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Article

Improving the Toolbox of Bioreductions by the Use of Continuous Flow Systems

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Packed bed reactors can be used as an interesting alternative on the bioreduction of β-ketoesteres mediated by immobilized microorganisms. Here in, we report our results on the bioreduction of ethyl 3-oxohexanoate by immobilized *Kluyveromyces marxianus* cells and *tert*-butyl 3-oxobutanoate by immobilized *Rhodotorula rubra* cells under continuous flow conditions leading the desired β-hydroxy esters corresponding in high yields and enantiomeric excess.

Keywords: whole cell bioreduction, packed bed reactor, continuous flow, and asymmetric reduction.

Introduction

Enantiosselective reduction reaction is an important transformation in organic synthesis to afford enantiomerically pure compounds used as synthons for important applications such as pharmaceutical intermediates. Different enzyme types can catalyze these reactions with high enantioselectivity, providing a good alternative to chemical catalysis.[1]

Bioreductions have already been studied for several years and recently, due to the advent of recombinant DNA technology, more easily introduced in many industrial processes for the synthesis of chiral alcohols, α and β-hydroxy acids and aminoacids.[2-4] In general, the enzymes that catalyze such reductions need the presence of a cofactor. Today, the developments of more efficient coupled systems for cofactor regeneration have upgraded the status of such methodology because the feasibility of the entire process depends on the efficient and economic supply of the expensive nicotinamide cofactor NAD(P)H which is required in stoichiometric amounts.[3,4]

Alternatively, the use of whole cells does not require cofactor regeneration but some improvements are still necessary. In this type of methodology, the living organism can metabolize substrate or products, the existence of parallel competing metabolic pathways is an issue, and the selective activation of the enzymatic system for the desired reaction must be reached.[5,6] Besides that, reactions catalyzed by whole cells microorganisms usually have long reactions times, high diluted media needed and sensibility to harsh reaction conditions (Figure 1).

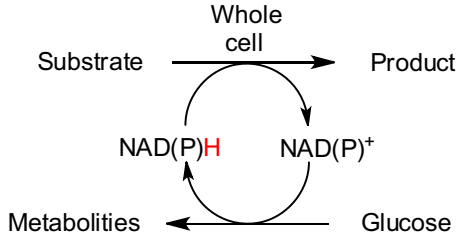

Figure 1. Whole cell biotransformation.

The use of packed bed reactors on biocatalyzed reactions or biotransformations is not new, but just a few examples can be found over literature about the use of this approach to the bioreduction of ketones.[7-12] In addition to the stated in the previous paragraphs concerning the use of whole cell, a key issue when working with these cells in packed bed reactors is the immobilization of the microorganism since the use of the free cell has several disadvantages, being the reduced recyclability of the system the most important one. The most simple and efficient way of immobilizing microorganisms is the calcium alginate entrapment.[13]

Recently, the continuous flow approach has attracted much attention from the organic chemistry community and in our continuous efforts towards the development of continuous flow biocatalyzed process here in we report the

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results on the bioreduction of acetoacetates derivatives for the production of chiral alcohols.[14-23]

Experimental

Materials

Ethyl 3-oxo-hexanoate (**1**) and *tert*-butyl 3-oxobutanoate (**2**) were purchased from Sigma and racemates were obtained by $NaBH_4$ reduction. The products were analyzed by ^1H and ^{13}C nuclear magnetic ressonance (NMR).

NMR analysis

rac-Ethyl 3-hydroxyhexanoate (**3**): ^1H NMR (200 MHz, $CDCl_3$, TMS) δ (ppm) 0.92 (t, 3H, H6), 1.26 (t, 3H, $COOCH_2C\underline{H}_3$), 1.43 (m, 4H, H4 and H5), 2.44 (m, 2H, H2), 3.15 (s, 1H, O<u>H</u>), 3.99 (m, 1H, H3), 4.16 (m, 2H, $COO\underline{C}H_2CH_3$); ^{13}C NMR (50 MHz, $CDCl_3$, TMS) δ (ppm) 14.1 (C6), 14.3 ($COOCH_2\underline{C}H_3$), 18.8 (C5), 38.8 (C4), 41.5 (C2), 60.8 ($COO\underline{C}H_2CH_3$), 68.0 (C3), 173.3 ($\underline{C}OOCH_2CH_3$); DEPT 135 (50 MHz, $CDCl_3$, TMS) δ (ppm) 14.1 (C6), 14.3 ($COOCH_2\underline{C}H_3$), 18.8 (C5), 38.8 (C4), 41.5 (C2), 60.8 ($COO\underline{C}H_2CH_3$), 68.0 (C3).

rac-tert-Butyl 3-hydroxybutanoate (**4**): ^1H NMR (200 MHz, $CDCl_3$, TMS) δ (ppm) 1.20 (d, 3H, *J* 6 Hz, H4), 1.74 (s, 9H, $COOC(C\underline{H}_3)_3$), 2.39 (d, 2H, *J* 4 Hz, H2), 3.17 (s, 1H, O<u>H</u>), 4.15 (m, 1H, H3); ^{13}C NMR (50 MHz, $CDCl_3$, TMS) δ (ppm) 22.5 (C4), 28.3 ($COOC(\underline{C}H_3)_3$), 44.0 (C2), 64.5 (C3), 81.4 ($COO\underline{C}(CH_3)_3$), 172.6 ($\underline{C}OOC(CH_3)_3$); DEPT 135 (50 MHz, $CDCl_3$, TMS) δ (ppm) 22.5 (C4), 28.3 ($COOC(\underline{C}H_3)_3$), 44.0 (C2), 64.5 (C3).

Microorganisms

Media, growth conditions and immobilization of cells in calcium alginate. *Kluyveromyces marxianus* belong to the collection of the 'Departamento de Engenharia Bioquímica, Escola de Química, UFRJ'. Cells were allowed to grow for 48 hours, under 150 rpm and 30 °C in a medium containing 1% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% $(NH_4)_2SO_4$ and 0.1% $MgSO_4.7H_2O$. After that period, the cells were centrifuged and 0.6 g (dry weight) was re-suspended in 3 mL of distilled water to obtain a cell-suspension. A 1.5% sodium alginate aqueous solution (20 mL) was added and this mixture (cell-suspension sodium alginate aqueous solution) was dropped in a $CaCl_2$ aqueous solution (0.1 mol L^{-1}) to form calcium alginate spheres. Spheres were filtered and washed with distilled water. Then, the column was filled with these spheres.

GC-MS analysis

Ethyl 3-oxohexanoate (**1**): conversions and enantiomeric excesses (e.e.) were determined by (chiral) gas chromatography (GC), on column Beta Dex325 (30 m × 0.25 mm × 0.25 μm) at 90 °C (23 min). The elution order was: ethyl (*S*)-3-hydroxyhexanoate (**3b**) (R_t = 18.8 min) followed by ethyl (*R*)-3-hydroxyhexanoate (**3a**) (R_t = 19.3 min). Substrate (**1**) was eluted at 15.3 min. The reaction product was characterized by NMR.

tert-Butyl 3-oxobutanoate (**2**): conversions and enantiomeric excesses were determined by (chiral) gas chromatography (GC), on column RTx-5MS (30 m × 0.25 mm × 0.25 μm) at 90 ºC (11 min). The elution order was: substrate (**2**) (R_t = 8.1 min), *tert*-butyl (*S*)-3-hydroxybutanoate (**4a**) (R_t = 8.6 min) and *tert*-butyl (*R*)-3-hydroxybutanoate (**4b**) (R_t = 9.0 min). The reaction product was characterized by NMR.

Optical rotation

Ethyl (*R*)-3-hydroxyhexanoate (**3a**): $[\alpha]_D^{25}$ –30.8° (*c* 1 g *per* 100 mL, $CHCl_3$); literature: $[\alpha]_D^{25}$ –26.9° (*c* 1.14 g *per* 100 mL, $CHCl_3$).[24] Optical rotations were measured from $CHCl_3$ solutions using a JASCO DIP-370 polarimeter at the sodium D line (589 nm) operating at room temperature and compared to literature.[24]

tert-Butyl (*S*)-3-hydroxybutanoate (**4a**): $[\alpha]_D^{25}$ +33.5° (*c* 1.64 g *per* 100 mL, $CHCl_3$, 95% optical purity); literature: $[\alpha]_D^{25}$ +26.3° (*c* 1.64 g *per* 100 mL, $CHCl_3$).[25] Optical rotations were measured from $CHCl_3$ solutions using a JASCO DIP-370 polarimeter at the sodium D line (589 nm) operating at room temperature and compared to literature.[25]

Continuous flow reaction procedure

A solution of 5 g of glucose in 100 mL of distilled water was prepared and ethyl 3-oxohexanoate (**1**) was added to the solution (0.013 mol L^{-1} or 2 g L^{-1}; 0.019 mol L^{-1} or 3 g L^{-1}; 0.025 mol L^{-1} or 4 g L^{-1}; 0.032 mol L^{-1} or 5 g L^{-1}). The starting mixture was stirred for 5 min, while the Asia (Syrris) instrument was equipped with the packed bed reactor containing immobilized cells from *Kluyveromyces marxianus* (volume: 12.3 mL). The temperature (30 °C) and 2 mL min^{-1} flow rate were selected on the flow reactor, and processing was started, whereby only pure solvent (glucose 5%) was pumped through the system until the instrument had achieved the desired reaction parameters and stable processing was assured. After that, the substrate was added to the glucose's solution and it was passed through the column with recycle. Aliquots were collected

in different times. The reaction mixture was extracted with ethyl acetate. The organic phase was dried (anhydrous Na_2SO_4), filtered, and concentrated under vacuum. Products were analyzed by (chiral) gas chromatography (GC). Asia Flow Reactor: a solution of 5 g of glucose in 100 mL of distilled water was prepared and the β-ketoester [ethyl 3-oxohexanoate (**1**) or *tert*-butyl 3-oxobutanoate (**2**)] were added to the solution (0.025 mol L^{-1} or 4 g L^{-1}). The starting mixture was stirred for 5 min while the instrument Asia Flow Reactor was equipped with Omnifit column (volume: 12.3 mL) containing the immobilized cells from *Kluyveromyces marxianus* [for the bioreduction of ethyl 3-oxohexanoate (**1**)] and *Rhodotorula rubra* [for the bioreduction of *tert*-butyl 3-oxobutanoate (**2**)]. The temperature (30 ºC) was selected on the flow reactor and for each flow tested (0.2 mL min^{-1}, 0.1 mL min^{-1} and 0.075 mL min^{-1}), first only the pure solvent (glucose 5%) was pumped through the system. At this point, the reaction mixture [ethyl 3-oxohexanoate (**1**) or *tert*-butyl 3-oxobutanoate (**2**)] was pumped through the system and aliquots were collected in different times depending on the flow rate tested (0.2 mL min^{-1} = 62 min; 0.1 mL min^{-1} = 123 min; 0.075 mL min^{-1} = 164 min). The reaction mixture was extracted with ethyl acetate. The organic phase was dried (anhydrous Na_2SO_4), filtered, and concentrated under vacuum. Products were analyzed by (chiral) gas chromatography (GC).

Results and Discussion

Based on previous results obtained by our group on the bioreduction of β-ketoester under batch conditions using *K. marxianus* and *Rhodotorula rubra* cells,[26,27] we decided to move forward towards a continuous flow methodology for such transformation. For such, microorganisms were immobilized on calcium alginate and packed on a glass column for the bioreduction of ethyl 3-oxohexanoate (**1**) and *tert*-butyl 3-oxobutanoate (**2**) under continuous flow conditions.

We began our work evaluating the effect of substrate concentration on the bioreduction of ethyl β-ketoester (**1**) and the results are shown in Table 1. In this first evaluation the packed bed worked in a loop system, the final operation time was set to 24 hours and the substrates pumped at a rate of 2 mL min^{-1} (Figure 2). This setup was chosen for in a previous report from our group on the bioreduction under batch conditions it demonstrated to be a very low process.

In general, the substrate concentration affects the conversion of the bioreduction process. The enantiomeric excess was up to 99% in all concentrations tested, however the conversion decreased drastically with concentrations

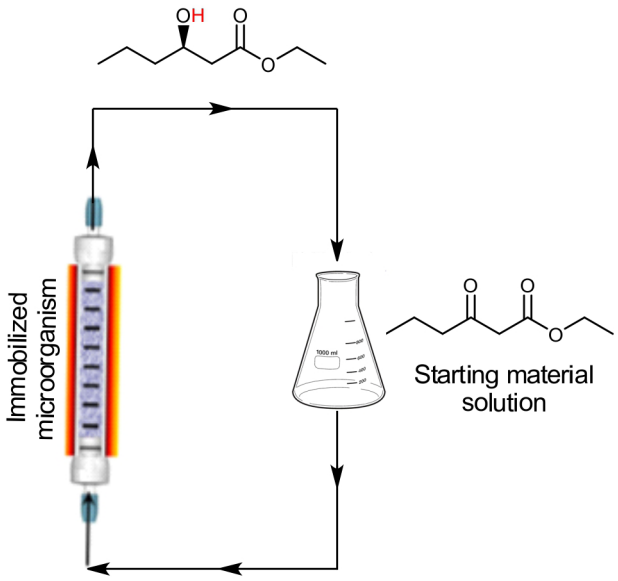


Figure 2. Packed bed reactor working on a loop system.

Table 1. Bioreduction of ethyl 3-oxohexanoate (**1**) to ethyl (*R*)-3-hydroxyhexanoate (**3a**) by immobilized *K. marxianus* cells

Concentration / (g L^{-1})	Conversion / %	e.e. / %
2	> 99	> 99 (*R*)
3	94	> 99 (*R*)
4	91	> 99 (*R*)
5	59	> 99 (*R*)

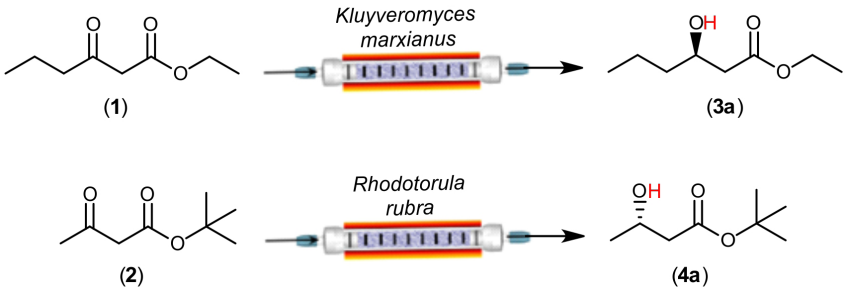
Reaction conditions: a solution of 5 g of glucose in 100 mL of distilled water was prepared and ethyl 3-oxohexanoate (**1**) was added to the solution (0.2; 0.3; 0.4 or 0.5 g). Conditions of flow reactor: volume of the column (12.3 mL); temperature (30 °C); flow rate (2 mL min^{-1}); pressure (10 bar); time (24 h).

higher than 4 g L^{-1} and the optimal substrate concentration was found to be in the range between 2-4 g L^{-1}.

When working on the loop system, we have followed the reaction conversion through time and observed that in order to achieve high conversions, starting material solutions needs to pass through the packed bed for several times (Figure 3) since at 2 mL min^{-1} the residence time is very short (ca. 6 minutes). As shown in Figure 3, with substrate concentration 4 g L^{-1}, after approximately 24 hours recirculating the solution through the system, the conversion arrives at the highest value (91%).

Aiming to obtain higher productivity and a more strain forward strategy towards the desired alcohol, we decided to reduce the flow rates used in the previous experiments and try to work in a system without the loop which means that the starting solution will pass through the packed bed reactor only once. Different flow rates were tested in order to find the best reaction condition, in Table 2 are shown the best results obtained. In this study we decided to use the

Table 2. Bioreduction of ethyl 3-oxohexanoate (**1**) by immobilized *K. marxianus* cells and *tert*-butyl 3-oxobutanoate (**2**) by immobilized *Rhodotorula rubra* cells



β-Ketoester	Flow rate / (mL min^{-1})	Conversion / %	e.e. / %
Ethyl 3-oxohexanoate (**1**)	0.2	58	> 99 *R*
	0.1	86	> 99 *R*
	0.075	98	> 99 *R*
tert-Butyl 3-oxobutanoate (**2**)	0.2	35	94 *S*
	0.1	57	96 *S*
	0.075	82	97 *S*

Reaction condition: a solution of glucose 5% in distilled water was prepared and the substrate (**1** or **2**) was added to the solution (0.4 g L^{-1}). Conditions of flow reactor: volume of the column (12.3 mL); temperature (30 °C); pressure (10 bar); residence time: 0.2 mL min^{-1} (62 min), 0.1 mL min^{-1} (123 min), 0.075 mL min^{-1} (164 min).

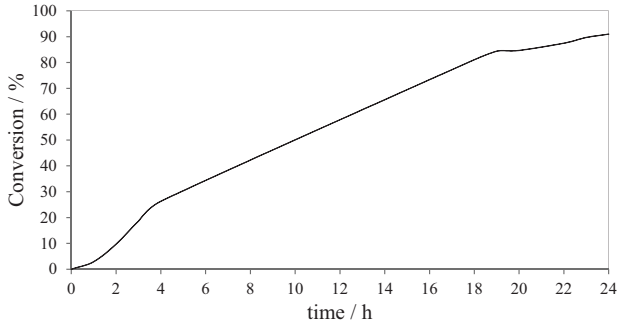


Figure 3. Conversion × time, on reaction loop system. Bioreduction of ethyl 3-oxohexanoate (**1**) to ethyl (*R*)-3-hydroxyhexanoate (**3a**) by immobilized *K. marxianus* cells. Reaction conditions: a solution of 5 g of glucose in 100 mL of distilled water was prepared and ethyl 3-oxohexanoate (**1**) was added to the solution (0.4 g). Conditions of flow reactor: volume of the column (12.3 mL); temperature (30 °C); flow rate (2 mL min^{-1}); pressure (10 bar).

4 g L^{-1} starting material solution in order to maximize the amount of product formed.

As shown in Table 2, increasing residence times (lower flow rate) lead to an almost quantitative reduction of the ethyl 3-oxohexanoate (**1**) with high e.e., while the *tert*-butyl 3-oxobutanoate (**2**) reach's 80% of conversion with also high e.e.

Conclusions

In conclusion a very simple procedure to obtain β-hydroxyesthers in high enantiomeric purity (> 99%) using immobilized cells of *K. marxianus* or *Rhodotorula rubra* was reported under continuous flow conditions. The reaction time was reduced significantly compared to the batch process and high yields of the corresponding β-hydroxyesthers in concentrations up to 4 g L^{-1}. High enantiomeric excess was observed and the absolute stereochemistry could be controlled varying β-ketoester structure and the microorganism immobilized.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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