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Este trabalho descreve a biorredução quimiosseletiva da (1E,4E)-1,5-bis(4-metoxifenil)-1,4-pentadien-3-ona (1) mediada por fermento de pão (BY, células de *Saccharomyces cerevisiae*) em sistema bifásico contendo água e n-hexano. A biotransformação deste composto foi quimiosseletiva, formando apenas a correspondente cetona saturada 1,5-bis(4-metoxifenil)-3-pentanona (2). Foi verificada a influência de vários fatores reacionais que podem afetar a biorredução de 1 tais como, tipo e porcentagem volumétrica do co-solvente, uso de seis diferentes leveduras S. cerevisiae (quatro comerciais e duas industriais), variação da concentração de substrato e levedura, temperatura, pH e volume das fases aquosa e orgânica. A melhor condição reacional foi obtida com 66,7 g L $^{-1}$ de BY da marca Fleischmann, $8,3 \times 10^{-3}$ mol L $^{-1}$ de substrato, pH de 6,5 a 35 °C na presença de 2,5% (v/v) de N,N-dimetilsulfoxido (DMSO) como aditivo e razão V_{aq}/V_{org} de 70/30. Nestas condições o produto 2 foi obtido com conversão de 82% em 5 h de reação.

This work describes the chemoselective bioreduction of (1E,4E)-1,5-bis(4-methoxyphenyl)-1,4-pentadien-3-one (1) mediated by baker's yeast (BY, *Saccharomyces cerevisiae* cells) in an aqueous/organic solvent biphasic system. The biotransformation of this compound was chemoselective and formed only the corresponding saturated ketone 1,5-bis(4-methoxyphenyl)-3-pentanone (2). The influence of various factors which may alter the bioreduction of 1, such as the type and percentage of co-solvents, use of six different *S. cerevisiae* yeast samples (four commercial and two industrial), variations in the substrate and yeast concentrations, temperature, pH and volume of aqueous and organic phases, was investigated. The best reaction conditions were 66.7 g L⁻¹ of Fleischmann BY, 8.3×10^{-3} mol L⁻¹ of substrate, pH 6.5 at 35 °C in the presence of 2.5% (v/v) of *N*,*N*-dimethyl sulfoxide (DMSO) as an additive and a V_{aq}/V_{org} ratio of 70/30. Under these conditions, the product 2 was recovered in conversions of 82% in 5 h reaction.

Keywords: Saccharomyces cerevisiae, α,β -unsaturated carbonyl compounds, bioreduction, biphasic system

Introduction

The use of whole cells or isolated enzymes in organic chemistry to mediate the 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 is the subject of extensive study. ¹⁻⁶ It is well known that biotransformation of an α , β -unsaturated carbonyl compound in many cases gives a mixture of saturated ketone or aldehyde, saturated alcohol or allylic alcohol, indicating that several enzymes may catalyze the reduction of C=C and C=O double bond competitively. However many studies on the enantioselective reduction

of enones by biocatalysts have revealed that several enone reductases can be used for the production of chiral ketones.^{7,8}

Isolated enzymes frequently lead to higher enantiomeric excesses (e.e.) since they avoid 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. 9-11 As an example, *Rhodotorula* sp AS2.2241 has been used as a biocatalyst for the asymmetric reduction of 4'-methoxyacetophenone to enantiopure (S)-1-(4-methoxyphenyl)ethanol, and

several water-immiscible ionic liquids (ILs) have been employed as green solvents. Also, biphasic wholecell biotransformations have been carried out for the asymmetric reduction of 4-chloroacetophenone to (R)-1-(4-chlorophenyl)ethanol and of 2-octanone to (R)-2-octanol. The evaluation of different hydrophobic ionic liquids has also been carried out. 10

Among the various possible biocatalysts, baker's veast (S. cerevisiae) is perhaps the most well-known whole-cell biocatalyst in the scientific and industrial world and is certainly the most commonly used yeast by organic chemists to mediate enantioselective reductions due to its high chemo-, regio- and enantioselectivity, high bioavailability, and ease of treatment and because it acts under mild reaction conditions. Microbial reactions are usually carried out in aqueous media where the cells are most active. Recently, the kinetics of the biotransformation of geraniol into citronellol by resting cells of S. cerevisiae (baker's yeast type II) was investigated in a continuousclosed-gas-loop bioreactor (CCGLB).¹² Unfortunately, many organic compounds of commercial interest which can potentially be transformed by enzymes or microorganisms are poorly soluble in this medium and display toxic effects on the biocatalyst. Thus, aqueous/organic solvent biphasic systems have been developed to overcome these limitations. These biphasic systems consist of an aqueous phase and a water immiscible organic phase where the microbial cells stay in the aqueous phase while the substrate and product(s) remain mainly in the organic phase. 13-14 The biphasic system offers numerous advantages, such as increased solubility of hydrophobic substrates, smaller reaction volumes, increased volumetric productivity and minimization of substrate/product inhibition of the biocatalyst. Enzyme inhibition/inactivation by the solvents is minimized due to their low concentration in the aqueous phase, where the reaction takes place, and the recovery of the product from the reaction medium is facilitated. 11,15 Modifying the reaction medium through the addition of water-miscible organic co-solvents is a method commonly used to improve the activity, selectivity and the stability of enzymatic reactions. 16-18 These co-solvents have the potential to increase the activity by increasing the dissolution of poorly soluble substrates; however, in some cases they may inhibit the enzyme by changing its conformation through denaturation.13,19

This paper reports a study on the baker's yeast-catalyzed bioreduction of an α,β -unsaturated carbonyl compound (1*E*,4*E*)-1,5-bis(4-methoxyphenyl)-1,4-pentadien-3-one. Firstly, the addition of various co-solvents to the buffer/*n*-hexane biphasic systems was investigated to find the most appropriate organic solvent to solubilize the substrate.

Different strategies to improve the conversion into product, varying the substrate and yeast concentrations, temperature, pH, and volume of aqueous and organic phases, were also considered.

Experimental

Yeasts

Six *S. cerevisiae* yeast strains were used in their active dry yeast form. Four samples were commercial baker's yeast (BY), named Fleischmann (AB Brasil Industria e Comércio de Alimentos LTDA, Brazil), Dona Benta (Pak Gida Uretim ve Pazarlam A. S., Turkey), Nordeste (Moinho do Nordeste S.A., Brazil) and Brugguemann (Algist Bruggeman NV, Belgium), and two were industrial yeast strains (CAT-1 and PE-2) which are currently used for ethanol production in Brazil.^{20,21} These two industrial fuel ethanol yeasts have been commercially available since the late 1990s, and are distributed by LNF Latino Americana Ltda. from Brazil.²² The pellets of dehydrated yeast cells were used directly in the reduction reactions as described below.

Materials

The following chemicals were used as received: potassium phosphate buffer purchased from Vetec; citric acid from Acros; sodium hydroxide from Grupo Química; acetone (99.5%) from Merck; 4-methoxybenzaldehyde (98%) and silica gel (70-230 mesh) from Sigma Aldrich; sodium borohydride (98%) from Merck; and deuterium chloroform (99.8%) from Cambridge Isotope Laboratories. All organic solvents were obtained from commercial sources and were of analytical grade.

General procedure for the bioreduction reactions employing yeasts

In a typical experiment, a mixture of 30 mL of n-hexane containing 290 mg of 1, 4 g of BY and 30 mL potassium phosphate/citric acid buffer was incubated at 35 °C in 250 mL Erlenmeyer flasks with magnetic stirring. Aliquots were periodically withdrawn from the aqueous and organic phases and extracted with dichloromethane (2 × 10 mL). The organic phase was combined, dried with MgSO₄ and the solvent was removed under vacuum. The residue was analyzed by GC to evaluate the percentage of conversion to product. Substrate and product peak areas were compared, and the sum of the two was considered as 100%. After the bioreduction of 1 with BY, the product 2 was isolated and characterized by 1H NMR and GC.

Details regarding the co-solvents, co-solvent percentage (%), substrate and yeast concentrations, buffer pH, temperature, and volumetric percentage of organic phase are specified for each assay in the results and discussion section.

Analytical methods

Proton nuclear magnetic resonance (¹H NMR) 400 MHz was carried out on a Varian 400 Mercury Plus spectrometer using CDCl₃ as the solvent and tetramethyl silane (TMS) as the internal standard. Infrared spectra (IR) were acquired with an ABB Boomer FTLA 2000-100 spectrometer using KBr pellets (range 4000-400 cm⁻¹) at the Center of Analysis, Department of Chemistry, UFSC (Florianópolis-SC, Brazil). Gas chromatography (GC) analysis was carried out with a Agilent 78202A GC-instrument equipped with an FID detector, using hydrogen as the carrier gas (19,84 psi), equipped with a Shimadzu CBP5 column $(25m \times 0.22 \text{ mm} \times 0.25 \text{ mm})$. The injector and detector temperatures were 280 and 290 °C, respectively, and the column temperature was 250 °C. The retention times observed for **1**, **2** and **3** were 12.5, 4.1 and 5.1 min, respectively. In the thin-layer chromatography (TLC) n-hexane:ethyl acetate (8:2 v/v) was used as the eluent.

Results and Discussion

In the first step of this study, (1E,4E)-1,5-bis(4methoxyphenyl)-1,4-pentadien-3-one (1) was synthesized by the aldol condensation reaction between the 4-methoxybenzaldehyde and acetone under basic conditions. This compound was used as a substrate in the bioreduction reaction using S. cerevisiae cells in aqueous/ organic solvent biphasic systems formed with n-hexane and potassium phosphate/citric acid buffer. The selection of *n*-hexane as the organic solvent was based on previous results obtained in the biohydrogenation of (2E)-1,3diaryl-2-propen-1-ones catalyzed by S. cerevisiae yeast.²³ The biocatalytic reduction of 1 by BY was completely chemoselective, producing only the corresponding saturated ketone 1,5-bis(4-methoxyphenyl)-3-pentanone (2) (Scheme 1). The alcohol (1E,4E)-1,5-bis(4-metoxyphenyl)-1,4-pentadien-3-ol (3) was not detected in any experiments,

and this finding was confirmed by comparison of the retention times (R_t) obtained from the analysis of 1, 2 and 3 by gas chromatography (GC), which were 12.5, 4.1 and 5.1 min, respectively. The structure of compound 2, obtained in the bioreduction reaction, was confirmed by IR and 1H NMR spectral analysis, as described in the experimental section.

After the spectroscopic identification of the product obtained in the bioreduction of **1** with *S. cerevisiae* yeast in the buffer/*n*-hexane biphasic system, the influence of various factors which may influence this reaction was investigated, as shown in the following sections.

Effect of addition of co-solvents and DMSO percentage

Since the substrate is not totally soluble in the media used to study the bioreduction of 1 catalyzed by BY in buffer/*n*-hexane biphasic systems, the influence of the addition of several co-solvents in the reaction medium, such as dichloromethane, ethanol, ethyl acetate, DMSO and *N*,*N*-dimethylformamide (DMF), was evaluated. The conversions to 2, as a function of the co-solvents added and the reaction time, are presented in Table 1.

Table 1. Effect of co-solvent on the bioreduction of **1** catalyzed by baker's yeast in buffer/*n*-hexane biphasic system^a

F4	Co-solvent (Log P)	Conversion ^b to 2 / %		
Entry		5 h	10 h	24 h
1	Dichloromethane (0.93)	nd ^c	nd ^c	nd ^c
2	Ethyl acetate (0.68)	10	14	14
3	Ethanol (-0.24)	29	29	39
4	DMF (-1.00)	nd^c	nd^{c}	3
5	DMSO (-1.30)	42	90	90

^aReaction conditions: dry Fleischmann BY (4 g), substrate (290 mg), 0.2 mol L¹ potassium phosphate/0.1 mol L¹ citric acid buffer (pH 5.5) (30 mL), *n*-hexane (24.6 mL), co-solvent (5.4 mL, equivalent to 9 % v/v), 35 °C. ^bThe conversion values were determined by GC. ^cnot detected.

As observed in Table 1, when ethyl acetate and ethanol were used as co-solvents the conversions were between 10-39% and were influenced by the reaction time (Table 1, entries 2 and 3). On using dichloromethane or DMF no product was detected in 10 h of reaction, and with the use

Scheme 1. Biotransformation of (1E,4E)-1,5-bis(4-methoxyphenyl)-1,4-pentadien-3-one catalyzed by S. cerevisiae yeasts in a biphasic system.

of DMF the conversion was only < 5% in 24 h of reaction (Table 1, entries 1 and 4). Better results were achieved with DMSO as the co-solvent, presenting conversions of 42, 90 and 90% in 5, 10 and 24 h of reaction, respectively (Table 1, entry 5). However, no direct relationship with the organic solvent polarity (expressed by Log P values) was observed.

These results indicate that DMSO was the most suitable co-solvent for the biosynthesis of **2** in a buffer/*n*-hexane biphasic system. Thus, the effect of varying the percentage of this co-solvent from 2.5% to 15% (v/v) was examined in 5 h of reaction. Higher percentages of DMSO were not feasible due to an increase in the reaction viscosity and experimental problems in the reaction work up, related to the reactant and product isolation. With the use of DMSO percentages of 2.5, 5, 9 and 15% the conversions were 38, 47, 42 and 38%, respectively, showing no significant changes as different quantities of DMSO were added to the reaction medium.

In several studies reported in the literature it was found that the addition of DMSO to the reaction medium leads to an increase in the conversion into products, as well as in the selectivity of reaction.²⁴⁻²⁶ In addition, the solvent toxicity must be considered and DMSO is well recognized as being non-toxic toward certain cells and can be used as a cryoprotectant, depending on its final concentration.²⁷⁻²⁹ For example, Wang et al.26 reported that the reduction of acenaphthenequinone using baker's yeast in a monophasic system provides an improvement in the conversion to the mono-alcohol 2-hydroxyacenaphthenones with the addition of 10% (v/v) DMF and DMSO as co-solvents, with conversions increasing from 72% to 97% and 98%, respectively. Thus, considering the above results, 2.5% (v/v) DMSO was used as the solvent in the subsequent experiments.

Screening of yeasts

In the next study, six different *S. cerevisiae* strains, four commercial (Fleischmann, Nordeste, Dona Benta and Brugguemann) and two industrial (CAT-1 and PE-2) were screened in order to identify which yeast strain was the most efficient in the bioreduction of (1*E*,4*E*)-1,5-bis(4-methoxyphenyl)-1,4-pentadien-3-one in a biphasic system (Table 2).

As observed in Table 2, the conversion to product was strongly influenced by the use of different BY and reaction times. When the reaction was carried out for 5 h, no significant differences in the conversions were observed using the commercial yeasts named Fleischmann, Nordeste, Dona Benta and Brugguemann, the values obtained being 38, 28, 31 and 34%, respectively.

Table 2. Screening of yeast strains for the bioreduction of 1 in buffer/*n*-hexane biphasic system with addition of DMSO

Entry	S. cerevisiae strain –	Conversion ^b to 2 / %		
		5 h	24 h	
1	Fleischmann	38	94	
2	Nordeste	28	66	
3	Dona Benta	31	68	
4	Brugguemann	34	80	
5	CAT-1	19	27	
6	PE-2	5	7	

^aReaction conditions: dry yeast (4 g), substrate (290 mg), 0.2 mol L⁻¹ potassium phosphate/0.1 mol L⁻¹ citric acid buffer (pH 5.5) (30 mL), n-hexane (28.5 mL), 2.5% (v/v) DMSO (1.5 mL), 35 °C. ^bThe conversion values were determined by GC.

However, when the bioreduction was carried with 24 h of reaction, the commercial BY from Fleischmann was the most efficient biocatalyst and the product **2** was obtained in a conversion of 94%. Low conversions to **2** (5 to 27%) were obtained when the industrial yeast samples CAT-1 and PE-2 were employed in 5 and 24 h of reaction. These were unexpected results considering that when these yeasts were used in the biohydrogenation of chalcones the conversions were similar to those obtained with BY from Fleischmann.²³ Based on these data, the commercial BY from Fleischmann was selected as the biocatalyst for the subsequent experiments.

Effect of BY concentration

An 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 efficient. Thus, the effect of yeast concentrations from 0 to 100.0 g L⁻¹ on the bioreduction of 1 catalyzed by Fleischmann BY was evaluated in order to determine the minimum yeast concentration required to obtain the 1,5-bis(4-methoxyphenyl)-3-pentanone with high conversions. The results are presented in Figure 1. The conversions increased linearly with an increase in the yeast concentration and that the highest conversion to 2 (61%) was obtained when the yeast concentration was 100.0 g L⁻¹. Although higher conversions into product could be obtained using more biomass, when the cell concentration was higher than 66.7 g L⁻¹ the mixture became too thick to be effectively stirred well and the reaction medium was not homogeneous. Thus, BY concentrations higher than 100.0 g L⁻¹ were not used, and a yeast concentration of 66.7 g L⁻¹ was employed in the subsequent experiments.

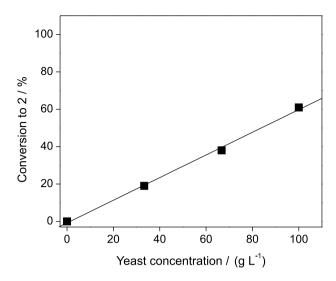


Figure 1. Effect of the yeast concentration on the bioreduction of 1 catalyzed by baker's yeast in water/n-hexane biphasic system with addiction of DMSO. Reaction conditions: BY (0-6 g), 1 (290 mg), 30 mL of citrate/phosphate/borate buffer (0.1 mol L⁻¹, pH 5.5), 28.5 mL n-hexane, 1.5 mL DMSO (2.5% v/v), 35 °C, 5 h, magnetic stirring.

Effect of substrate concentration

It is extensively reported in the literature that a high substrate concentration may lead to a decrease in the product formation owing to the possible inhibitory effects on the cells. 30,31 Thus, in order to verify this effect in the bioreduction of 1, a substrate concentration range of 8.3 to 41.7 \times 10⁻³ mol L⁻¹ was assayed. The conversion values obtained in 5 h of reaction are given in Figure 1. The conversion to 2 decreases from 69 to 21% with an increase in the substrate concentration from 8.3 to 41.7×10^{-3} mol L⁻¹. Similar results were obtained by Wang et al.9 in the asymmetric reduction of 4'-methoxyacetophenone catalyzed by immobilized Rhodotorula sp. AS2.2241 cells in a C₄MIM·PF₆/buffer biphasic system. They observed a decrease in the maximum substrate conversion when the substrate concentration was above 40×10^{-3} mol L⁻¹, probably due to the inhibition of the reaction caused by the product. In this study the optimal substrate concentration was 8.3×10^{-3} mol L⁻¹ and this value was chosen for the subsequent experiments to investigate the effects of the temperature, pH and volume ratio of aqueous to organic phase.

Effect of reaction temperature

The influence of temperature on bioreduction of 1 employing BY was also investigated. Earlier reports have described that the enzyme activity and stability, as well as the stereoselectivity, can be affected by the reaction temperature. ^{32,33} As observed in Figure 3, the conversions into 2 after 5 h of reaction increased as the temperature

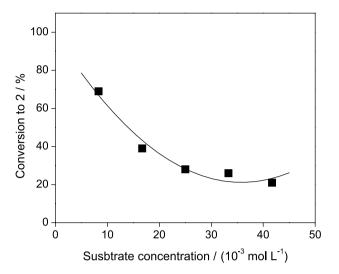


Figure 2. Effect of the substrate concentration on the bioreduction of 1 catalyzed by baker's yeast in water/*n*-hexane biphasic system with addiction of DMSO. Reaction conditions: BY (4 g), 1 (140-720 mg), 30 mL of citrate/phosphate/borate buffer (0.1 mol L⁻¹, pH 5.5), 28.5 mL *n*-hexane, 1.5 mL DMSO (2.5% v/v), 35 °C, 5 h, magnetic stirring.

increased from 20 °C to 40 °C, but when the temperature was further increased from 40 °C to 45 °C, a small decrease in the conversion was observed (from 89% to 82%). These results are interesting because they show that BY enzymes responsible for the reduction of 1 maintained they catalytic activity even at high temperatures. Although the highest conversion was achieved at 40 °C, the temperature of 35 °C (with a conversion of 65%) was chosen to evaluate the effect of the buffer pH and the volume ratio of aqueous to organic phase (V_{aq}/V_{org}). At lower temperatures, the organic solvent evaporation is minimized, important to keep the initial total

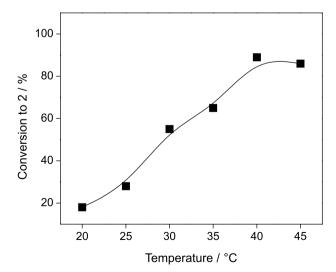


Figure 3. Effect of reaction temperature on the bioreduction of **1**catalyzed by baker's yeast in water/*n*-hexane biphasic system with addition of DMSO. Reaction conditions: BY (4 g), **1** (140 mg), 30 mL of citrate/phosphate/borate buffer (0.1 mol L⁻¹, pH 5.5), 28.5 mL *n*-hexane, 1.5 mL DMSO (2.5% v/v), 20-45 °C, 5 h, magnetic stirring.

reaction volume. Meitan *et al.*³⁴ reported similar results in relation to optimal reaction temperature, in the asymmetric synthesis of (2S,5S)-2,5-hexanediol catalyzed by BY number 6 in aqueous media with the addition of glucose, where the highest yield into product was achieved at 40 °C. However, a decrease in the e.e. value from >95.7 to ca. 90% was observed at this temperature.

Effect of buffer pH

The effect of reaction medium pH on the bioreduction of (1E,4E)-1,5-bis(4-methoxyphenyl)-1,4-pentadien-3-one in a biphasic system mediated by BY was investigated for an aqueous phase pH range from 3.5 to 8.5 at 35 °C. The conversion into 2 was found to be strongly dependent on pH (Figure 4), and under the experimental conditions used, the highest conversion (74%) to product was achieved at the pH 6.5, while the decrease in the conversion was more pronounced at more acidic. These results are in agreement with data reported in the literature, where it is well documented that all enzymes have an optimal pH at which the reaction rate is maximized. Deviations in pH from the optimal value can lead to decreased activity due to changes in the ionization of amino acid residues at the active site of the enzymes, while larger deviations in pH lead to denaturation of the enzyme protein itself. 35,36

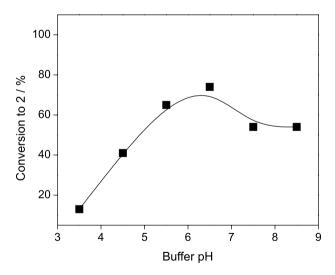


Figure 4. Effect of buffer pH on the bioreduction of 1catalyzed by baker's yeast in water/n-hexane biphasic system with addition of DMSO. Reaction conditions: BY (4 g), 1 (140 mg), 30 mL of citrate/phosphate/borate buffer (0.1 mol L¹, pH 3.5-8.5), 28.5 mL n-hexane, 1.5 mL DMSO (2.5% v/v), 35 °C, 5 h, magnetic stirring.

Effect of volume ratio of aqueous to organic phase (V_{ao}/V_{org})

The effect of V_{aq}/V_{org} on the bioreduction of **1** employing BY in a buffer/n-hexane biphasic system with addition of

2.5 % (v/v) DMSO was studied varying the organic phase volumetric percentage from 0 to 100%, maintaining a total reaction volume of 60 mL. The results are presented in Figure 5.

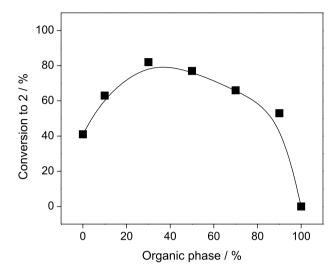


Figure 5. Effect of V_{aq}/V_{org} on the bioreduction of **1** catalyzed by baker's yeast in water/n-hexane biphasic system with addition of DMSO. Reaction conditions: BY (4 g), **1** (140 mg), 0.0-58.5 mL of citrate/phosphate/borate buffer (0.1 mol L⁻¹, pH 6.5), 0.0-58.5 mL n-hexane, 1.5 mL DMSO (2.5% v/v), 35 °C, 5 h, magnetic stirring.

As can be observed, the volume ratio of aqueous to organic phase influenced the conversion into product. The conversion to 2 increased from 41 to 82% with an increase in the organic phase up to 30%. A further increases in the organic phase led to a decline in the conversion from 82 to 0%. These results show the importance of using a biphasic system in reactions catalyzed by yeasts in order to increase the substrate solubility and reduce the possible toxic effects of organic solvents on the enzymes present in this microorganism. The highest conversion (82%) was achieved when a ratio of V_{aq}/V_{org} 70/30 (v/v) was used in 5 h of reaction. A similar result was obtained by Bie et al. 37 in the bioconversion of methyltestosterone to methandienone by Arthrobacter simplex AS.1.94 in a carbon tetrachloridephosphate buffer two-phase system with the addition of menadione, where the highest biocatalytic rate was obtained with a volumetric phase ratio of 70/30 V_{ag}/V_{org}.

Conclusions

The 1,5-bis-(4-methoxyphenyl)-3-pentanone was obtained in good conversion (82%) after the optimization of the experimental conditions using baker's yeast-mediated bioreduction of (1E,4E)-1,5-bis(4-methoxyphenyl)-1,4-pentadien-3-one in a buffer/n-hexane biphasic system with the addition of DMSO as a co-solvent. From the

synthetic point of view, the use of BY in the bioreduction of the C=C double bonds of an α , β -unsaturated carbonyl compound showed considerable advantages since the product was obtained with high chemoselectivity under mild, eco-friendly reaction conditions.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

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