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Improvement of alcoholic fermentations by simultaneous extraction and enzymatic esterification of ethanol

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Abstract

An extractive biocatalysis process consisting of a biphasic aqueous–organic system was studied for the enzymatic esterification of ethanol. Oleic acid was used simultaneously as organic extractant and substrate for the esterification reaction which was catalysed by a free and immobilized lipase from *Rhizomucor miehei*. The immobilization method used was the adsorption in a polyamide support—Accurel EP 700 [A.C. Oliveira, M.F. Rosa, J.M.S. Cabral, M.R. Aires-Barros, Bioprocess Eng. 16 (6) (1997) 349–353.]. Some relevant parameters for the lipase catalytic activity such as ethanol concentration, oleic acid concentration and pH were studied using a $2^{(3)}$ factorial design expanded further to a composite design (CCD) [G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistics for Experiments, Wiley, New York, 1985; T.B. Barker, Quality by Experimental Design, Marcel Dekker, New York, 1985]. The response surface methodology was applied to the results. This biochemical process could be applied to the extraction of ethanol from fermentation broths of high glucose concentration [A.C. Oliveira, M.F. Rosa, J.M.S. Cabral, M.R. Aires-Barros, Bioprocess Eng. 16 (6) (1997) 349–353]. In this work, the performance of extractive fermentations of 300 g/l of glucose using a flocculent *Saccharomyces cerevisiae saké* strain and a non-flocculent *Saccharomyces cerevisiae* strain were compared.

Keywords: *Rhizomucor miehei* lipase; Biphasic system; Esterification

1. Introduction

The use of aqueous–organic two-phase systems has been applied to solve many biotechnological problems such as the reduction of product toxicity in alcoholic fermentations. In this case, the fermentation process and the recovery of ethanol are integrated into a single step, overcoming the end-product inhibition effects, in a process called extractive fermentation. The choice of the organic solvent (extractant) plays an important role as it could affect both the performance of the microorganism and the efficiency of the ethanol extraction. However, as it is usually difficult to find a solvent with high affinity and biocompatibility, the association of an enzymatic reaction with the extractive fermentation improves the product extraction by

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modification of its affinity to the solvent – extractive biocatalysis.

In this work, extractive biocatalysis of ethanol from fermentation broths of high glucose concentration using a free and immobilized lipase from *Rhizomucor miehei* was evaluated in order to optimize the whole fermentation process. The performance for two different *Saccharomyces cerevisiae* strains were compared. The effect of ethanol and oleic acid concentrations, as well as the pH, on the specific activity of the free and immobilized lipase was also studied using as model the ethanol–oleic acid biphasic system.

2. Materials and methods

2.1. Enzyme

A commercial lipase from *R. miehei*, Palatase M1000L, a gift from Novo-Nordisk, Denmark, was used in the esterification reactions of ethanol with oleic acid.

2.2. Enzyme immobilization

The lipase was immobilized by adsorption in Accurel EP700, a polyamide support. The enzyme solution was added to the immobilization support (34 mg protein/g support). After being vortex-mixed for 1 min, the enzyme-support contact time was 1 h. The preparation was washed with buffer and vacuum-filtered.

2.3. Esterification reaction and lipase activity

Lipase-catalysed esterifications in the biphasic system, at 30°C and 150 rpm, were carried out in 100 ml Erlenmeyer flasks containing a total volume of 20 ml. The aqueous phase was a potassium phosphate solution (100 mM) with ethanol, and oleic acid was used as the extracting solvent ($[\text{Oleic acid}]_i = 0.2–2$ M; $[\text{Ethanol}]_i = 0.2–2$ M).

The ethanol concentration on aqueous phase was followed by gas liquid chromatography for 48 h. Specific activity was defined as the millimolar concentration of ethanol consumed per minute and milligram of protein.

2.4. Microorganism

Flocculent (saké) and non-flocculent (DER 24) yeast strains of *S. cerevisiae* were used for ethanol production.

2.5. Growth medium and culture conditions

The fermentation and growth medium used for flocculent and non-flocculent yeast strain were, in g/l: glucose (variable), yeast extract (1; 5), KH_2PO_4 (5; 5), $(NH_4)_2SO_4$ (2; 5) and $MgSO_4 \cdot 7H_2O$ (0.4; 1). The fermentation experiments were carried out at 30°C in a rotary shaker (150 rpm) inoculated with 0.1 g dry weight/l from an exponential growing culture. The pH was controlled at 4.5 and 4 when using, respectively, the non-flocculent and the flocculent strain. Glucose consumption and ethanol production were monitored.

2.6. Analytical methods

2.6.1. Ethanol determination

Ethanol concentrations in the aqueous phases were determined by gas–liquid chromatography using a Philips chromatograph with flame ionization detector ($T_{col} = 165$°C; $T_{inj} = 170$°C and $T_{det} = 250$°C), and the samples injected in a Porapack Q column.

2.6.2. Glucose determination

Glucose was determined by the dinitrosalicylic acid method [1].

3. Results and discussion

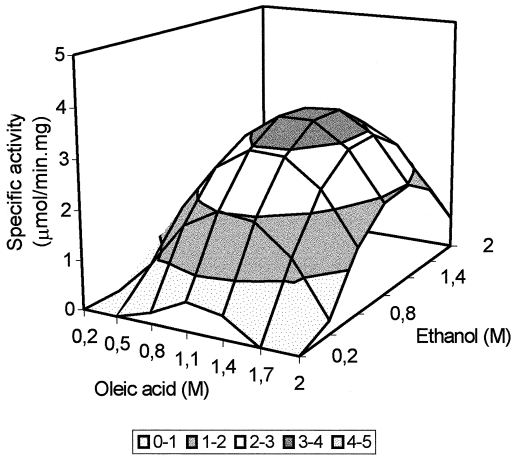
3.1. Optimization of ethanol–oleic acid esterification reaction

To optimize the esterification reaction system, the influence of pH, ethanol and oleic acid

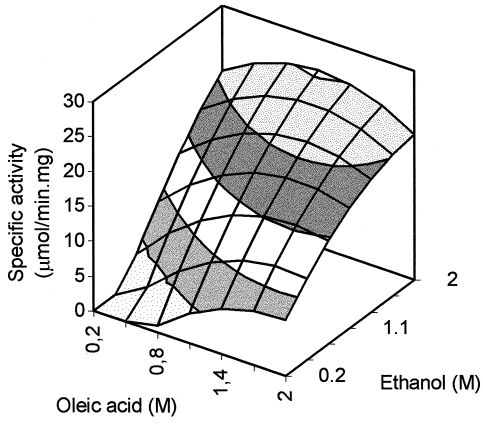
concentrations on the specific activity of free and immobilized *R. miehei* lipase was studied on a factorial design.

3.1.1. Effect of oleic acid and ethanol concentration

Oleic acid and ethanol concentrations were studied in the range 0.2–2 M. The response surface shows that in the system with free enzyme (Fig. 1A), the specific activity is dependent on the two substrates. An optimum value of 3.50 μmol/min mg for the specific activity was obtained when the oleic acid and ethanol concentration was 1.25 M. High concentrations of those two substrates have an inhibitory effect on the esterification reaction. This could be explained, with respect to ethanol, by its high solubility in the aqueous solution, which can cause enzyme inhibition or inactivation, probably due to lipase tridimensional structure modification [2]. For high concentrations of oleic acid ($>$ 1.25 M), the observed decrease of specific activity could be due to lipase inhibition originated by the acid substrate (oleic acid) and/or to mass transfer diffusional limitations.



(A)



(B)

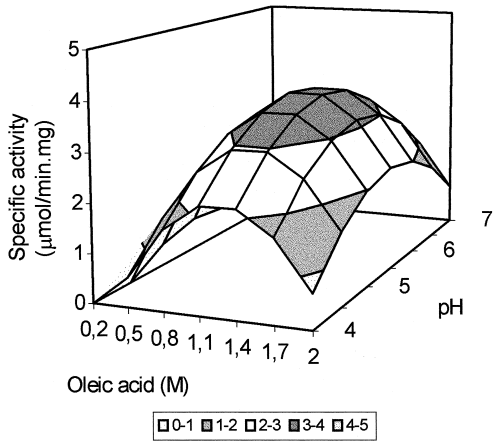
Fig. 1. Influence of oleic acid and ethanol concentrations on the specific activity of free (A) and immobilized (B) lipase at pH 5.5.

The use of the immobilized lipase in the biphasic system led to an increase of its specific activity (Fig. 1B). This could be related to the immobilization support hydrophobicity (Accurel EP700) which causes its aggregation at the aqueous–organic interface, making easy the action of the lipase, that is an interfacial enzyme, in the ethanol–oleic acid esterification reaction. In this system, the increase of the ethanol concentration proportionally enhanced the specific activity while for high oleic acid concentrations ($>$ 1.5 M), an inhibitory effect was observed.

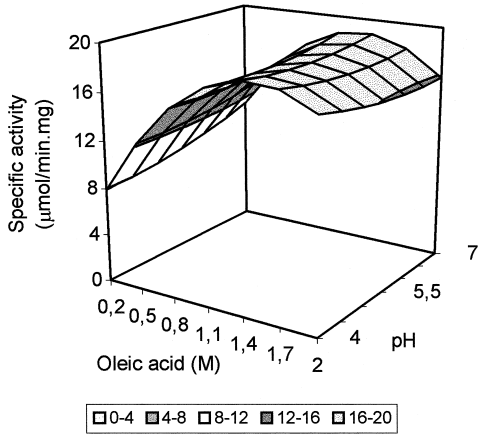
The specific activity value obtained for an ethanol concentration of 2 M and an oleic acid concentration of 1.15 M was 32.4 μmol/min mg.

3.1.2. Effect of pH

The pH effect was studied in the range 4–7. The pH value of aqueous phase affected the specific activity of free lipase (Fig. 2A), with a maximum activity of 3.70 μmol/min mg observed at pH 5.6. However, with the immobilized form (Fig. 2B), no significant differences on the specific activities values were observed with the pH for a fixed oleic acid concentration. Klibanov [3] explained this behaviour as due to the 'pH memory' of the enzyme which acquires the ionization state corresponding to the pH of the first aqueous solutions in its presence (in the present case, the pH corresponding to the immobilization process, pH = 5). Furthermore, the



(A)



(B)

Fig. 2. Influence of pH on the specific activity of free (A) and immobilized (B) lipase for an ethanol concentration of 1.1 M.

increase of the acid substrate concentration leads to an acidification of the microenvironment and, consequently, to a different ionization state of the enzyme.

3.2. Extractive fermentations

Extractive fermentations of 300 g/l of glucose by a flocculent and a non-flocculent *S. cerevisiae* strain were carried out in the presence and in the absence of a *R. miehei* lipase (Fig. 3). The oleic acid concentration was 1.58 M, and the organic phase/fermentation medium ratio was 1.

For non-flocculent strain, the presence of a free lipase in the extractive fermentation system decreased the specific uptake rate of glucose. Despite of the longer lag phase, a decrease in the ethanol level in the aqueous phase is also being observed (Fig. 3; Table 1). In those fermentation systems, the glucose was almost completely and more rapidly consumed due probably to the more effective extraction of ethanol from the aqueous phase and, consequently, to the decrease of product inhibition. The lower ethanol concentration value obtained in those cases may be explained by its esterification with oleic acid.

Comparing the results (Table 1), it can also be seen that for the non-flocculent strain (DER24), a better productivity and a similar

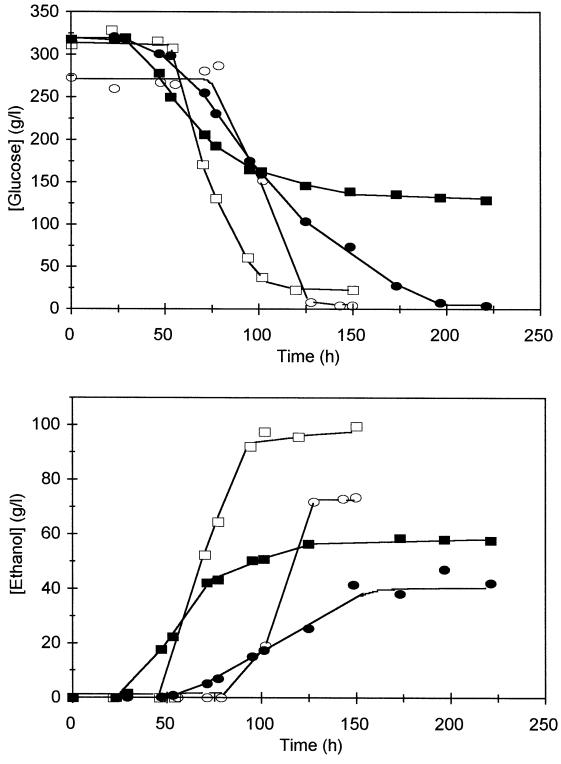


Fig. 3. Extractive fermentation of 300 g/l of glucose by the flocculent (■, ●) and the non-flocculent (□, ○) *Saccharomyces cerevisiae* strains, in the presence (●, ○) and in the absence (■, □) of a *Rhizomucor miehei* lipase.

Table 1
Extractive fermentation parameters obtained with different yeast strains and with or without enzymatic esterification

	Without enzyme		With enzyme	
	DER24	Saké	DER24	Saké
Residual glucose (g/l)	23	134	4.30	4.46
Ethanol in aqueous phase (g/l)	97.7	58.3	73.4	47.3
Ethanol in organic phase (g/l)	16.6	9.91	n.d.	n.d.
Total ethanol (g/l)	114.3	68.2	n.d.	n.d.
Ethanol yield (g/g)	0.396	0.411	n.d.	n.d.
Productivity (g/l h)	0.957	0.459	n.d.	n.d.
Specific uptake rate of glucose (h-1)	0.040	0.011	0.027	0.016

ethanol yield were obtained. This fact could be due to different inhibitory effects or different secondary products production by the two *S. cerevisiae* strains.

Experiments have also been done using the *R. miehei* lipase in the immobilized form instead of the free one. Similar extractive fermentation performances were observed in both cases [4].

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