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Immobilized microorganisms in the reduction of ethyl 4-chloro acetoacetate

Joyce Benzaquem Ribeiro [a,b,*], Aline de Souza Ramos [a,c], Sorele Batista Fiaux [d], Selma Gomes Ferreira Leite [a], Maria da Conceição Klaus V. Ramos [b], Francisco Radler de Aquino Neto [b], Octavio A. C. Antunes [b]

[a] *Escola de Química, Universidade Federal do Rio de Janeiro, CT Bloco E, Cidade Universitária, Rio de Janeiro RJ 21949-909, Brazil*
[b] *Instituto de Química, Universidade Federal do Rio de Janeiro, CT Bloco A, Cidade Universitária, Rio de Janeiro RJ 21949-909, Brazil*
[c] *Farmanguinhos, Fundação Oswaldo Cruz, Rua Sizenando Nabuco 100, Manguinhos, Rio de Janeiro RJ 21041-250, Brazil*
[d] *Faculdade de Farmácia, Universidade Federal Fluminense, Rua Mário Viana, 523, Santa Rosa, Niterói, Rio de Janeiro RJ 24241-000, Brazil*

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ABSTRACT

Microorganisms were used to reduce ethyl 4-chloroacetoacetate (CAAE) to ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE]. *Mucor ramannianus* provided 98% conversion with 84% ee. Free cells of *Kluyveromyces marxianus* led to 95% conversion with 81% ee. After a fractionary factorial design to study the reaction conditions, calcium alginate immobilized cells of *K. marxianus* furnished the product with 99% conversion with 91% ee.

1. Introduction

Asymmetric reductions of ketoesters can provide versatile chiral building blocks in organic synthesis. These procedures are preferred over the kinetic resolution of racemates, due to the possibility of producing only one enantiomer with high enantiomeric excess and chemical yields.[1] Chiral metal complexes such as Ru-BINAP and related systems can be used as catalysts in asymmetric hydrogenations, but these routes employ drastic conditions such as high temperature and pressure.[2] Biocatalytic transformations are an advantageous alternative, especially because of their high selectivity (chemo-, regio-, and stereo-selectivity), mild reaction conditions, and environmental aspects.[3,4]

Isolated enzymes and whole cells can be used as biocatalysts in enantioselective reductions. The use of isolated enzymes frequently leads to higher enantiomeric excess while avoiding problems associated with competing catalysts with differing stereoselectivities. Unfortunately, reduced cofactors must be regenerated in situ in a second catalytic cycle or provided in stoichiometric amounts to sustain catalytic activity.[5,6] Hence, whole cells are used more often because of their own cofactor regeneration system.[7] *Saccharomyces cerevisiae* has been widely used to obtain hydroxyesters, due to its low cost, ease of use, and good prediction for the stereochemistry of the product, however the desired configuration cannot always be achieved.[8,10]

Ethyl 4-chloro-3-hydroxybutanoate is a very useful chiral building block for enantiomerically pure pharmaceuticals. For example, the (*R*)-enantiomer can be converted to (*R*)-carnitine, an essential factor for the metabolism of fatty acids,[11] while the (*S*)-enantiomer is a key intermediate for HMG-CoA reductase inhibitors, a compound class used in the treatment of hypercholesterolemia.[12] A racemic mixture of such compounds cannot be used; ethyl 4-chloro acetoacetate is an inexpensive substrate for asymmetric reduction, the best way to produce optically active ethyl 4-chloro-3-hydroxybutanoate.[13]

In this study, 12 microorganisms were employed to catalyze the asymmetric reduction of ethyl 4-chloroacetoacetate (CAAE) to (*S*)-ethyl 4-chloro-3-hydroxybutanoate [(*S*)-CHBE] (Fig. 1) in whole-cell processes. Some strains were also used immobilized in calcium alginate spheres. This immobilization technique can influence the product configuration, enantiomeric excess, and conversion level.[14,15] Some reaction's conditions were studied by an experimental design as recommended by Taguchi to enhance conversion.[16]

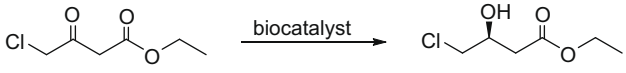

Figure 1. Microbial reduction of ethyl 4-chloro acetoacetate (CAAE) to ethyl (*S*)-4-chloro-3-hydroxybutanoate (*S*-CHBE).

2. Results and discussion

In the first step of this work, the free cells of twelve microorganisms (nine yeast strains and three filamentous fungi strains) were tested for CAAE reduction ability (Table 1). All microorganisms were able to reduce the substrate with excess of (*S*)-CHBE under the experimental conditions used.

Only *Aspergillus niger* gave lower than 50% conversion. *Rhodotorula rubra* and *Rhodotorula minuta* furnished 100% conversion,

* Corresponding author. Tel.: +55 21 2562 7248.
E-mail address: joyce_benzaquem@yahoo.com.br (J. B. Ribeiro).

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Table 1
Microbiological reduction of CAAE to *S*-CHBE with free cells

Microorganism	Conversion (%)	ee (%)
Saccharomyces cerevisiae 40	62.9	81.5
Saccharomyces cerevisiae 60	63.8	79.5
Saccharomyces cerevisiae 80	55.8	82.1
Pichia sp.	66.5	75.8
Hansenula sp.	86.1	57.5
Candida sp.	82.6	56.4
Kluyveromyces marxianus	94.7	80.8
Rhodotorula rubra	100	48.1
Rhodotorula minuta	100	50.2
Trichoderma harzianum	95.0	82.8
Mucor ramannianus	98.5	83.7
Aspergillus niger	36.1	90.0

Incubation: 30 °C, 150 rpm, 24 h.

but the enantiomeric excess was moderate (48.1% and 50.2%, respectively). *Mucor ramannianus* and *Trichoderma harzianum* were considered the best biocatalysts, with high conversion levels (98.5% and 95.0%, respectively) and up to 82% ee. *Kluyveromyces marxianus* also provided good results, with 94.7% conversion and 80.7% ee. However, when ethyl acetoacetate was used as a substrate, *K. marxianus*, *A. niger* and *T. harzianum* produced an excess of the (*R*)-hydroxy ester,[9,17] hence these microorganism are sensitive to changes in the substrate's structure.

Several microorganisms such as yeasts, molds, bacteria, and actinomycetes have been found to catalyze this reaction.[18] Most of them predominantly produce the (*S*)-enantiomer, as we found in this work. We confirmed the (*S*)-configuration by using the (*S*)-isomer purchased from Aldrich. It is sometimes difficult to find a good microorganism by screening whole cells because the coenzyme NADPH is frequently required in this reduction and there is not always an efficient NADPH-recycling system and an CAAE-reducing enzyme in the same cell.[18,19]

Numerous approaches have been documented to improve the stereospecificity of whole-cell bioconversions.[6,8,12,19,20] Better results were obtained with the addition of allyl alcohol,[6] higher biomass concentrations (150–400 g wet weight/L),[6,8] heating cells,[20] heating acetone dried cells,[19] and the substitution of glucose by 2-propanol as a co-substrate.[12] Recombinant microorganisms have also been used with good results.[7,21–23] However, wild-type strains, such as the ones used in this work, are generally preferred to industrial processes mainly because of their robustness.[24] Our results are compatible with wild-type strains reported in the literature, but in our case without toxic inhibitor or cell pretreatment. This suggests that some approaches could enhance the conversion levels and enantiomeric excess.

To evaluate the entrapment effects on microorganisms, *S. cerevisiae* 40, *Pichia* sp., *Hansenula* sp., *Candida* sp., *K. marxianus*, *R. rubra* were immobilized in calcium alginate spheres and tested again for CAAE reduction ability (Table 2). After immobilization, *Candida* sp. and *R. rubra* provided lower conversion levels and enantiomeric excesses in comparison with free yeasts used in the first step of this study. Immobilized cells of *S. cerevisiae* 40, *Hansenula* sp, and *K. marxianus* led to higher enantiomeric excess than the free cells, with a decrease in conversions. The maximum conversion level was obtained with immobilized cells of *R. rubra*, which provided 99.3% of conversion, but only 31.9% ee. *K. marxianus* is considered as the best biocatalyst after immobilization, with 84.2% ee of (*S*)-CHBE at moderate conversion level (77.5%) after 24 h.

Table 2
Microbiological reduction of CAAE to *S*-CHBE with immobilized cells

Microorganism	Conversion (%)	ee (%)
S. cerevisiae 40	41.7	86.2
Pichia sp.	75.4	78.1
Hansenula sp.	51.9	62.8
Candida sp.	48.8	48.6
K. marxianus	77.5	84.2
R. rubra	99.3	31.9

Incubation: 30 °C, 150 rpm, 24 h.

In addition to the good enantiomeric excess obtained with *K. marxianus*, product recovery was much easier after immobilization than the product obtained when free cells were used. Hence, a 2^{5-2} fractional factorial design was used to enhance conversion levels and enantioselectivity of the reaction with this strain.[16,25] Five variables were studied: concentrations of CAAE, $MgCl_2$, glucose, biomass (Cell), and volume of sodium alginate solution (V). Three central points replicates were accomplished. Experimental domain and results are shown in Table 3. Response variables were % conversion and % ee after 18 h-incubation-period. All experimental runs furnished an excess of (*S*)-CHBE.

According to Table 3, in entries 3 and 5 (when the maximum biomass concentration and minimum substrate concentration were used) 100% conversion and high enantioselectivity was obtained (95.0% ee and 94.3% ee, respectively). In both conditions, conversion and enantioselectivity were better than that obtained with free cells of *K. marxianus* (94.7% conversion and 80.8% ee Table 1). The maximum biomass/substrate ratio also furnished good results in the bio-reduction of CAAE by the fungus *Aureobasidium pullulans*.[13] The high stereoselectivity achieved in run 5 with immobilized cells of *K. marxianus* is shown in Figure 2.

It was not possible to establish a model to explain the enantiomeric excess, but the same conditions that furnished the highest

Table 3
Experimental design to improve reaction conditions of CAAE reduction to *S*-CHBE by immobilized cells of *K. marxianus*

Entries	Factors[a]					Responses (18 h)	
	V (mL)	Cell (gdw/L)	CAAE (mM)	$MgCl_2$ (g/L)	Glucose (g/L)	Conversion (%)	ee (%)
1	30	1.9	35	0	10	38.6	86.3
2	30	1.9	70	2	50	11.8	84.9
3	30	5.6	35	2	50	100	94.3
4	30	5.6	70	0	10	46.2	85.5
5	70	5.6	35	0	50	100	95.0
6	70	5.6	70	2	10	53.6	86.3
7	70	1.9	35	2	10	26.2	91.0
8	70	1.9	70	0	50	10.8	100
9	50	3.7	52.5	1	30	38.4	88.4
10	50	3.7	52.5	1	30	34.8	87.7
11	50	3.7	52.5	1	30	36.4	88.0

Incubation: 30 °C, 150 rpm, 18 h.
[a] V: volume of 1.5% w/v sodium alginate solution in 100 mL of medium + cells; Cell (biomass concentration—g dry weight/L).

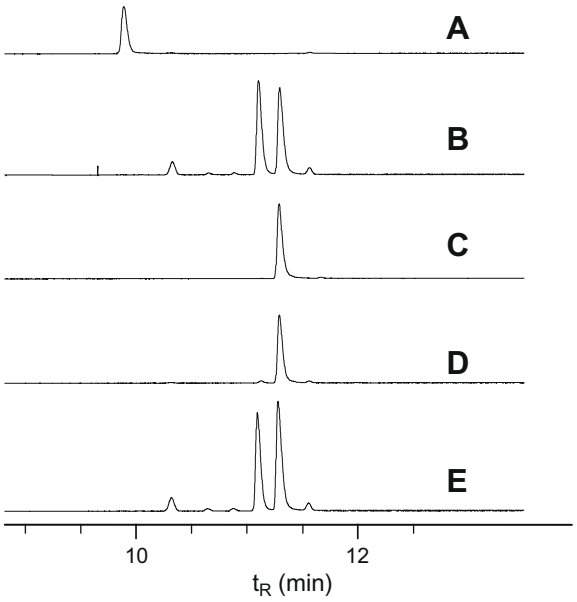

Figure 2. Chiral GC analysis on column Lipodex-E (25 m × 0.25 mm × 0.25 μm), at 70–130 °C (5 min) at 5 °C/min: (A) CAAE; (B) ethyl 4-chloro-3-hydroxybutanoate (racemate obtained via $NaBH_4$ reduction); (C) (*S*)-CHBE (Aldrich); (D) immobilized *K. marxianus* reduction (experimental design—entry 5);and (E) co-elution of (B) and (D).

Table 4
Results of regression analysis for the fractionary factorial design to response variable, % conversion (normalized variables)

Term[a]	Coefficient	*t*-Value	*p*-Value
Intercept	45.4	83.4	0.0001
V	−0.75	−1.18	0.3606
Cell	26.6	41.8	0.0006
CAAE	−17.8	−27.9	0.0013
$MgCl_2$	−0.50	−0.78	0.5151
Glucose	7.25	11.4	0.0076
V × Cell	2.60	4.08	0.0552
V × CAAE	2.35	3.69	0.0664

Coefficient of determination, $r^2 = 0.96$.
[a] V: volume of 1.5% w/v sodium alginate solution in 100 mL of medium; Cell (biomass concentration).

conversions also provided the highest stereoselectivity, except entry 8 that provided 100% ee and only 10.8% conversion. Then, only conversion was maximized in the experimental domain studied. The regression coefficients and *t*-values of terms are presented in Table 4. Analysis of variance was performed in order to validate the regression model. The fit of the model is expressed by r^2, which was calculated to be 0.96. This indicates that the model explains 96% of the variability in the data.

Biomass and glucose had a positive effects on the conversion, while the substrate showed a negative effect. The addition of the salt $MgCl_2$ did not affect conversions, as also observed by Houng et al.[8] in the same reaction but employing baker's yeast as biocatalyst. The volume of sodium alginate solution and interactions studied had no significant effect (*p*-value >0.05) in response. Based on these results, the following conditions were selected for the bioreduction of ethyl 4-chloroacetoacetate by *K. marxianus* in 100 mL of medium: volume of 1.5% sodium alginate solution, 30 mL; biomass, 5.6 gdw/L; substrate 35 mM; glucose 50 g/L.

Experiments performed in triplicate were conducted in order to verify the conversion level and enantiomeric excess in the reaction conditions described above. After 18 h, 99% conversion was obtained (SD = 1.7%) and 91% ee (SD = 2.5%) of (*S*)-CHBE. Such a result is rarely obtained with wild microorganisms in the absence of toxic enzymatic inhibitors. Optimization studies are currently in progress.

3. Conclusions

Twelve strains of yeast and filamentous fungi were used in the reduction of CAAE. Some of them provided good enantiomeric excess of (*S*)-CHBE with conversion higher than 50% when used as free cells. *M. ramannianus* provided 98.5% conversion with 83.7% ee. Despite providing good and easy recovery of the product, immobilized cells in calcium alginate spheres led to lower conversion levels in comparison with free cells used in most cases. Good enantiomeric excess was obtained with *K. marxianus* and product recovery was much easier after immobilization than when free cells were used. A fractionary factorial design to study some reaction conditions with *K. marxianus* allowed us to improve conversion level to 99% with 91% ee. Further optimization studies can improve these results.

4. Experimental

Microorganisms, media, growth conditions, and biotransformation with free cells: *Hansenula* sp., *K. marxianus*, *Candida* sp., *Pichia* sp., 3 strains of *S. cerevisiae*, *R. rubra*, *R. minuta* and filamentous fungi, *A. niger*, *T. harzianum* and *M. ramannianus*, belong to the collection of the 'Departamento de Engenharia Bioquímica, Escola de Química, UFRJ' and are freely available upon request. Cells were allowed to grow for 48 h, under 150 rpm at 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 this period, the cells were harvested by centrifugation, re-suspended in water and then used for the reaction. After centrifugation, the cells (4 g/L, dried weight) were added to the reduction's medium containing: glucose (5%), $MgCl_2$ (0.1%) in a final volume of 100 mL. After 30 min of addition of the microorganisms, the substrate (0.5%) in the aqueous-ethanol was added to the medium. 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. Products were analyzed by (chiral) gas chromatography (GC), on column Lipodex-E (25 m × 0.25 mm × 0.25 μm), at 70 °C to 130 °C (5 min) at 5 °C/min.

Immobilization of cells (S. cerevisiae 40, K. marxianus, R. rubra, Candida sp., Hansenula sp, and Pichia sp.) in calcium alginate and biotransformation: Cells grown for 48 h in the medium described before were centrifuged and re-suspended in distilled water to obtain a cell-suspension. Sodium alginate aqueous solution was added and this mixture (cell-suspension + 1.5% w/v sodium alginate aqueous solution) was dropped into $CaCl_2$ aqueous solution (0.1 M) to form calcium alginate spheres. Spheres were filtered, washed with distilled water and then added to the medium for reduction. Reduction was carried out in 500 mL cotton plugged Erlenmeyer flasks at 30 °C, 150 rpm for 24 h. After that period, medium was filtered to separate the biocatalyst and the liquid phase was treated as described above.

Experimental design: A 2^{5-2} fractionary factorial design[16] was used to study five variables in 8 runs with 3 replicates of the central point. Variables and domain were: volume of 1.5% w/v sodium alginate solution with cells of *K. marxianus* (V): 30–70 mL in 100 mL of medium; Biomass concentration (Cell): 1.9–5.6 g dry weight/L CAAE: 35–70 mM; $MgCl_2$: 0–2 g/L; Glucose: 10–50 g/L. Response variables were % conversion and % ee. Cells of *K. marxianus*, obtained as described above, was re-suspended in sodium

alginate solution according to the biomass and total volume of sodium alginate solution described in the experimental domain. After 30 min of immobilization, the substrate in aqueous-ethanol was added to the medium. The reaction was carried out in 500 mL cotton plugged Erlenmeyer flasks containing 100 mL of medium for 18 h at 30 °C and 150 rpm. After that period, medium was filtered to separate the biocatalyst and the liquid phase was treated as described above. Statistical analyses were performed using Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA).

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