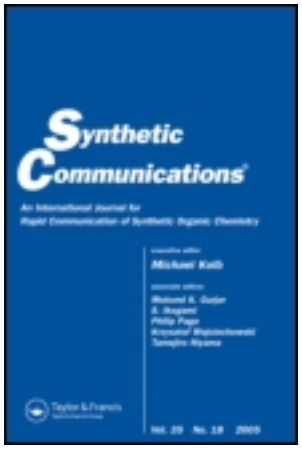

Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lsyc20

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Accepted author version posted online: 17 May 2012.Version of record first published: 06 Mar 2013.

To cite this article: Aline de Souza Ramos , Joyce Benzaquem Ribeiro , Raquel de Oliveira Lopes & Rodrigo Octavio Mendonça Alves de Souza (2013): Whole Cells in Enantioselective Reduction of tert-Butyl Acetoacetate, Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry, 43:12, 1611-1618

To link to this article: http://dx.doi.org/10.1080/00397911.2012.655685

Synthetic Communications®, 43: 1611–1618, 2013

ISSN: 0039-7911 print/1532-2432 online
DOI: 10.1080/00397911.2012.655685

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WHOLE CELLS IN ENANTIOSELECTIVE REDUCTION OF *tert*-BUTYL ACETOACETATE

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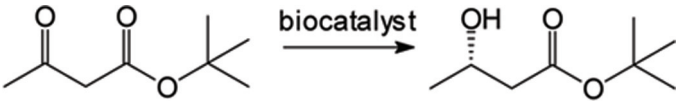
GRAPHICAL ABSTRACT



Abstract *The β-ketoester* tert*-butyl acetoacetate was enantioselectively reduced to* tert*-butyl (S)-3-hydroxybutanoate by seven microorganism strains. The best result using free cells was obtained with the yeast* R. rubra*, which furnished 97.6%* ee *and higher than 99% of conversion within 24 h. After immobilization in calcium alginate spheres,* R. rubra *furnished 96%* ee *and higher than 99%* ee *within 24 h, even if substrate concentration was 58 mM. Immobilized cells were reused three times without loss of enantioselectivity.*

Keywords Calcium alginate; chiral reduction; immobilization; β-ketoester; *R. rubra*

INTRODUCTION

Both enantiomers of the hydroxyester *tert*-butyl 3-hydroxybutanoate are important chiral building blocks in the synthesis of bioactive natural products[1,2] and β^3-aminoxy peptides.[3] The asymmetric reduction of β-ketoesters to β-hydroxyesters is very useful for organic synthesis, and much attention has been paid to biotransformation reactions, particularly because of their easy accomplishment, high stereoselectivity, and ecofriendly appeal.[4–6]

For an economically viable bioreduction process, reduced nicotinamide cofactors must be regenerated in a second catalytic cycle to sustain catalytic activity. Whole cells have their own cofactor regeneration system and therefore are usually preferred to purified enzymes. *Saccharomyces cerevisiae* is often used in the biotransformation of carbonyl compounds because it is easily available. However, the desired

Received November 30, 2011.
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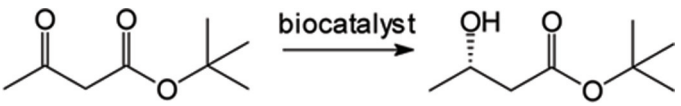

Figure 1. Microbial reduction of *tert*-butyl acetoacetate to *tert*-butyl (*S*)-3-hydroxybutanoate.

configuration cannot always be achieved and competing enzymes within cells can lead to poor stereoselectivity.[5–8] Several techniques have been reported to enhance stereospecificity of whole-cell bioconversions, such as screening for other microorganisms besides *S. cerevisiae*,[5,7] addition of enzymatic inhibitors,[9] biocatalyst immobilization,[10–13] heating acetone dried cells,[14] the use of organic solvents,[15,16] and the modification of reaction conditions.[6,10,11]

Over the past few years, we have been investigating the reduction of various β-ketoesters by microorganisms.[7,10,11,17–20] In the present work we report our studies on the reduction of *tert*-butyl acetoacetate (Fig. 1) using wild yeasts and filamentous fungi previously tested on other bioreductions. Some yeast strains were immobilized in calcium alginate spheres before assays and evaluated for recyclability.

RESULTS AND DISCUSSION

The low cost of the bioreduction process using whole cells and the abundance of microorganisms in nature encouraged us in the search for microorganisms, in addition to the well-known *Saccharomyces cerevisiae* and *Aspergillus niger*, that are capable of conducting the reduction of *tert*-butyl acetoacetate with good conversion and enantioselectivity. Thus, we started this study with a screening step using free cells of five yeasts (*Saccharomyces cerevisiae* 40*, Hansenula* sp., *Geotrichum candidum, Kluyveromyces marxianus*, and *Rhodorotula rubra*) and two filamentous fungi (*Trichoderma harzianum* and *Aspergillus niger*). Conversions and enantiomeric excesses (*ee*) obtained after 24 h, with substrate concentration at 29 mM, are shown in Table 1.

All tested strains were able to catalyze the reaction with excess of the (*S*)-hydroxyester, the one predict by Prelog's rule[21] and the most frequent configuration found in ketoester microbial reductions described in the literature.[7,8] The product configuration was determined by measurement of optical rotation, which

Table 1. Bioreduction of *tert*-butyl acetoacetate (29 mM) to *tert*-butyl (*S*)-3-hydroxybutanoate by free cells

Microorganism	Conversion (%)[a]	*ee* (%)[a]
Saccharomyces cerevisiae 40	57.6	73.9
Hansenula sp.	>99	84.1
Geotrichum candidum	>99	96.5
Kluyveromyces marxianus	64.2	51.5
Rhodotorula rubra	>99	97.6
Trichoderma harzianum	>99	53.2
Aspergillus niger	76.4	46.2

Note. Incubation: 30 °C, 150 rpm, 24 h.
[a]Conversion and enantiomeric excess (*ee*) determined by GC analysis.

was $[\alpha]_D^{23}$ +33.5° (95% *ee*, *c* 1.64 g/100 mL, $CHCl_3$), and compared to the literature: $[\alpha]_D^{23}$ +26.3° (c 1.64 g/100 mL, $CHCl_3$)[22a] and $[\alpha]_D^{20}$ +36.0° (c 1.08 g/100 mL, $CHCl_3$).[22b]

It is worth noting that *S. cerevisiae* and *A. niger*, the most used microorganisms in biotransformations, furnished the reaction product in moderate enantimeric excess (73.9% *ee* and 46.2% *ee*, respectively), which highlights the need to seek new biocatalysts for this reaction. Conversions varied from 57.6% (with *S. cerevisiae*) to more than 99% (with *Hansenula* sp., *G. candidum, R. rubra*, and *T. harzianum*). Enantiomeric excesses varied from 46.2% (with *A. niger*) to 97.6% (with *R. rubra*). Among the tested biocatalysts as free cells, the most promising was *R. rubra*, which led to the excellent conversion (>99%) and high enantiomeric excess (97.6% *ee*). *Geotrichum candidum* also showed very good results, with more than 99% conversion and 96.5% *ee*. Examples of chromatograms obtained are shown in Fig. 2.

Compared to our previous studies, free cells of *K. marxianus* had greater conversion and enantioselectivity in the reduction of ethyl 4-chloroacetoacetate[11] and ethyl 3-oxohexanoate,[20] although product configuration changed according to the substrate. However, *R. rubra* seemed to give better results than *K. marxianus* on the conversion of bulkier substrates, as ethyl benzoylacetate[10] and *tert*-butyl acetoacetate. Salvi and Chattopadhyay[23] observed in their work on microbial reductions of ketones by the filamentous fungus *Rhizopus arrhizus* that the elongation of the alkyl chain (methyl to *tert*-butyl) in the alkoxy group led to a gradual increase in yields and enantioselectivities of the reduced products. In our study, we also observed that free cells of *R. rubra* exhibited better conversion and enantioselectivity in the reduction of *tert*-butyl acetoacetate compared to the reduction of methyl and ethyl acetoacetate, but we did not detect such correspondence with other biocatalysts, and even different product configurations were found with *K. marxianus* and *A. niger*.[7]

Several microorganisms have already been assayed as biocatalysts in the reduction of *tert*-butyl acetoacetate. North[15] obtained high (*S*)-enantioselectivity (>98% *ee*) and 75% of conversion with *S. cerevisiae* in a medium containing petrol/water 25:4, but this author employed very high biomass concentration (approximately 200 g freeze-dried/L). Medson et al.[16] also used *S. cerevisiae* in petroleum and reported isolated yield of 68% and 98% *ee* for the same isomer when the substrate concentration was 20 mM. Salvi and Chattopadhyay[23] used the filamentous fungi *Rhizopus arrhizus* in the reaction and achieved 71% of isolated yield and 94% *ee* after 4 days, but the substrate concentration in medium was only 0.66 g/L. Bernardi et al.[24] obtained low to moderate excess of the (*S*)-hydroxyester when using *A. niger* (48% *ee*) and *G. candidum* (85% *ee*), whereas 92% *ee* of the *R* configuration was achieved with the actinomycete *Streptomyces* sp. as biocatalyst. Acetone powder of *G. candidum* was used in the asymmetric reduction of *tert*-butyl acetoacetate and furnished the corresponding (*S*)-alcohol in high enantiomeric purity (>99%) and more than 99% conversion.[25] Afterward, Matsuda et al.[26] developed a method using ionic liquids as reaction media for asymmetric reduction by acetone-dried cells of *G. candidum* immobilized on water-absorbing polymer containing water. The substrate was added at 20 mM, and 87% of conversion with 98% *ee* was achieved for the (*S*)-hydroxyester. In both cases in which dried *G. candidum* cells were used, it was necessary to add NAD+ and 2-propanol as cosubstrate. Our data obtained with free cells of *R. rubra* and *G. candidum* are comparable with the greatest conversions

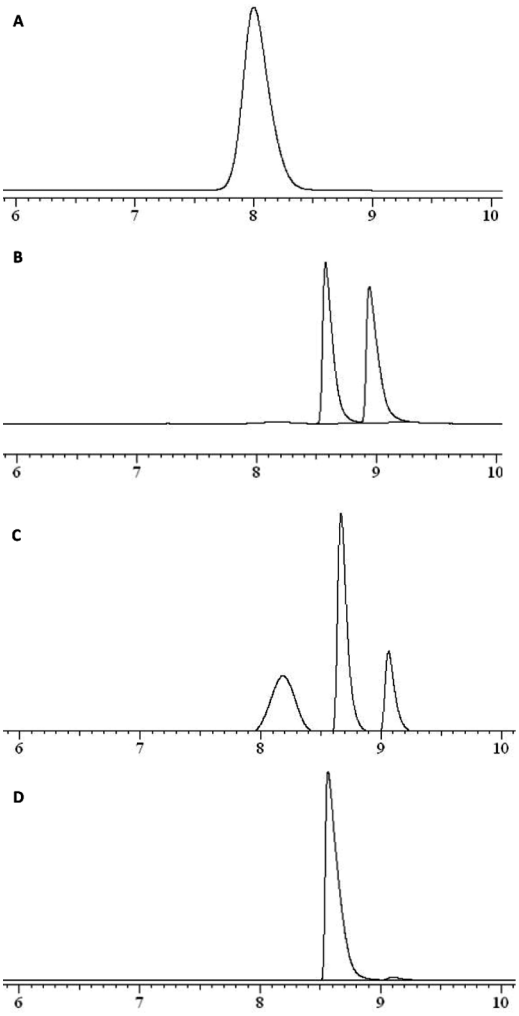

Figure 2. Typical chromatograms showing the selectivities achieved. Chiral GC analysis on column RTx-5MS (30 m × 0.25 mm × 0.25 μm), at 90 °C (11 min): (a) *tert*-butyl acetoacetate (substrate); (b) *tert*-butyl 3-hydroxybutanoate (racemate obtained via NaBH4 reduction); (c) *Aspergillus niger* reduction; (d) *Rhodotorula rubra* reduction. Retention times (tR): tR (substrate) = 8.1 min, tR ((*S*)-enantiomer) = 8.6 min, tR ((*R*)-enantiomer) = 9.0 min.

and enantioselectivities described in the literature for whole cells, but have some advantages, such as lower biomass concentration (12 g/L dry weight), greater substrate concentration (29 mM), and no need to add reduced cofators to reaction medium.

In the studies with plant cells, Speicher et al.[27] obtained greater than 95% *ee* of *tert*-butyl (*S*)-3-hydroxybutanoate with cultured cells of *Riccia fluitans*, with 70–90% yield within 1–2 days. In another study with plant cells, Kojima et al.[28] carried out the asymmetric reduction of ketones, including *tert*-butyl acetoacetate, using cultured cells of *Nicotiana tabacum* as biocatalyst and showed that the reduction of

Table 2. Bioreduction of *tert*-butyl 3-oxobutanoate (29 mM) to *tert*-butyl (*S*)-3-hydroxybutanoate by immobilized cells

	First cycle		Second cycle (after 14 d)	
Microorganism	Conversion (%)[a]	*ee* (%)[a]	Conversion (%)[a]	*ee* (%)[a]
K. marxianus	97.0	51.6	>99	72.4
G. candidum	97.4	90.8	97.0	95.0
Hansenula sp.	98.0	77.5	77.5	79
R. rubra	>99	95.9	>99	94.1

Note. Incubation: 30 °C, 150 rpm, 24 h.
[a]Conversion and enantiomeric excess (*ee*) determined by GC analysis.

tert-butyl acetoacetate gave high *ee* of the corresponding (*S*)-alcohol (98% *ee*) under illumination of fluorescent light and high CO_2 concentration, with 98% conversion. In contrast, under dark conditions, low CO_2 concentration and with the addition of glucose, the (*R*)-hydroxyester was achieved with 82% *ee* and 83% yield. Although interesting, plant cells hardly have been used until now for asymmetric reductions because of the lack of knowledge about this kind of process, mainly on a large scale. Microbial transformation processes are better known, but they are difficult to apply in biotransformations using plant cells.

In a second step of this study, *K. marxianus, G. candidum, Hansenula* sp., and *R. rubra* were immobilized in calcium alginate spheres to evaluate the entrapment effects on the bioreduction of *tert*-butyl acetoacetate. This immobilization technique makes product recovery easier, allows biocatalyst recycling, and makes continuous processes possible.[10–13,17,18] Moreover, cell immobilization in calcium alginate spheres sometimes improves conversion and enantioselectivity.[10,13] To evaluate biocatalyst recycling, immobilized yeasts were used twice: the first cycle was carried out immediately after the immobilization, and the second cycle was performed after a storage for 14 days in $CaCl_2$ solution at 4 °C (Table 2).

Immobilized cells of *K. marxianus* exhibited better conversion and enantioselectivity in the second cycle.

Compared to free cells, the entrapment of *K. marxianus* increased conversion level, while the immobilization decreased conversion level and enantioselectivity of *Hansenula* sp. (especially in the second cycle) and *G. candidum*. High conversion (>99%) and enantioselectivity (>94% *ee*) were maintained with immobilized *R. rubra* in both cycles, although a slight decrease in enantioselectivity had been observed compared to the use of free cells. Based on results showed in Tables 1 and 2, *R. rubra* was chosen for further studies.

Immobilization of cells in calcium alginate sometimes allows adding substrate to reaction medium at greater concentrations because of the diffusion barrier, which could protect cells from substrate toxicity.[13] As high substrate concentration is always desired in industrial biotransformation processes,[13,29] we evaluated the catalytic ability of immobilized cells of *R. rubra* in three cycles when *tert*-butyl acetoacetate was added at 58 mM. In the first cycle, the biocatalyst was used immediately after the immobilization; second and third cycles were performed after storage at 4 °C, at intervals of 7 days (Table 3). In previous studies with other substrates,

Table 3. Bioreduction of *tert*-butyl 3-oxobutanoate (58 mM) to *tert*-butyl (*S*)-3-hydroxybutanoate by immobilized cells of *R. rubra*

Biomass (g dry weight/L)	First cycle		Second cycle (after 7 days)		Third cycle (after 14 days)	
	Conversion (%)[a]	*ee* (%)[a]	Conversion (%)[a]	*ee* (%)[a]	Conversion (%)[a]	*ee* (%)[a]
12	>99	95.5	>99	93.0	54.5	94.5
24	>99	96.0	>99	96.0	>99	96.0

Note. Incubation: 30 °C, 150 rpm, 24 h.
[a]Conversion and enantiomeric excess (*ee*) determined by GC analysis.

we verified that biomass concentration can influence the reaction.[10,11] In addition, Medson et al.[16] observed that the amount of yeast (*S. cerevisiae*) required to effect complete consumption of the same amount of starting material increased with the size of the ester group: Methyl and ethyl acetoacetate were reduced with just 1 g of yeast whereas the most sterically demanding *tert*-butyl group required 11 g. So, to evaluate whether the biomass amount could affect the reaction, cells were employed at two concentrations: 12 g dry weight/L and 24 g dry weight/L.

In the first two cycles, the biomass concentration did not affect conversion, which was the same with free cells of *R. rubra* (>99%). High conversion was maintained in the third cycle when biomass was used at 24 g/L, but at 12 g/L only 54.5% of conversion was achieved. Enantioselectivity was high in all tested conditions, especially when biomass was added at 24 g/L (96.0% *ee*), showing that cells do not need to be used immediately after cultivation and immobilized biomass could be reused at least three times. These findings suggest robustness, an important characteristic for continuous process and industrial applications.

In summary, we described here a search for biocatalysts to be used in the asymmetric reduction of *tert*-butyl acetoacetate to *tert*-butyl (*S*)-3-hydroxybutanoate. In the screening step, free cells of seven microorganism strains were tested, and the yeast *R. rubra* led to best results. The biocatalytic potential of this yeast was demonstrated by the high enantioselectivity (96%) and excellent conversion (>99%) obtained within 24 h even after immobilization of cells in calcium alginate spheres during up to three cycles, when substrate concentration was 58 mM and biomass was used at 24 g/L. This is the first use of *R. rubra* in this reaction.

EXPERIMENTAL

Saccharomyces cerevisiae, *Hansenula* sp., *Geotrichum candidum*, *Kluyveromyces marxianus*, *Rhodotorula rubra*, *Aspergillus niger*, and *Trichoderma harzianum* belonged to the collection of the Departamento de Engenharia Bioquímica, Escola de Química, Universidade Federal do Rio de Janeiro. Cells were grown for 48 h 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% $MgSO_4 \cdot 7H_2O$. After that period, they were harvested by centrifugation, resuspended in water, and used for the reaction. After centrifugation, the cells (12 g/L, dry weight) were added to 50 mL of the reduction medium containing glucose (5%) and substrate (29 mM). The reaction was carried

out in 500-mL, cotton-plugged Erlenmeyer flasks for 24 h at 30 °C and 150 rpm. After 24 h, the medium was centrifuged again to separate the cells, and the liquid phase was extracted with ethyl acetate. The organic phase was dried (anhydrous Na_2SO_4), filtered, and concentrated under vacuum. 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 °C (11 min). The elution order was substrate (tR = 8.1 min), *tert*-butyl (*S*)-3-hydroxybutanoate (tR = 8.6 min) and *tert*-butyl (*R*)-3-hydroxybutanoate (tR = 9.0 min). The reaction product was characterized by nuclear magnetic resonance (NMR) and mass spectroscopy. 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 the literature.[22]

Cells grown during 48 h in the medium described were centrifuged, and 0.6 g (dry weight) was resuspended in 3 mL of distilled water to obtain a cell suspension. A sodium alginate aqueous solution (1.5%, 20 mL) was added, and this mixture (cell-suspension sodium alginate aqueous solution) was dropped in a $CaCl_2$ aqueous solution (0.1 M) to form calcium alginate spheres. Spheres were filtered, washed with distilled water, and added to 50 mL of medium containing glucose (5%) and substrate (29 or 58 mM). Reduction was carried out in 500-mL, cotton-plugged Erlenmeyer flasks, at 30 °C and 150 rpm during 24 h. After that period, the medium was filtered to separate the biocatalyst, and the liquid phase was treated as described previously. Immobilized cells were washed with distilled water and maintained at 4 °C in a $CaCl_2$ aqueous solution (0.1 M) for 7 or 14 days. Thus, immobilized cells were incubated in growth medium under 150 rpm and 30 °C for 2 h, washed with distilled water again, and reused under the same reaction conditions.

Compound *tert*-Butyl-3-oxo-butanoate

^{1}H NMR (200 MHz; $CDCl_3$): 1.47 [s, 9H, COOC($C\underline{H}_3$)]; 2.25 (s, 3H, H_4); 3.35 (s, 2H, H_2).

Compound *tert*-Butyl (*S*)-3-Hydroxybutanoate

^{1}H NMR (200 MHz; $CDCl_3$): 1.19 and 1.22 (d, 3H, H_4); 1.74 [s, 9H, COOC($C\underline{H}_3$)]; 2.40 and 2.38 (d, 2H, H_2); 3.17 (s, 1H, O$\underline{H}$); 4.07, 4.10, 4.13, 4.16, 4.19, 4.23 (m, 1H, H_3). ^{13}C NMR (50 MHz; $CDCl_3$): 22.54 (C_4); 28.34 [COOC($\underline{C}H_3$)]; 44.04 (C_2); 64.59 (C_3); 81.45 [COO$\underline{C}$(CH_3)]; 172.63 [$\underline{C}$OOC(CH_3)]. DEPT 135 (50 MHz; $CDCl_3$): 22.54 (C_4); 28.34 [COOC($\underline{C}H_3$)]; 44.04 (C_2); 64.59 (C_3). CGMS m/z 57: tert-butyl; m/z 103: RCOO.

ACKNOWLEDGMENTS

Financial support from FAPERJ, CAPES, and CNPq-BRAZIL is acknowledged.

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