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Asymmetric reduction of (4*R*)-(−)-carvone catalyzed by Baker's yeast in aqueous mono- and biphasic systems

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ABSTRACT

(1*R*,4*R*)-dihydrocarvone (**2**), an important renewable building block, was prepared with good conversions and excellent diastereoisomeric excess through the reduction of the α,β-unsatured carbon–carbon double bond of (4*R*)-(−)-carvone (**1**) mediated by Baker's yeast (BY) in aqueous mono- and biphasic systems. Some parameters that may alter this bioreduction reaction, such as the concentrations of yeast and substrate, temperature, and pH, were evaluated. The effect of the addition of different additives on the course of **1** biotransformation was also investigated. The results showed that the conversion and diastereoisomeric excesses were strongly dependent on these variables. The optimum reaction conditions were 100 g L^{-1} of BY, 16.6 mM of substrate, and pH 7.5 at 26 °C in the presence of DMSO, trehalose, or sucrose as additives. Under the optimum conditions, the (1*R*,4*R*)-dihydrocarvone was recovered with diastereoisomeric excesses of 92–99% and with conversions of 70–74%.

1. Introduction

Chemical transformation using biocatalysts represents an effective and sometimes preferable alternative to the standard synthesis of fine chemicals and optically active compounds [1–4]. The use of biocatalysts is an advantageous alternative, primarily because of their high selectivity (chemo-, regio-, and stereo-selectivity), act under mild conditions and are environmentally acceptable [5–7]. Isolated enzymes and whole cells can be used as biocatalysts in asymmetric synthesis. The use of isolated enzymes frequently leads to higher enantiomeric excess while avoiding problems associated with competing catalysts of different stereoselectivity. Unfortunately, reduced cofactors must be regenerated *in situ* in a second catalytic cycle or provided in stoichiometric amounts to sustain the catalytic activity. Thus, the use of whole living cells is attractive since they do not require the addition of exogenous cofactors because of their own cofactor regeneration systems [3,7–9]. *Saccharomyces cerevisiae* (Baker's yeast) is perhaps the most well-known whole-cell biocatalyst in the scientific and industrial world and is certainly the yeast most commonly used by organic chemists to mediate the enantioselective reduction of ketones, β-ketoesters, imines, and α,β-unsaturated systems with C=C activated by strongly polarizing groups, such as nitro, carbonyl, or hydroxyl groups [10–16].

The microbial and enzymatic biotransformation of widely available monoterpenoids, such as carvone, menthol, citronellol, and geraniol, into derivatives with added value has long been of interest to researchers because of their economical potential for the perfume, food, and pharmaceutical industries [17,18]. Carvone (5-isopropenyl-2-methyl-2-cyclohexenone), a monocyclic monoterpene ketone, is produced by over 70 different plants. It is found basically in two stereoisomeric forms: (4*R*)-(−)-carvone, which is the main component in the essential oil of spearmint (*Mentha spicata*); and (4*S*)-(+)-carvone, which is the main component in the essential oils of caraway (*Carum carvi*) and dill (*Anethum graveolens*). Both enantiomers can be used in the asymmetric synthesis of natural products [19]. The stereoselective reduction of (4*R*)-(−)-carvone and (1*R*,4*R*)-dihydrocarvone has been previously carried out using various cell types including bacteria, fungi, yeast, plant cell cultures, and marine microalgae [17–21]. Data available in the literature indicate that the microbiological reduction of these compounds in many cases gives a mixture of saturated ketone, saturated alcohol, and more rarely the allylic alcohol, indicating that several enzymes may catalyze the reduction of C=C and C=O double bonds competitively [17–19,21]. For example, Shimoda and Hirata [22] studied the reduction of (4*S*)-(+) and (4*R*)-(−)-carvone by cultured cells of *Astasia longa*. In the latter case, the cells reduced the C=C double bond adjacent to the carbonyl group and then the carbonyl group to give (1*R*,4*R*)-dihydrocarvone and (1*R*,2*S*,4*R*)-dihydrocarveol (**4**) with degrees of conversion of ∼0–60% and ∼0–38%, respectively. In the case of (4*S*)-(+)-carvone, both (1*R*,4*S*)-dihydrocarvone and (1*R*,2*S*,4*S*)-dihydrocarveol were obtained with

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conversions of ~0–62% and ~0–25%, respectively. On the other hand, when the reduction of (4*R*)-(−)- and (4*S*)-(+)-carvone by two enone reductases (reductase I and II) isolated from cells of *A. longa* was investigated, (1*R*,4*R*)- and (1*R*,4*S*)-dihydrocarvones (**3**) were obtained as single products.

Several additives can be used in reactions mediated by purified enzymes or microorganisms to improve the activity and also to increase the conversion and selectivity. While some alcohols or sugars (e.g. glucose) can act as electron donors for recycling the cofactors through metabolism [23,24], the disaccharide trehalose can act as a protector for the cell membrane and other structures [25,26]. Ionic liquids and co-solvents have the potential to improve the activity by increasing the dissolution of poorly soluble substrates or products [27,28].

In this study, commercially available Baker's yeast (BY) was used as a biocatalyst in the reduction of (4*R*)-(−)-carvone in aqueous monophasic or aqueous/organic biphasic systems to obtain (1*R*,4*R*)-dihydrocarvone as a pure diastereoisomer. This compound can be used as a precursor for the synthesis of some molecules of biological interest, such as (−)-thujopsene [29], 10α-acetoxy-1αH,7αH-guaia-4,11-dien-3-one [30], and (+)-decipienin [31]. The operational conditions for the reduction of **1**, such as concentrations of BY and substrate, temperature and pH values, and the addition of different additives, were optimized under univariate approach in order to improve both the conversion and the diastereoisomeric excess values of **2**.

2. Materials and methods

2.1. Yeast

Dry Baker's yeast (BY) was purchased from Fleischmann Ltd. and was stored in a refrigerator at 2–8 °C.

2.2. Chemicals

The following chemicals were used as received. (4*R*)-(−)-carvone (98%) and (−)-dihydrocarveol [*n*-, ~75%, *iso*-, ~6%, *neo*-, ~3%, *neoiso*-, ~13%] were purchased from Sigma–Aldrich; potassium phosphate buffer from Vetec; citric acid from Acros; sodium hydroxide from Grupo Química; metallic zinc from Riedel-De Haen AG; and deuterium chloroform (99.8%) from Cambridge Isotope Laboratories. The ionic liquids, 1-butyl-3-methyl imidazolium hexafluorphosphate [BMIm][PF_6] (≥96%), 1-butyl-3-methyl imidazolium tetrafluoroborate [BMIm][BF_4] (≥97%), and 1-butyl-3-methylpyridinium tetrafluoroborate [BMPy][BF_4] (≥96%) were purchased from Fluka. All organic solvents were obtained from commercial sources and were of analytical grade.

2.3. Preparation of dihydrocarvones

Dihydrocarvones were prepared from (4*R*)-(−)-carvone through reduction with metallic zinc and ethanolic potassium hydroxide as described in the literature [32], and were used as standard compounds in the chiral-GC and GC–MS analyses.

The dihydrocarvones were obtained as a mixture of diastereoisomers of (1*S*,4*R*)-**3** and (1*R*,4*R*)-**2** in a 1:4.6 ratio, with 30% yield after purification in a chromatographic column on silica gel using a mixture of *n*-hexane and ethyl acetate (9:1, v/v) as the eluent. GC–MS t_R: 8.2 min (1*R*,4*R*)- and 8.4 min (1*S*,4*R*)-isomer; m/z (relative intensity): 152 $[M]^+$ (26), 123 (6), 109 (46), 95 (80), 67 (100), and 41 (49). Chiral-CG t_R: 6.4 for diastereoisomers (1*S*,4*R*)- and 6.5 min for diastereoisomer (1*R*,4*R*)- ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.03 (d, 3H, J = 6.4 Hz, CH_3), 1.10 (d, 3H, J = 6.4 Hz, CH_3), 1.73 (s, 3H, =CCH_3), 1.74 (s, 3H, =CCH_3), 1.3–2.5 (m, 16H) 4.69–4.83 (m, 4H, C=CH_2).

2.4. General procedure for the biotransformation of (4*R*)-(−)-carvone

The (4*R*)-(−)-carvone (0.08 mL, 0.5 mmol) and BY (3.0 g) were added to an Erlenmeyer flask (125 mL) containing potassium phosphate/citric acid buffer solution (30 mL) adjusted to the desired pH. The reaction mixture was incubated at 22–40 °C with constant orbital stirring. Aliquots were withdrawn at specified time intervals from the reaction mixture, extracted with dichloromethane (2 × 10 mL). The crude product was subjected to chiral-GC analysis and the conversion and diastereoisomeric excess were determined. Retention time (minutes) for these products in the chiral-GC analysis were as follows: (1*S*,4*R*)-dihydrocarvone (6.4), (1*R*,4*R*)-dihydrocarvone (6.5), (1*R*,2*S*,4*R*)-dihydrocarveol (7.9), (1*R*,2*R*,4*R*)-dihydrocarveol (8.8), and (1*S*,2*R*,4*R*)-dihydrocarveol (9.1). The final products were identified by comparison of chiral-GC, GC–MS, and ^{1}H NMR spectra with the standard compounds prepared and characterized as previously described and also with commercially pure samples.

The conversions to products were calculated by comparison of the peak areas of the substrate and products. The sum of the two areas was considered as 100%, and thus the percentage of diastereoisomeric excess (% *d.e.*) of (1*R*,4*R*)-dihydrocarvone was calculated as described in Eq. (1).

$$\%\,d.e. = \frac{(1R,\,4R)\text{isomer} - (1R,\,4S)\text{isomer}}{(1R,\,4R)\text{isomer} + (1R,\,4S)\text{isomer}} \times 100 \quad (1)$$

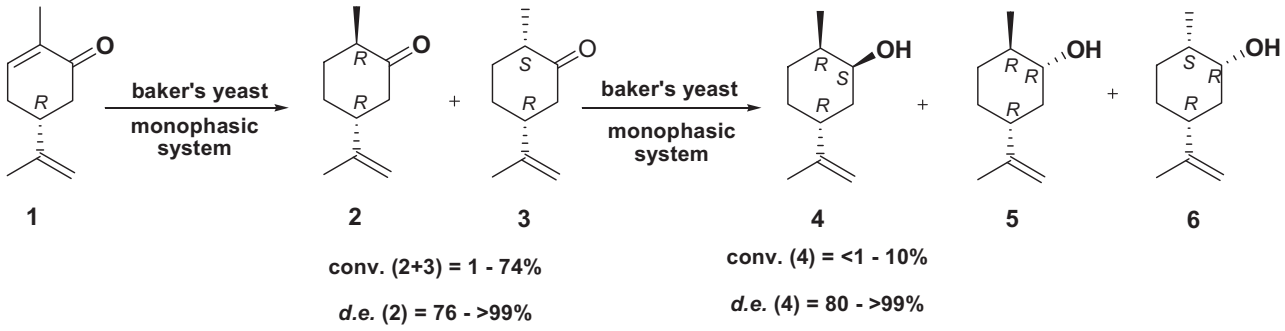
The main parameters which influence on bioreduction reactions mediated by BY are well reported in the literature [14,24], and thus the studies herein presented were carried out using an univariate approach.

2.5. Analytical methods

The (4*R*)-(−)-carvone reduction reactions were monitored by thin-layer chromatography (TLC) using *n*-hexane:ethyl acetate (9:1, v/v) as the eluent, and gas chromatograph (GC-14B Shimadzu) equipped with a chiral column (RT-BetaDEX-SM, 30 m × 0.32 mm × 0.25 μm, Restek) and H_2 was used as the carrier gas with a pressure of 75 kPa. The temperatures of the injector and detector were 230 °C. A column was set to temperature ramps of 90–109 °C (10 °C/min), 109–116 °C (1 °C/min), and 116–220 °C (10 °C/min). The substrate and products were characterized by gas chromatography–mass spectrometry (GC–MS) (Shimadzu GC-MS 2010 Plus), equipped with a fused silica capillary column Rtx-5MS (5% diphenyl–95% dimethylpolysiloxane 30 m × 0.25 mm × 0.25 μm, Restek), where helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature program and the injector temperature were the same as previously described for the GC except for the temperature of the injector and detector which was 280 °C. The quadrupole mass detector was operated at 260 °C in the electron impact mode at 70 eV. The ion source temperature was set at 200 °C, and the transfer line was set at 250 °C. The mass acquisition range was 40–400 m/z. The peaks were identified on the basis of their fragmentation patterns using the NIST Mass Spectral Search Program 05 (NIST, Washington, D.C.). The substrate and products were also characterized by proton nuclear magnetic resonance (^{1}H NMR) (Varian Ac 400F-400MHz).

3. Results and discussion

The biotransformation of the α,β-unsaturated ketone (4*R*)-(−)-carvone catalyzed by commercially available Baker's yeast (BY) in aqueous media was investigated. BY reduces **1** at the endocyclic double bond to give the corresponding (1*R*,4*R*)-dihydrocarvone (**2**) and (1*S*,4*R*)-dihydrocarvone (**3**) as the major and minor products,



Scheme 1. Biotransformation of (*R*)-carvone catalyzed by Baker's yeast.

respectively. The preferential formation of these products indicated that the reduction of (4*R*)-(−)-carvone occurred stereospecifically resulting in the products with the configuration of *R* at C-1 and *R* at C-6. These observations showed that the hydrogen attack at the conjugated C=C double bond occurred stereospecifically from the *si*-face at C-1 and *re*-face at C-6 by anti-addition. The hydrogen atoms participating in the reduction at C-1 and C-6 originated from the medium and the *pro*-4*R* hydrogen of NADH, respectively [22]. The formation of (1*R*,2*S*,4*R*)-dihydrocarveol (**4**) in the biotransformation of **1** mediated by BY was also observed, whereas only traces of (1*R*,2*R*,4*R*)- and (1*S*,2*R*,4*R*)-dihydrocarveol (**5** and **6**, respectively) were sometimes observed (Scheme 1). No product derived from the reduction of the methylethenyl group was observed. Similar reductive transformation of (*R*)-(−)-carvone was reported by Carballeira et al. [33] using *Gongronella butleiri* under growth conditions, and by Hirata et al. [34] in the presence of cultured cells of *Nicotiana tabacum* in aqueous media.

After the characterization by ^{1}H NMR, chiral-GC, and GC–MS of the products obtained in the reduction of **1**, and in order to improve the conversion and diastereoisomeric excess of (1*R*,4*R*)-dihydrocarvone, some other reaction parameters were evaluated, such as yeast and substrate concentration, temperature, pH, and the addition of co-additive. Although a multivariate optimization approach is useful to minimize the number of experiments and/or verify possible correlations between two or more sets of variables, as discussed below we decided to use an univariate approach since most of the parameters which influence the bioreduction reactions mediated by BY are well reported in the literature [14,24].

3.1. Influence of reaction medium

Firstly, the reduction of (4*R*)-(−)-carvone to the corresponding dihydrocarvones in an aqueous/organic biphasic system and aqueous monophasic system at 35 °C and pH 5.5 was investigated. The diastereoisomeric excess (*d.e.*) in (1*R*,4*R*)-dihydrocarvone and the conversions to dihydrocarvones **2** and **3** obtained after 48 h of reaction are presented in Fig. 1.

As can be observed in Fig. 1, when the aqueous monophasic system was used in the reduction of **1** the conversions to dihydrocarvones **2** and **3** were higher than those obtained in the aqueous/organic biphasic system in 48 h of reaction (59% and 22%, respectively). This was probably due to the increased mass transfer limitation and lowered substrate concentration in the case of the biphasic system, or due to damage to cell membranes caused by the organic solvent leading to denaturation of the enzyme [35]. When the reaction was performed in a biphasic system, the diastereoisomeric excess values of **2** were higher (99%) than those obtained for the reaction carried out in the aqueous monophasic system (72%). When the latter system was used in the reduction of **1**, (1*R*,2*S*,4*R*)-dihydrocarveol also was obtained but with lower conversions (3–8% with *d.e.* >99% in 48 h reaction).

Thus, considering that the conversions to dihydrocarvones **2** and **3** were higher when the reaction was performed in aqueous monophasic system, this was chosen for the subsequent experiments to improve the diastereoisomeric excess of **2**.

3.2. Influence of yeast concentration

The optimum yeast concentration is an important experimental parameter in terms of shortening the reaction time and also increasing the product yield, making the process more attractive. Thus, the effect of yeast concentration (0–200 g L^{-1}) on the asymmetric reduction of (4*R*)-(−)-carvone catalyzed by BY was studied in order to determine the minimum yeast concentration required to obtain the (1*R*,4*R*)-dihydrocarvone in high conversions and diastereoisomeric excess.

As observed in Fig. 2, no spontaneous conversion of (4*R*)-(−)-carvone to products was detected in the absence of biocatalyst. In 24 h of reaction, the conversions to **2** and **3** changed from 37 to 53% when the yeast concentration was increased from 66.7 to 100 g L^{-1}, but a small decrease in the *d.e.* values for **2** (from 91 to 89%) was also observed. At higher concentrations (>100 g L^{-1}) there was no significant variation in the conversions to dihydrocarvones (56–57%) or in the diastereoisomeric excess (93–94%). However, as the yeast concentration increased, an increase in the conversion to (1*R*,2*S*,4*R*)-dihydrocarveol **4** from 6 to 36% was observed. In this study the formation of dihydrocarveols **5** and **6** was detected with conversions of 2 and 3%, respectively (see Scheme 1).

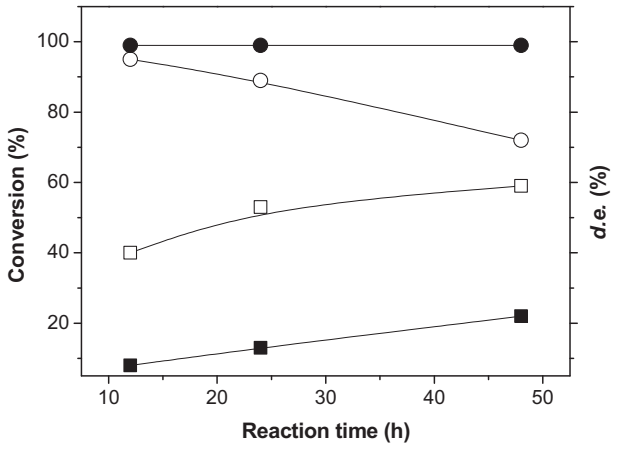


Fig. 1. Time course of asymmetric reduction of (*R*)-carvone catalyzed by BY in aqueous/organic biphasic system (full symbols) and in aqueous monophasic system (open symbols). Symbols: (■, □) conversion into dihydrocarvones **2** and **3**; (●, ○) *d.e.* of (1*R*,4*R*)-dihydrocarvone **2** [Reaction conditions: 3.0 g of BY (100 g L^{-1}), 80 mg (0.5 mmol) of substrate, in 30 mL potassium phosphate/citric acid buffer (0.2 M/0.1 M, pH 5.5) without organic solvent (monophasic system) or in the presence of 30 mL of *n*-hexane (biphasic system), 35 °C].

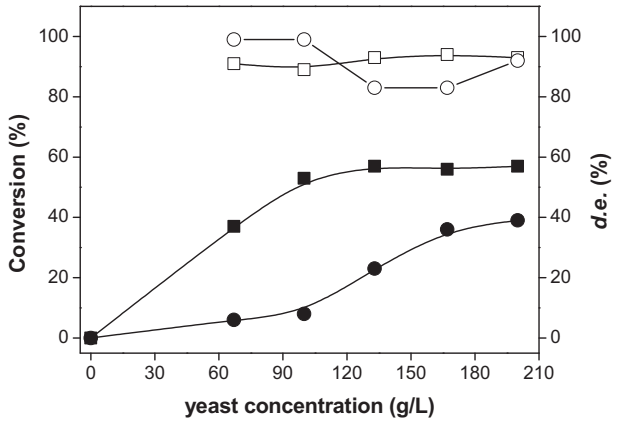


Fig. 2. Effect of the yeast concentration on the conversion into dihydrocarvones **2** and **3** (■) and dihydrocarveols **4, 5,** and **6** (●) and diastereoisomeric excess of **2** (□) and **4** (○) for the asymmetric reduction of (*R*)-carvone catalyzed by Baker's yeast in aqueous monophasic system at 24 h [Reaction conditions: BY, 80 mg (0.5 mmol) of substrate, 30 mL potassium phosphate/citric acid buffer (0.2 M/0.1 M, pH 5.5), 35 °C].

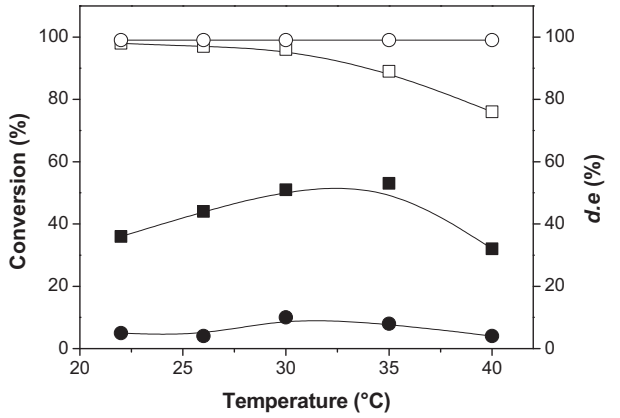


Fig. 4. Effect of the temperature on the conversion to dihydrocarvones **2** and **3** (■) and dihydrocarveol 4 (●) and on the diastereoisomeric excess of **2** (□) and **4** (○) for the asymmetric reduction of (*R*)-carvone catalyzed by Baker's yeast in the aqueous monophasic system at 24 h [Reaction conditions: 3.0 g of BY (100 g L^{-1}), 80 mg (0.5 mmol) of substrate, 30 mL potassium phosphate/citric acid buffer (0.2 M/0.1 M, pH 5.5)].

Based on these results, a yeast concentration of 100 g L^{-1} was selected for the subsequent experiments, considering that the (1*R*,4*R*)-dihydrocarvone was obtained in good conversions with high *d.e.* values.

3.3. Influence of substrate concentration

It is well reported in the literature that high concentrations of substrate may lead to the inhibition of different enzymes present in whole cells [36]. Thus, in order to evaluate the effect of substrate concentration on the bioreduction of **1** in an aqueous monophasic system, substrate concentrations ranging from 16.6 to 166.4 mM were studied.

Fig. 3 shows that, in general, the substrate concentration affects the reduction of (4*R*)-(−)-carvone. The conversions to **2** and **3** decreased from 53 to 6% when the concentration increased from 16.6 to 166.4 mM, while the conversions to **4** decreased from 8 to 0%. The initial rate for different substrate concentrations was determined. The values obtained indicated a decrease in the initial rate of reaction (from 11.7 into 7.1×10^{-3} mmol L^{-1} min^{-1}) as the substrate concentration increased to 100 mM, results that are in agreement with the conversion values (results not shown in Fig. 3). These results indicate that, in general, with an increase in the substrate concentration there was inhibition of the BY enzymes. The diastereoisomeric excess values of (1*R*,4*R*)-isomer remained practically constant in this concentration substrate range (87–91%) and the (1*R*,2*S*,4*R*)-dihydrocarveol was obtained with *d.e.* > 99%.

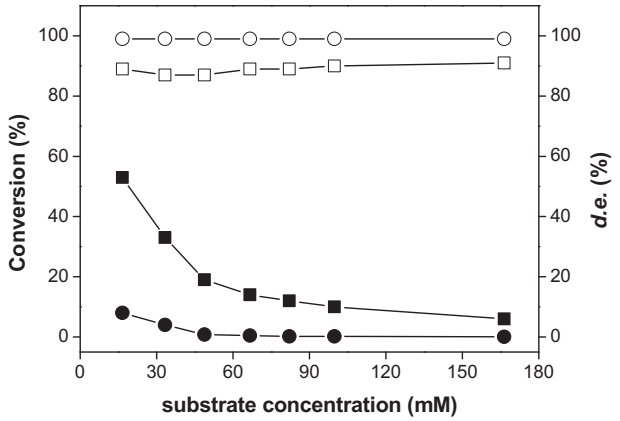


Fig. 3. Effect of the substrate concentration on the conversion into dihydrocarvones **2** and **3** (■) and dihydrocarveols **4, 5,** and **6** (●) and diastereoisomeric excess of **2** (□) and **4** (○) for the asymmetric reduction of (*R*)-carvone catalyzed by Baker's yeast in aqueous monophasic system at 24 h [Reaction conditions: 3.0 g of BY (100 g L^{-1}), 30 mL potassium phosphate/citric acid buffer (0.2 M/0.1 M, pH 5.5), 35 °C].

Zhang et al. [23] described a similar effect of substrate concentration on biocatalytic synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate with *Candida krusei* SW2026 in aqueous medium. With an increase of substrate concentration from 2.5 to 20 g L^{-1}, the yield and *e.e.* of product decreased from 95.1% to 45.8% and 99.7% to 87.5%, respectively.

In support to the use of an univariate approach, if we consider the results presented in Fig. 2 (the effect of cell concentration) with the ones presented in Fig. 3 (the effect of substrate concentration), the effect on the conversions for these two variables can be directly related to a single one, the relation between substrate and enzyme concentration. For example, for similar conversions of 36% (Fig. 2) to 37% (Fig. 3), the substrate/enzyme ratio is 0.28–0.30, while for conversions of 50% (both figures) the substrate/enzyme ratio is 0.18–0.20, indicating that using the univariate or the multivariate tool, the results would be practically the same. Thus, regardless the fixed substrate concentration used to obtain the results presented in Fig. 2, the optimal enzyme concentration was the one that leads to an optimal substrate/enzyme ratio, and the same conclusion can be applied to the results presented in Fig. 3 with a fixed cell concentration, showing that the conversions are not really related to total substrate or enzyme concentration, but to the ratio between them. Thus, considering the above results, 16.6 mM of substrate was used in the subsequent experiments.

3.4. Effect of temperature

In general, the reaction temperature has a strong influence on enzymatic reactions because higher temperatures may accelerate the molecular collisions between the enzyme and substrate, but inactivation of the enzyme can also occur at higher temperatures [37]. We used reaction temperatures from 22 to 40 °C to evaluate the influence of temperature on the reduction of **1** catalyzed by BY in an aqueous monophasic system.

Fig. 4 shows that both the conversions to products and the diastereoisomeric excess were dependent on this parameter. When the temperature increased from 22 to 35 °C the conversion to dihydrocarvones increased. The maximum conversion to **2** and **3** was 53% at 35 °C. However, a small decrease in the *d.e.* value for **2** was observed (98–89%) when the temperature increased. Above 35 °C

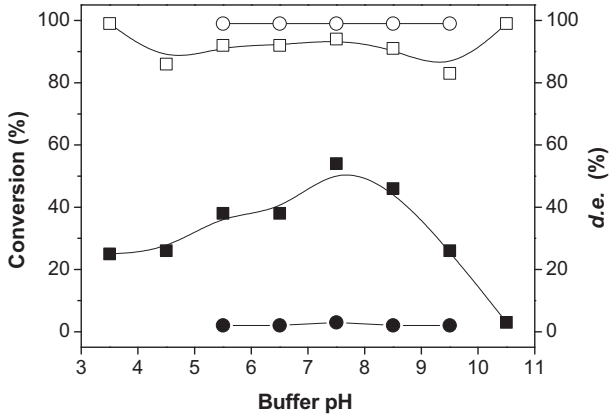


Fig. 5. Effect of the buffer pH on the conversion to dihydrocarvones **2** and **3** (■) and dihydrocarveol 4 (●) and on the diastereoisomeric excess of **2** (□) and **4** (○) for the asymmetric reduction of (*R*)-carvone catalyzed by Baker's yeast in the aqueous monophasic system at 24 h [Reaction conditions: 3.0 g (100 g L^{-1}) of BY, 80 mg (0.5 mmol) of substrate, 30 mL buffer, 26 °C].

the conversion to **2** and **3** and the *d.e.* value for **2** decreased, these being of 32 and 76%, respectively. These results indicate a possible inactivation of the enzymes present in BY caused by the increase in the reaction temperature. (1*R*,2*S*,4*R*)-dihydrocarveol was obtained with conversions of **4** to 10% and *d.e.* ≥ 99% within the temperature range analyzed.

Similar results were obtained by Hu et al. [24] in the reduction of 2-hydroxy-1-phenylethanone using *S. cerevisiae* JUC15 in aqueous media with glucose as co-substrate. The highest yield in (*R*)-phenyl-1,2-ethanediol (80.5%) was obtained at 30 °C, and a further increase in the temperature up to 40 °C led to a 6.3% decrease in the yield of product. The *e.e.* of product remained above 99.9% within the temperature range analyzed.

Thus, a temperature of 26 °C was selected to further evaluate the effect of pH and the addition of co-additives on the reduction of this substrate.

3.5. Effect of pH

Another important parameter which must be evaluated in biocatalyzed reactions is the pH of the reaction medium, because a variation in this value may alter the ionic state of the substrate and the enzymes involved in the reactions, particularly those catalyzed by enzymes for which the enantioselectivity varies with pH, leading to changes in the yields and in *e.e.* or *d.e.* values. Therefore, pH is among the most significant factors affecting enzyme-catalyzed reactions [38,39]. Thus, the effect of pH on the asymmetric reduction of (4*R*)-(−)-carvone mediated by BY was also investigated. In this study, five different buffers with various pH values at a concentration of 0.1 mol L^{-1}, that is, citrate (pH 3.5), acetate (pH 4.5–5.5), potassium phosphate (pH 6.5–7.5), borate (pH 8.5), and carbonate (pH 9.5–10.5) were used.

Fig. 5 shows that when the pH buffer increased from 3.5 to 7.5, the conversions to dihydrocarvones **2** and **3** increased from 25 to 54%, and the *d.e.* values for (1*R*,4*R*)-dihydrocarvone ranged from 86 to 99%. A further increase in the pH buffer from 7.5 to 10.5 caused a decrease in the conversions to **2** and **3** (from 54 to 3%), but the *d.e.* values remained in the range of 83 to >99%. The dihydrocarveol **4** was obtained with low conversions (2–3%); however, the *d.e.* values were ≥99%. From these results, it is clear that pH 7.5 is the optimal value for the reduction of **1**.

Chen et al. [39] studied the influence of pH (pH 5.5–8.5) on the asymmetric reduction of ethyl 4-chloro-3-oxobutyrate catalyzed by Baker's yeast in aqueous media. A strong influence of pH on the activity was observed, and the highest yields (91.5%) were also obtained at pH 7.5.

3.6. Effect of the addition of additives

It is well known that different additives can significantly influence the conversion and the selectivity of BY-mediated reactions [40]. Thus, after optimizing some experimental parameters, a series of additives, including polar and nonpolar solvents, ionic liquids, and a 10% solution of sucrose or trehalose, were evaluated in the asymmetric reduction of (*R*)-(−)-carvone catalyzed by BY in an aqueous monophasic system.

In Table 1 it can be observed that the conversions to products were strongly influenced by the addition of additives. When using polar and non-polar solvents as additives (CH_2Cl_2, DMF, THF, acetonitrile, *t*-butanol, and *n*-hexane; Table 1, entries 2–7) no improvement in the conversions to dihydrocarvones were observed (1–39% with *d.e.* values of 67–99%), compared with the values obtained in the absence of an additive (54% with *d.e.* of 94%; Table 1, entry 1). However, better results for the conversions and/or *d.e.* values were obtained when glycerol (53% with *d.e.* of 97%), ethanol (57% with *d.e.* of 91%), and the sulfur compounds DMSO (70% with *d.e.* of 97%) and L-cysteine (63% with *d.e.* of >99%) were used (Table 1, entries 8–11).

It has been extensively reported in the literature that sulfur compounds, such as L-cysteine and DMSO, have the ability to improve the selectivity and reactivity of BY reductions because these compounds contain a mercapto group which can act as a hydride source to regenerate NAD(P)H, and may also interact with the active site of the enzyme present in BY [41,42]. Recently, Podea et al. [43] investigated the influence of L-cysteine as an additive in the synthesis of (*R*)- and (*S*)-heteroaryl-ethane-1,2-diols mediated by BY. These authors found that the presence of this additive in the cell suspension raised the enantiopurity of the isolated products. For example, the *e.e.* values for (*S*)-1-(benzo[*b*]thiophen-2-yl)ethane-1,2-diol increased from 88% to 96% in the presence of L-cysteine.

Another class of additives which has been widely used in biocatalysis is the ionic liquids (ILs), due to the fact that the presence of ILs in the appropriate concentration can boost the activity and enantioselectivity of enzymes [27,28,44]. In this study, the results showed that when the reduction of **1** with BY was performed using [BMIm][BF_4] and [BMPy][BF_4], both the conversions (22–36%) and *d.e.* values for **2** and **3** (83–86%) were lower than those obtained in aqueous monophasic systems (Table 1, entries 1 and 12–13). However, when [BMIm][PF_6] was used as an additive, the (1*R*,4*R*)-dihydrocarvone was obtained as the only product with a conversion and *d.e.* value of 56% and >99%, respectively (Table 1, entry 14). This result showed that [BMIm][PF_6] had a positive influence on the asymmetric reduction of (*R*)-(−)-carvone catalyzed by BY.

Bräutigam et al. [45] obtained similar results in the asymmetric reduction of 4-chloroacetophenone in biphasic ionic liquid/water-systems using recombinant *Escherichia coli*. These authors observed that the enantiomeric excess of (*R*)-1-(4-chlorophenyl)-ethanol for the reactions with ionic liquids was ≥99.5% in all cases, which represents an improvement when compared to the aqueous system (*e.e.* 96%).

It is well documented in the literature that the non-reducing disaccharide trehalose protects cellular structures against destabilization due to stress or denaturing agents [44,45]. On the other hand, many sugars and alcohols can be used as co-substrates to supply redox equivalents for biosynthetic process [31,33]. Thus, in order to observe the effect of sugars, sucrose and trehalose were added to the asymmetric reduction of (*R*)-(−)-carvone mediated by BY. When a solution of sucrose or trehalose was used good conversions to dihydrocarvones **2** and **3** were obtained (71 and 74%, respectively) (Table 1, entries 15 and 16). However, lower *d.e.*

Table 1
Effect of additives on the reduction of **1** using Baker's yeast.[a]

Entry	Additives	Conversion (**2**+**3**) (%)[b]	*d.e.* (**2**) (%) [b]	Conversion (**4**) (%)[b]	*d.e.* (**4**) (%) [b]
1	None	54	94	3	99
2	CH_2Cl_2	1	99	0.3	99
3	DMF	2	99	–	–
4	THF	4	67	–	–
5	Acetonitrile	23	81	–	–
6	*t*-Butanol	25	75	0.4	99
7	*n*-Hexane	39	99	–	–
8	Glycerol	53	97	8	99
9	Ethanol	57	91	2	99
10	DMSO	70	97	6	99
11	L-cysteine	64	99	–	–
12	[BMPy][BF_4]	22	83	–	–
13	[BMIm][BF_4]	36	86	–	–
14	[BMIm][PF_6]	56	99	–	–
15	Sucrose	71	92	7	99
16	Trehalose	74	99	8	99

[a] Reaction conditions: substrate (80 mg, 16.6 mM), water (27 mL), additives (3 mL) (10%), dry Baker's yeast (3.0 g, 100 g L^{-1}), reaction time 4.0 h, 30 °C.
[b] The conversion and *d.e.* values were determined by chiral-GC using a Restek column (RT-BetaDEX-SM, 30 m × 0.32 mm × 0.25 μm).

values were obtained for **2** when the sucrose solution was used (92%), compared with the solution of trehalose (99%). Thus, the results indicated that trehalose was a good additive in the reduction of **1** in aqueous monophasic system, where good conversions to **2** and **3** and a high *d.e.* value for **2** were obtained.

Albuquerque et al. [26] studied the enantioselective bioreduction of ethyl acetoacetate to *n*-hexane by a haploid laboratory *S. cerevisiae* strain grown under different conditions with the addition of sucrose or trehalose solutions. The best conversions (∼50%) to the chiral alcohol ethyl (S)-(+)-3-hydroxybutanoate (*e.e.* > 99%) were obtained when trehalose was added to the reaction medium using the cells grown with glycerol–ethanol. When sucrose was added to the reaction medium, using cells grown in glucose, the best conversion to the product was found to be ∼15% with *e.e.* values ranging from 30 to 50%. These results indicated that trehalose may be an efficient protector for immobilized or free yeast cells during enantioselective reductions in organic solvent, especially since this sugar was not consumed by the cells, while sucrose acts as an electron donor for the cofactor recycling because the sugar was consumed by the yeasts.

It is important to note that in this study the formation of **4** was also observed when some additives were used. This compound was obtained with a conversion of 0.3–8% and *d.e.* >99%.

4. Conclusions

In summary, the bioreduction of (*R*)-carvone catalyzed by Baker's yeast in an aqueous monophasic system is an efficient way to prepare (1*R*,4*R*)-dihydrocarvone with good conversion and excellent *d.e.* values. The results were dependent on some experimental parameters, such as the concentration of yeasts and substrate, temperature and pH as well as the addition of different additives. The highest conversions and *d.e.* values for (1*R*,4*R*)-dihydrocarvone were achieved when L-cysteine (64% conversion; *d.e.* 99%), trehalose (74% conversion; *d.e.* 99%), [BMIm][PF_6] (56% conversion; *d.e.* 99%), and DMSO (70% conversion; *d.e.* 97%) were used as additives.

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