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Highly enantioselective bioreduction of 4-bromoacetophenone

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ABSTRACT

Microorganisms were used to reduce 4-bromoacetophenone to (*S*)-4-bromophenylethanol and (*R*)-4-bromophenylethanol. After a fractional factorial design to identify the important variables for this reaction, *Geotrichum candidum* provided a 98.9% conversion with >99% ee of the (*R*)-isomer, while *Rhodotorula rubra* led to a 97.6% conversion with a 98.8% ee of the *S*-isomer.

1. Introduction

Biocatalysis has become an increasingly valuable tool for synthetic chemists. Bioreductions are attractive methods, mainly due to high enantioselectivity, mild and safe reaction conditions and lower environmental impact compared to conventional reactions in organic chemistry.[1–4] Chiral alcohols with additional functional groups are important intermediates in the synthesis of enantiomerically pure pharmaceuticals and other important chemicals. For example, optically active phenylethanols and their derivatives are useful building blocks for the synthesis of complex molecules because the alcohol functionality can be easily transformed, without racemization, into other useful functional groups.[5] Chiral phenylethanols [(*R*) or (*S*)] are interesting compounds with a number of potential applications, particularly in the drug industry. These alcohols are used as building blocks for the synthesis of bioactive compounds, such as pharmaceuticals, agrochemicals, and natural products.[6] The reduction of acetophenone to chiral phenylethanol has also been widely studied as a model reaction for ketone bioreductions in order to produce a specific chiral alcohol.[7] The use of microbial whole cells is advantageous because they contain the necessary co-factors (NADH and NADPH) while the metabolic pathways for their regeneration is inexpensive.[8]

Herein we report the use of 14 microorganisms (10 yeasts strains and 4 filamentous fungi strains) for the asymmetric reduction of 4-bromoacetophenone (Fig. 1). Some reaction conditions were studied by a fractional factorial design.

2. Results and discussion

2.1. Bioreduction of 4-bromoacetophenone using resting cells

At first, we tested 14 microorganisms (10 yeasts strains and 4 filamentous fungi strains) for the asymmetric reduction of 4-bromoacetophenone using whole cells after growing. The results are shown in Table 1. All microorganisms were able to reduce 4-bro-

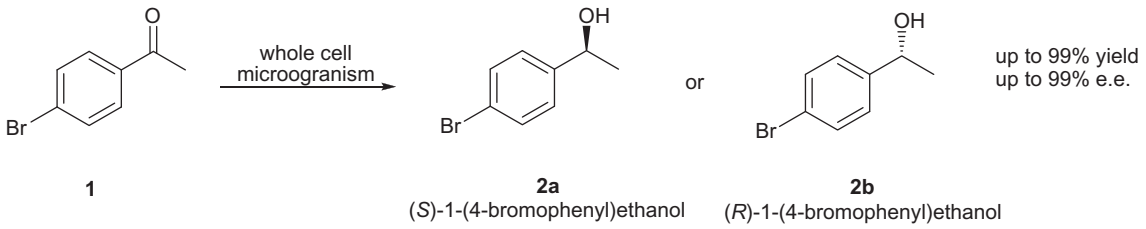

Figure 1. Reduction of 4-bromoacetophenone.

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Table 1
Bioreduction of 4-bromoacetophenone

Microorganism	Conversion[a] (%)	ee[a] (%)	Configuration
K. marxianus	13.2	51	(*R*)
Hansenula sp.	6	84	(*R*)
Pichia sp.	64.5	89.8	(*S*)
G. candidum	91.9	97.4	(*R*)
Candida sp.	59.1	53.8	(*R*)
A. niger	98.9	>99	(*R*)
M. ramannianus	>99	20	(*S*)
T. harzianum	98.5	98	(*R*)
S. cerevisiae 40	6	93.2	(*S*)
S. cerevisiae 60	3.5	>99	(*S*)
S. cerevisiae 80	0	0	—
P. chrysosporium	13.2	31.6	(*R*)
R. rubra	96.1	98.8	(*S*)
R. minuta	99.3	98.2	(*S*)

[a] Measured by GC with the chiral column BETA DEX 225.

Table 2
Monitoring of the reaction time of 4-bromomacetophenone reduction

Microorganism	Time's reaction (h)	Conversion[a] (%)	ee[a] (%)
A. niger	5	79.9	95.3
	10	91.1	98.2
	15	94.2	97.4
	20	83.4	95.6
G. candidum	5	95.7	81.2
	10	98.9	>99
	15	99.1	>99
	20	98.2	98
R. minuta	5	74.4	97.6
	10	96.8	98.4
	15	98.9	98
	20	98	98
R. rubra	5	48.9	>99
	10	94.4	>99
	15	99.2	97.4
	20	97.8	>99

[a] Measured by GC with the chiral column BETA DEX 225.

moacetophenone, but some microorganisms, such as *Kluyveromyces marxianus*, *Hansenula* sp., 3 strains of *Saccharomyces cerevisiae*, and *Phanerochaete chrysosporium* showed low conversions (between 3% and 13%). All reactions were conducted over 24 h.

Geotrichum candidum, *Aspergillus niger*, and *Trichoderma harzianum* were able to produce the (*R*)-enantiomer with excellent conversions and enantiomeric excess (ee), while *Rhodotorula rubra* and *Rhodotorula minuta* gave the (*S*)-enantiomer. Numerous approaches have been documented to improve the stereospecificity of whole-cell bioconversions. Although recombinant microorganisms have also been used with good results, wild-type strains, such as the ones used herein, are generally preferred for industrial processes mainly because of their robustness.[9] Our results are compatible with the wild-type strains reported in the literature. Kurbanoglu et al.[10] described the reduction of 4-bromoacetophenone using *A. niger*; they obtained a 100% conversion and higher than a 99% ee of the (*R*)-enantiomer, within 48 h while we obtained a 98.9% conversion and higher than a 99% ee within 24 h. In another work, Kurbanoglu et al.[5] described the reduction of the same substrate but using immobilized cells of *Rhodotorula glutinis*; they obtained the (*S*)-isomer with a 100% conversion and higher than a 99% ee. Immobilization can influence the enantiomeric excess and conversion level, as verified in previous studies with other substrates.[8,9,11] We used two species of *Rhodotorula* genus, *R. rubra* and, *R. minuta*, and obtained the (*S*)-isomer with a 99.3% conversion and a 98.2% ee without immobilization. This suggests that some approaches could enhance the conversion levels and enantiomeric excess.

2.2. Monitoring of reaction time

A. niger and *G. candidum*, which produce the (*R*)-enantiomer and *R. rubra* and *R. minuta*, which produce the (*S*)-enantiomer, were selected for monitoring the reaction time. It was possible to verify high conversion and ee within 10 h with all microorganisms tested. Thus, it was not necessary to keep the reaction for 24 h. We did not observe inversion of the configuration. Nakamura et al.[12] observed that the use of *G. candidum* and acetophenone as the substrate gave the (*S*)-isomer in the first 12 h, while a longer reaction time (40 h) led to the formation of the (*R*)-enantiomer (Table 2).

2.3. Fractional factorial design to simplify reaction conditions to obtain (*R*)-4-bromophenylethanol

Fractional factorial designs are frequently used to evaluate the reaction conditions of bioconversions and thus improve the methods, conversions, and ee[8,9,13] *G. candidum* was considered to be the best biocatalyst for obtaining (*R*)-4-bromophenylethanol, with a 98% ee at high yields (98%) after 20 h. A 2^{5-2} fractional factorial design was employed to simplify the reaction conditions while maintaining the high conversion and high enantiomeric excess. Five variables were studied: biomass (Cell), pH, and concentrations of 4-bromoacetophenone, glucose, and $MgCl_2$. Three central point replicates were accomplished. The experimental domain and results are shown in Table 3. Response variables were % conversion and % ee after 20 h-incubation-periods.

According to Table 3, all experimental runs using *G. candidum* led to an excess of (*R*)-4-bromophenylethanol. Conversions obtained after 20 h varied from 40.4% to 99.6% while enantiomeric excesses varied from 20.6% to higher than 99%. When minimum substrate concentration was used (runs 1, 2, 5, and 6), higher than 98% conversion and high enantioselectivity were obtained (>99% ee). Under these conditions, the conversions and enantioselectivities were similar to those obtained with *G. candidum* (Table 1).

A high conversion is important for a promising industrial process, but a high enantioselectivity is essential for obtaining a chiral building block. Furthermore, the conditions that furnished the highest stereoselectivities also provided the highest conversions. Hence only the enantiomeric excess was maximized in the experimental domain studied. Data were evaluated by means of analysis of variance (ANOVA) and the confidence level was set at 95% (Table 3). Only the substrate concentration was the significant parameter (p-value <0.05). Other variables were not significant parameters in the experimental domain (p >0.05) and were not considered in the model below (normalized variables):

$$\%\text{ee} = 67.9 - 31.6 \times S \quad (1)$$

where S = substrate concentration (4-bromoacetophenone).

The fit of the model is expressed by R^2, which was calculated to be 0.88. This indicates that the model explains 88% of the variability in the data. The adjusted R^2 statistic was (0.87). Analysis showed that the curvature was not important to the model (p-value = 0.89) and that the linear model obtained to describe the enantiomeric excess was suitable for the observed data.

Since the substrate concentration showed a negative effect on enantioselectivity while the other variables had no effect on the response, the conditions selected for 4-bromoacetophenone reduction to 4-bromophenylethanol by *G. candidum* were: substrate, 1 g/L; biomass, 4.0 gdw/L; glucose, 30 g/L; pH, 5.

Experiments (performed in triplicate) were conduced to verify conversion level and enantiomeric excess under the reaction conditions described as above. After 20 h, the product was obtained in a 98.9% conversion (SD = 0.5%) and greater than a 99% ee. This result matched the model prediction.

Table 3
Experimental design to simplify the reaction conditions of 4-bromoacetophenone reduction to give (*R*)-4-bromophenylethanol by *G. candidum*

Run	Factors[a]					Responses (20 h)	
	Cell (gdw/L)	S (g/L)	pH	Glucose (g/L)	$MgCl_2$ (g/L)	Conversion[b] (%)	ee[b] (%)
1	4	1	4	30	0	99.4	>99
2	4	1	6	50	1	98.7	>99
3	4	3	4	50	1	40.4	30.4
4	4	3	6	30	0	63.2	20.6
5	12	1	4	30	1	99.4	>99
6	12	1	6	50	0	99.6	>99
7	12	3	4	50	0	94.5	45.6
8	12	3	6	30	1	80	46.2
9	8	1.5	5	40	0.5	89.9	76.6
10	8	1.5	5	40	0.5	92	49
11	8	1.5	5	40	0.5	89.4	82

Incubation: 30 °C, 150 rpm, 20 h.
[a] Cell (biomass concentration–g dry weight/L); *S* (substrate–4-bromoacetophenone).
[b] Measured by GC with the chiral column BETA DEX 225.

For experimental designs, the fit of the model is considered acceptable when R^2 is greater than 0.7 for biological data and greater than or equal to 0.8 for data of chemical origin.[14]

2.4. Fractional factorial design to simplify the reaction conditions to obtain (*S*)-4-bromophenylethanol

R. rubra was chosen to obtain the other isomer, due to the high conversion of 4-bromoacetophenone to (*S*)-4-bromophenylethanol (a 96% yield with a 98.8% ee). The same variables studied in the reduction of 4-Br-acetophenone catalyzed by *G. candidum* were also investigated by a 2^{5-2} fractional factorial design in the reduction catalyzed by *R. rubra*. Three central point replicates were accomplished. The variables, experimental domain, and results are shown in Table 4. Response variables were % conversion and % ee after a 20 h-incubation-period.

According to Table 4, all experimental runs using *R. rubra* led to up to 99% ee of (*S*)-4-bromophenylethanol with conversions obtained after 20 h ranging from 26.4% to 97.4%. Only the conversion level should be maximized in the experimental domain studied. The highest conversions ($\geqslant$96.9%) were achieved in the experimental runs 5 and 6, when maximum biomass concentration and minimum substrate concentration were used. Under both conditions, conversion and enantioselectivity were similar to the results in Table 1 with the same amount of substrate and *R. rubra* biomass.

Data were evaluated by means of analysis of variance (ANOVA) and are shown in Table 4. The confidence level was set at 95%. Biomass concentration, substrate concentration, and the interaction between both variables were significant parameters (p-value <0.05). Other variables were not significant parameters in the experimental domain (p >0.05) and thus were not considered in the model below (normalized variables):

$$\%\text{Conversion} = 55.1 + 18.3 \times \text{Cell} - 16.7 \times S - 6.3 \times \text{Cell} \times S \quad (2)$$

where Cell = biomass concentration; S = substrate concentration (4-bromoacetophenone).

The coefficient of determination (R^2) was 0.99, which means that the model explains 99% of variability in response. The adjusted R^2 statistic was also high (0.98). Analysis showed that the curvature was not important to the model (p-value = 0.17) and that the linear model obtained to describe the enantiomeric excess was suitable for the observed data.

Biomass had a positive effect on conversion within the experimental domain. Quite the contrary, substrate and the interaction between biomass and substrate concentration showed negative effects. The addition of $MgCl_2$ had no effect on the reduction of 4-bromoacetophenone catalyzed by *R. rubra* and *G. candidum*; this was also observed in previous studies on the reduction of ethyl benzoylacetate by *R. rubra*[8] and ethyl 4-chloroacetoacetate by *K. marxianus*.[9] Variation of glucose concentration had no significative effect on response and it was used at a minimum level. Based on these results, the following conditions were selected for the bioreduction of 4-bromoacetophenone with *R. rubra*: biomass, 12 gdw/L; substrate, 1 g/L; glucose 30 g/L; pH 5.

Experiments were performed in triplicate in order to verify conversion level and enantiomeric excess under the reaction

Table 4
Experimental design to simplify the reaction conditions for 4-bromoacetophenone reduction to (*S*)-4-bromophenylethanol by *R. rubra*

Run	Factors[a]					Responses (20 h)	
	Cell (gdw/L)	S (g/L)	pH	Glucose (g/L)	$MgCl_2$ (g/L)	Conversion[b] (%)	ee[b] (%)
1	4	1	4	30	0	47.2	>99
2	4	1	6	50	1	48.6	>99
3	4	3	4	50	1	26.4	>99
4	4	3	6	30	0	27.9	>99
5	12	1	4	30	1	97.4	>99
6	12	1	6	50	0	96.9	>99
7	12	3	4	50	0	49.5	>99
8	12	3	6	30	1	52.8	>99
9	8	1.5	5	40	0.5	55.4	>99
10	8	1.5	5	40	0.5	51.9	>99
11	8	1.5	5	40	0.5	52.2	>99

Incubation: 30 °C, 150 rpm, 20 h.
[a] Cell (biomass concentration–g dry weight/L); *S* (substrate–4-bromoacetophenone).
[b] Measured by GC with the chiral column BETA DEX 225.

conditions described above. After 20 h, the product was obtained in a 97.6% conversion (SD = 0.3%) and a 98.8% ee (SD = 0.2%). These results matched the prediction model.

3. Conclusions

A screening study of some wild fungi strains has been carried out with different enantioselectivities in the reduction of 4-bromoacetophenone being obtained. The yeast *G. candidum* highlighted in the production of (*R*)-4-bromophenylethanol and *R. rubra* were selected for obtaining the other isomer, (*S*)-4-bromophenylethanol. In the reduction catalyzed by *G. candidum*, the reaction conditions selected by an experimental design were simpler than those used in the initial studies and led to excellent conversions (98.9%) and enantioselectivities (>99% ee) for the (*R*)-isomer. Biomass, glucose, $MgCl_2$, and pH were not significant parameters, which are a good characteristic for industrial processes, while allowing some variation of the parameters during process without affecting the enantiomeric excess. In the 4-bromoacetophenone reduction with *R. rubra*, the reaction conditions selected by the experimental design led to a 97.6% conversion and a 98.8% ee for the (*S*)-4-bromophenylethanol isomer. Further optimization studies could improve these results.

4. Experimental

Microorganisms, media, growth conditions, and biotransformation. Hansenula sp., *K. marxianus*, *Candida* sp., Pichia sp., *G. candidum*, 3 strains of *S. cerevisiae*, *R. rubra*, *R. minuta* and filamentous fungi, *A. niger*, *T. harzianum*, *Mucor ramannianus*, and *P. chrysosporium*, belong to the collection of the 'Departamento de Engenharia Bioquímica, Escola de Química, UFRJ' (Cidade Universitária, CT Bloco E, Rio de Janeiro, Brazil, e-mail selma@eq.ufrj.br) and are freely available upon request. Cells were allowed to grow 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, re-suspended in water, and used for the reaction. After centrifugation, the cells (12 g/L, dried weight) were added to the reduction's medium containing: glucose (5%), $MgCl_2$ (0.1%) in a final volume of 50 mL. After 30 min of addition of the microorganisms, the substrate (50 mg diluted in 1 mL of ethanol 96%) 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 the column Beta DEX 225 (30 m × 0.25 mm × 0.25 μm), at 130 °C (35 min). We confirmed the configuration by using the (R)-isomer purchased from Aldrich.

In order to study the reaction conditions on the reduction of 4-bromoacetophenone by *G. candidum* and *R. rubra*, $2^{5,6}$ fractional factorial designs[13] were used to evaluate five variables in 8 runs with 3 replicates of the central point. Variables and domain were: Biomass concentration (Cell): 4–12 g dry weight/L; substrate (4-bromoacetophenone): 1–3 g/L; pH, 4–6; glucose: 30–50 g/L; $MgCl_2$: 0–1 g/L. Response variables were: % conversion and % ee Reactions were carried out in 500 mL cotton plugged Erlenmeyer flasks containing 50 mL of medium for 20 h at 30 °C and 150 rpm. After that period, the medium was treated as described above. Statistical analyses were performed using Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA).

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