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A greener bioreduction using baker's yeast cells in supercritical carbon dioxide and glycerol system

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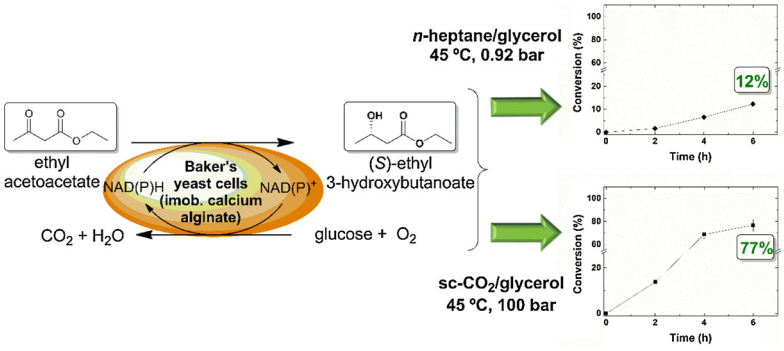
GRAPHICAL ABSTRACT



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ABSTRACT

This study reports results of an entire green methodology combining bioreduction by baker's yeast supported cells and a medium formed by glycerol and $scCO_2$ to a β-ketoester biotransformation. Fermented cells of *Saccharomyces cerevisiae*, immobilized in calcium alginate beads, were chosen to catalyze ethyl acetoacetate reduction in a biphasic system of $scCO_2$ and glycerol 20% (vol/vol) (T = 45 °C and P = 100 bar). The bioreduction was also carried out at normal conditions (T = 45°C and P = 0.92 bar) in *n*-heptane and glycerol 20% (vol/vol). Using $scCO_2$/glycerol the reaction reached 77% of conversion in 6 h, and the enantiopure (*S*)-ethyl-3-hydroxybutyrate (enantiomeric excess > 99%) was observed as the unique product. The same reaction under normal conditions led to only 12% conversion in 6 h. The approach evaluated in this study showed a great potential to be employed as a greener alternative to conventional bioreduction process.

1. Introduction

Nowadays, it has been increased the interest by greener and cleaner approaches in organic synthesis, mainly because of the environmental impact of the waste generated with this kind of process. One of the most sustainable ways to achieve highly enantiopure alcohols, which are required as important intermediates for the introduction of chiral center into several chemicals [1], is the bioreduction of carbonyl compounds mediated by alcohol dehydrogenases (E.C. 1.1.1.1), both in whole cells or isolated form [2]. This class of enzymes exhibits crucial

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advantages to green chemistry proposes as highly selective reactions, environmentally benign processes, catalytic efficiency under mild reaction conditions and energy-effective operations [3]. Despite their similarities, both biocatalytic approaches show differences, mainly regarding to the cofactor regeneration issue: whole cells dismiss this step because they already present cofactors needed to complete the reaction, NAD(P)H. On the other hand, alcohol dehydrogenases do not present these cofactors being necessary to add them in stochiometric amounts, what increases the enzymatic bioreduction costs [4].

Employing whole cells as biocatalysts, however, displays some drawbacks as they are susceptible to mechanical stress, unstable to the reaction conditions and present lower reaction rates as compared to the enzymes. One way to overcome these drawbacks without increasing the process costs is the cells immobilization in cheap matrices, as calcium alginate, carrageenan or celite. Among these, calcium alginate has been the most employed matrix to immobilize cells in biocatalysis, it is cheaper and readily available, having ability to form gels under mild conditions and the fact it is non-toxic and non-pathogenic, which makes it attractive for applications in pharmaceutical industry [5].

Although biocatalysis by whole cells might be a good option to enantioselective bioreduction, these reactions should be carried out in organic solvents because many reagents are insoluble in water. Employing organic solvents in biocatalysis produces volatile organic compounds (VOCs) and decreases the green status of the adopted methodology. One alternative to overcome this disadvantage is the application of supercritical fluids (SFCs) as solvents, due their ability to solubilize organic compounds, and be a harmless medium to biocatalyst. Supercritical carbon dioxide ($scCO_2$) is a medium to biocatalysis that plays this role very well because it is bioavailable, cheap, non-toxic and inert [6]. This neoteric solvent was already evaluated in enzymatic reactions, enhancing conversions when compared with organic solvents [7–9]. Even though $scCO_2$ shows advantages as cited before, the high pressures employed could be a handicap in using it with whole cells reactions, mainly because is already known that $scCO_2$ causes cell death [10–12]. One way to enhance cell viability is employing a solvent to keep stability of the cell without harm the “greenness” of the process.

Glycerol has been considered a green solvent that could be used in chemical reactions showing advantages over other solvents. Its utilization in biocatalysis is an innovative trend that has been recently used taking the advantages of glycerol as high potential to solubilize hydrophobic compounds, due to its biodegrability, low vapor pressure, and non-toxicity [13–15]. On the other hand, the glycerol/$scCO_2$ system presents very limited solubility and forms a two-phases system at very high pressures and a wide composition range, allowing further development of biphasic reaction systems. Medina-Gonzalez et al. [16] observed that the glycerol solubility in CO_2 is extremely low (in the range of 10^{-5} in mole fraction) and that the glycerol rich phase dissolved CO_2 reaching concentrations up to 0.13 (mole fraction). The use of glycerol and $scCO_2$ as solvents for chemical and biochemical reactions have been used as a way to enhance the performance of biocatalysts [17], for partially or even totally substitution of organic solvents, however studies employing the glycerol as a supporting to $scCO_2$ medium (glycerol/$scCO_2$ system) in biocatalysis has been not found in the open literature. Therefore, the main subject of this study was to investigate the potential of immobilized baker’s yeast cells to be applied in the bioreduction of a model compound (ethyl acetoacetate) in a system formed by glycerol and $scCO_2$ system. Moreover, the effect of $scCO_2$ on the baker’s yeast cells was evaluated, as well as the operational stability of the biocatalyst under successive pressurization/depressurization steps.

2. Material and methods

2.1. Materials

Carbon dioxide (99.9 wt% in the liquid phase) was supplied by White Martins S.A. (Osasco, SP, Brazil). Ethyl acetoacetate (EAA), glycerol (≥99.5%), dichloromethane, baker’s yeast, glucose, yeast extract, tryptone, propidium iodide were obtained from Sigma-Aldrich (St Louis, MO, USA). All other reagents were used in analytical grade.

2.2. Fermentation and harvest

Yeast cells were grown in 250 mL shake flasks containing 200 mL of culture medium, which consisted of 20 g L^{-1} glucose, 10 g L^{-1} yeast extract, 20 g L^{-1} tryptone, 0.5 g L^{-1} $MgSO_4$, 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl. Medium sterilization was conducted at 121 °C for 20 min at 1 bar in autoclave [18]. The medium was inoculated with 0.2 g of yeast and incubated at 30 °C using a shaker orbital set at 150 rpm for 66 h. The cells were harvested by centrifugation at 4000 rpm for 20 min [19].

2.3. Immobilization of baker’s yeast

After centrifugation, the supernatant was removed, and the yeast cells were re-suspended in 35 mL of culture medium. Thus, this cellular suspension (1.375 g dry weight) was added to 30 mL of sodium alginate solution 4% (w V^{-1}). The cell-alginate suspension was extruded through a 22 G needle dropwise into a chilled bath of 0.2 moL L^{-1} calcium chloride solution (previously sterilized at 120 °C for 15 min), which caused the alginate to cross-link and form hardened beads. The beads remained in calcium chloride solution without stirring for 60 min to ensure finishing the gelling process. The beads were, therefore, washed in distillated water to remove un-reacted material and low molecular weight byproducts and dried for 2 h on paper towels.

2.4. Reaction in organic solvent

A scheme of the EAA reduction by immobilized baker’s yeast (IBY) both essayed either in *n*-heptane/glycerol or in $scCO_2$/glycerol is depicted in Fig. 1. Batch reactions in *n*-heptane and glycerol 20% (V V^{-1}) were carried out in 250 mL flasks containing 0.1 mmoL of EAA and 0.2 mmoL of glucose as cosubstrate. In order to catalyze the reduction of substrate to (*S*)-ethyl-3-hydroxybutyrate ((*S*)-E-3-HB) 32 g of supported cells were employed. The reaction was incubated in an orbital shaker for 48 h at 45 °C and 150 rpm. Samples were withdrawn from the flasks and analyzed by GC (as described in item 2.8) to determine conversion and enantiomeric excess (ee).

2.5. Reaction in supercritical CO_2 ($scCO_2$)

The experimental set-up used in this work is shown in Fig. 2. The experimental apparatus consists basically of a CO_2 cylinder, a cooler, a high-pressure pump, a Parr reactor of 100 mL (model 4848) equipped with a mechanical stirring system and a heating mantle. The reactor was loaded with reactants (0.1 mmoL of EAA and 0.2 mmoL of glucose), glycerol 20% (V V^{-1}) and free baker’s yeast (FBY: 2 g of lyophilized cells) or (IBY: 32 g of calcium alginate beads containing immobilized cells), and then the desired temperature (35, 45 and 55 °C) was set. The stirring speed was set to 175 rpm. Thus, the reactor was pressurized to the desired pressure (85, 100 and 115 bar) with pre-cooled CO_2 (the syringe pump jacket was maintined at 15 °C for all experiments during the CO_2 feed) A pressure gauge and a temperature indicator were installed to ensure that the system operated at constant pressure and temperature throughout the reaction. The reaction was finished by depressurizing the reactor at a rate around 3 bar min^{-1}. The reaction mixture was collected with water, filtered to separate the beads and the substrate and product were extracted with dichloromethane (3 × 10 mL). Before the samples analysis by GC, they were concentrated in a rotary evaporator and dried with anhydrous $MgSO_4$. Duplicate runs were carried out for each experimental condition.

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