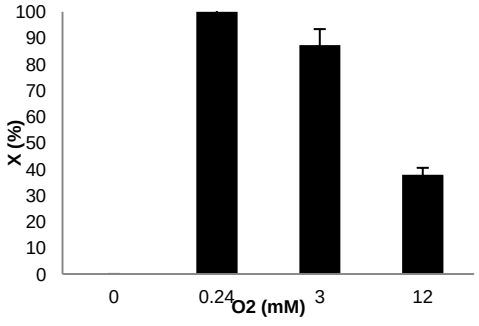

Figure 6 - Activity of cholesterol oxidase in the presence of different oxygen concentrations using 10 ml, magnetically stirred, biphasic microreactors.

The oxygen concentrations were determined through a calibration curve (oxygen concentration vs. time), which was measured with an oxygen probe, as stated in Marques et

al., (2010). The probe was calibrated to measure the oxygen concentrations not only in aqueous media but also in organic solvents. The results revealed that higher conversion rates were obtained with an open reactor and 3 mM of oxygen, providing a satisfactory enzyme activity. The same did not happened with a 12 mM concentration of oxygen, where the enzyme activity was low and inhibition may have occurred. The difference between supplying additional oxygen and use an open reactor relies on the fact that, with the open reactor, the evaporation of the organic solvent will occur, especially in a long term operation.

Continuous mode operation

Prior to the continuous mode operation, the evaluation of the effect of different hydrogen peroxide concentrations (from 0 to 10M) over the cholesterol oxidase behavior was assessed. The tested concentrations revealed a reduction in the enzyme activity as the hydrogen peroxide concentration was increased. When 10 M was present in the reaction media, the enzyme had lost over 50% of the activity, comparing with free-hydrogen peroxide media (data not shown).
Table 2 resumes the determined parameters to proceed to the construction of the continuous setups, presented in Figures 1 and 2.

Table 2 – Resume of the determined conditions defined during the previous assays

Temperature = 30ºC
Aqueous media composed of a 100 mM, pH 7 Phosphate Buffer
Enzymes concentration = 0.1 $g.l^{-1}$
Cholesterol concentration = 1 $g.l^{-1}$
Immobilization of the enzymes is essential (PVA-PEG method)
n-heptane is the suitable organic solvent to use as substrate pool
Hydrogen peroxide has an inhibition effect over cholesterol oxidase function
Bioconversion efficiency is favored if the two enzymes are immobilized separately
The system has to be oxygenated (3 mM)
The system must be closed to avoid evaporation

The continuous mode setup was composed by a packed-bed reactor (PBR) and a fluidized-bed reactor (FBR). The PBR was chosen to cholesterol oxidase since it increases the residence time and the specific mass transfer area but the same could not be used for catalase since the formed hydrogen peroxide is a gas and to flow properly inside the reactor needs spaces to diffuse. To avoid clogging or vessel burst problems, the entrance of the liquid was provided from the bottom to the top in the case of the FBR, containing catalase. The reactors had a 2 ml (PBR) and 3 ml (FBR) capacity and the residence time were 4 and 6 minutes, respectively.

During the assays performed with the continuous setup low bioconversion values were achieved. There was an apparent evolution for a stationary after 40 hours of operation and, between 70 and 100 hours of operation, the conversions obtained were approximately constant (though at a value far from the desired). This must be due to: i) the refrigeration system incorporated in the system that may have lowered the temperature inside the reaction and the enzymes could be working out of its optimum range, ii) the precipitation of the product due to the low temperatures imposed, iii) losses of the solvent through the system linkages, iv) short residence times inside the reactors, v) lack of an aqueous phase inside the reactors (and possible toxicity of long-term exposure to *n*-heptane).
Despite the unsatisfactory results obtained to cholesterol oxidase activity, catalase had demonstrated to be effective in the hydrogen peroxide removal using this system (Figure 7).

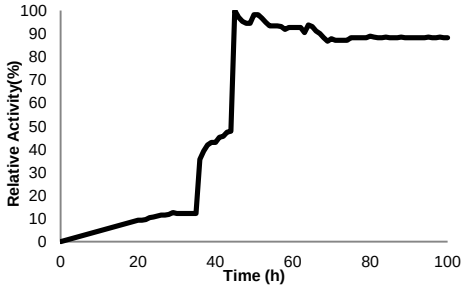

Figure 7 - On-line measurement of the hydrogen peroxide content in the continuous mode system using a spectrophotometer U-2000 from Hitachi (Japan)

Although working without an aqueous phase inside the reactor, the enzyme had lowered the toxic compound from the aqueous phase of the reservoir. This result suggests that catalase can be integrated with cholesterol oxidase for cholesterol oxidation in a continuous operation. The use of catalase enables the removal of hydrogen peroxide of the system that may inhibit cholesterol oxidase proper function. Moreover, the online monitoring system assembled was proved effective throughout the entire reaction period.

Conclusions

The main objective of this work was to characterize an efficient system for the oxidation of cholesterol, using microreactors. A continuous mode setup was obtained using the reaction of oxidation of cholesterol by cholesterol oxidase. This steroid biotransformation also yields hydrogen peroxide thus the incorporation of catalase into the system to degrade the hydrogen peroxide was envisaged. The results demonstrated that further modifications have to be made to achieve suitable bioconversions using both enzymes in the same system, although, it was established that cholesterol oxidation coupled to hydrogen peroxide removal can be performed with cholesterol oxidase and catalase. Despite the need of further optimization, this proposed base line strategy gave an extended and more realistic view over a scale-up protocol, since the removal of all by-products within the reaction process may facilitate the downstream, making it faster, easier and cheaper, inserting it in an high throughput approach, enabling the reuse of both enzymes and providing effective inside view on the application of green practices and industrial viability.

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