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Production of 2-phenylacetic acid and phenylacetaldehyde by oxidation of 2-phenylethanol with free immobilized cells of *Acetobacter aceti*

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Abstract

Acetobacter aceti MIM 2000/28 was employed for the oxidation of 2-phenyl-1-ethanol. Oxidation in aqueous systems gave phenylacetic acid with high yields, while the use of a two-liquid phase system (composed of water and isooctane) allowed for the production of the corresponding aldehyde. Free cells showed poor operational stability and were immobilized in calcium alginate; immobilized cells had specific activity, substrate tolerance and stability higher than that obtained with free cells. Simple fed-batch operation with immobilized cells in an air-lift reactor allowed for the production of 23 g l^{-1} phenylacetic acid in 9 days without foam formation.

Keywords: Acetobacter aceti; Immobilized cells; 2-Phenyl-l-ethanol; Phenylacetic acid; Phenylacetaldehyde; Flavours

1. Introduction

Acetic acid bacteria have an incomplete oxidative metabolism, being able to oxidize primary alcohols to the corresponding carboxylic acids, while aldehydes are normally not accumulated [1–3]. Production of aldehydes may be obtained by using strains with low aldehyde dehydrogenase activity [4–6] or two-liquid phase systems where the more hydrophobic aldehyde is removed before its further oxidation [7,8]. Strains belonging to the genus *Acetobacter* proved very efficient in the bio-oxidation of primary alcohols, showing high conversions and selectivity [9,10]. Recently a newly isolated *Acetobacter aceti* MIM 2000/28 has been employed for the enantioselective oxidation of 2-phenyl-l-propanol [11].

Biotransformations can be employed on a preparative scale providing that the biocatalyst has good stability under the operational conditions; the immobilization of the microorganisms can strongly enhance the stability of the enzymatic activities and simplifies biocatalyst recovery. Despite interest in applications of *Acetobacter* strains, their immobilization has been rarely studied [12–14].

In the present paper we describe the performance of *Acetobacter aceti* MIM 2000/28 for the biotransformation of 2-phenyl-1-ethanol in aqueous and two-liquid phase systems and its immobilization by entrapment in calcium alginate gel beads. The products of the biotransformation (phenylacetaldehyde and phenylacetic acid) are valuable components of flavours and their production by biocatalysis starting from the naturally occurring 2-phenylethanol is of interest in the flavour industry [15].

2. Materials and methods

2.1. Microorganism and culture conditions

Acetobacter aceti 2000/28 MIM was routinely maintained on GYC slants (glucose 50 g l^{-1}, yeast extract 10 g l^{-1}, $CaCO_3$ 30 g l^{-1}, agar 15 g l^{-1}, pH 6.3) at 28 °C. The strain, grown on GYC slants for 24 h at 28 °C, was inoculated into 500-ml Erlenmeyer flasks containing 50 ml of liquid

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medium GLY (glycerol 25 g l^{-1}, yeast extract 10 g l^{-1}, pH 5, distilled water) and incubated on a reciprocal shaker (100 spm). Dry weights were determined by centrifugation of 100 ml of submerged cultures, washing of the cells with distilled water and dried at 110 °C for 24 h.

2.2. Immobilization procedures

Cells obtained by centrifugation (4000×*g* for 15 min at 4 °C) of the culture broth were suspended in different amounts (10–40 ml) of sodium alginate (Sigma Chemicals) dissolved in distilled water at different concentrations (0.5–2.5% w/w). The resulting homogeneous mixture was degassed and extruded through a thin injection needle into 50 ml of an iced calcium chloride solution (0.1 M). The bead size had an average diameter in the range of 2.5–2.8 mm. After hardening for 3 h at 4 °C, the beads were filtered under vacuum and either used for biotransformation or stored at 4 °C in fresh calcium chloride (0.01 M) solution. The amount of biocatalyst in the beads was estimated by dissolving 10 g of beads in 2% sodium polyphosphate solution; the suspension was then filtered through a pre-dried weighed filter paper (Whatman No. 1) and dried at 110 °C to a constant weight.

2.3. Biotransformation conditions

Biotransformations with freely suspended cells were accomplished using the bacterium grown directly in Erlenmeyer flasks or used after centrifugation for 10 min at 6000 rpm and re-suspension in different aqueous solution Immobilized cells (10 g of wet beads) were suspended in 50 ml of different butters. In experiments with two-liquid phase systems, solvents were added to reach a 1:1 volume ratio. Substrate was directly added into suspensions.

Biotransformations were also performed in a stirred tank reactor (STR) and in an air-lift reactor (ALR), The STR was a Chemap FZ 3000 fermentor having a working volume of 1 l. The bioreactor was maintained at 28 °C under constant stirring (150 or 300 rpm) and aeration (1.5 or 2.0 vvm); no antifoaming agent was used in experiments with immobilized cells. The ALR had 1 l of working volume and has been described before [9]. The air was supplied through a porous sparger after sterilization through a 0.2 μm microfiltration membrane; the air flow rate was 1.5 or 2.0 vvm. The control of pH was performed by continuous addition of aqueous NaOH via a multichannel Watson-Marlow 503 U/R peristaltic pump connected to a pH controller (pH/ORP Controller 3675, Jenco Electronics).

2.4. Partition experiments

The partitioning of 2-phenylethanol, phenylacetaldehyde and phenylacetic acid between alginate beads and aqueous carbonate solution were measured after mixing the alginate beads and aqueous solution for 3 h in the presence of each compound. The concentration of each compound in the beads was analyzed after resolving the beads in 5% (w/v) sodium polyphosphate solution.

2.5. Repeated use of cells

Both free and immobilized cells were reused in successive cycles of biotransformation. At the end of 24 h, free cells were centrifuged and resuspended in fresh reaction mixture and 2-phenylethanol (2.5 g l^{-1}) was added; immobilized cells were filtered on paper and reused after washing with a saturated $CaCO_3$ solution. The reaction was discontinued if the yield of phenylacetic acid was less than 15% after 24 h.

2.6. Analytical methods

Substrate and product concentrations were determined by GLC on a Carlo Erba Fractovap G1 gaschromatograph equipped with a hydrogen flame ionization detector. The column was packed with Carbowax 1500 (10% on Chromosorb W 80–100 mesh) with the column temperature kept at 150 °C. Samples (0.5 ml) were taken at intervals. The aqueous phase was brought to pH below 2 with 5 M HC1 and extracted with an equal volume of a $CHC1_3$ solution of an internal standard (1-phenylethanol). The organic extracts were directly analyzed to determine 2-phenylethanol and phenylacetaldehyde, while the determination of phenylacetic acid was performed after treatment with diazomethane [14].

3. Results and discussion

3.1. Activity of free cells of Acetobacter aceti 2000/28 MIM

The correlation between growth and activity of *Acetobacter aceti* MIM 2000/28 towards 2-phenylethanol (2.5 g l^{-1}) showed that the highest conversions were obtained with cells grown for 20–24 h when the average cell dry weight was 4.1–4.2 g l^{-1} Phenylacetic acid was the main product of the biotransformation with complete conversion within 5 h, while a maximum concentration of 0.5 g l^{-1} of the intermediate aldehyde was observed in the first hour. Fig. 1 shows the profile of the whole cell biotransformation.

Cells grown for 24 h were employed in further experiments aimed at cells immobilization.

3.2. Preparation and activity of immobilized cells

Different amounts of cells of *Acetobacter aceti* MIM 2000/28 grown for 24 h were immobilized in calcium alginate beads using different percentages of sodium alginate (0.5–2.5% w/w). Beads obtained using 2% alginate and a cell concentration of 7 $mg_{cells}\ g_{wetbeads}{}^{-1}$ resulted to be optimal and were used for the optimization of the biotransformation with immobilized cells.

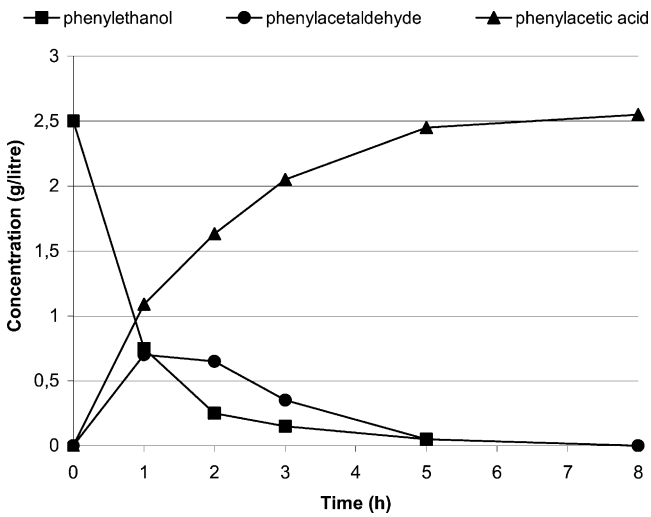

Fig. 1. Time course of the oxidation of 2-phenylethanol (2.5 g l^{-1}) with *Acetobacter aceti* MIM 2000/28.

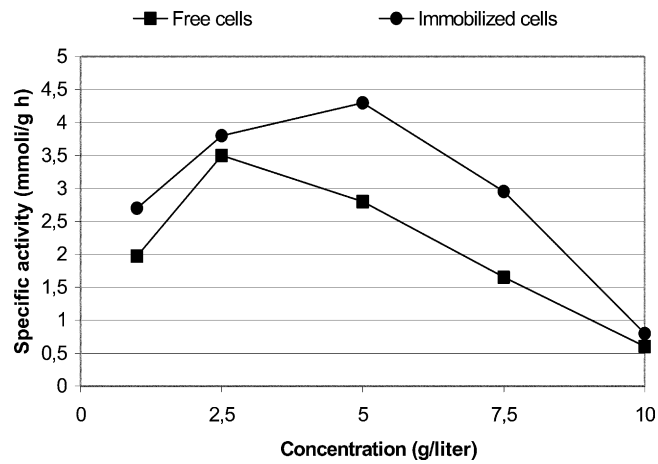

Fig. 2. Activity of freely suspended and immobilized *Acetobocter aceti* MIM 2000/28 towards 2-phenylethanol at different initial concentrations of 2-phenyl-1-ethanol.

The medium composition (ionic strength, buffer molarity, pH, and salts) often has critical importance for the stability of gel beads [16]; therefore, various buffers and aqueous solutions were checked as reaction media (Table 1).

The nature of the buffer had some influence on the process, the best results being obtained with a saturated solution of $CaCO_3$. Phosphate buffers gave good specific activity and yield, but the alginate beads are destroyed at prolonged reaction times by the phosphate ion that removes Ca^{2+} from the matrix; media containing an excess of Ca^{2+} ions are needed in prolonged operations to maintain the physical consistency of calcium alginate beads.

Biotransformation with immobilized cells in carbonate solution was accomplished with different substrate concentrations; the results obtained with freely suspended cells are also reported (Fig. 2).

The specific activity of immobilized cells was generally higher than that obtained with free cells; moreover, immobilized cells showed a better tolerance towards high substrate concentration. The partitioning of 2-phenylethanol, phenylacetaldehyde and phenylacetic acid between gel beads and $CaCO_3$ solution showed that more than 95% of the three compounds was found dissolved in the aqueous phase, while only traces could be detected in the gel beads.

Therefore, the real concentration of the components in the beads and therefore surrounding the cells is much lower than that experienced by free cells and toxicity towards the cells is therefore reduced in the immobilized biocatalyst.

Table 1
Oxidation of 2-phenylethanol with immobilized *Acetobacter aceti* MIM 2000/28 in different aqueous media

Medium	pH	Specific activity (mmol (g h)$^{-1}$)	Molar conversion (%)
Tap water	6.9	2.0	45
Phosphate buffer	6.0	3.4	>95
Phosphate buffer	7.0	3.1	>95
Citrate buffer	6.0	2.9	90
Citrate/phosphate	6.0	2.3	55
Acetate buffer	5.6	2.7	>95
Tris–HCl	7.0	2.7	90
$CaCO_3$ solution	7.2	3.8	>95

Molar conversions refer to phenylacetic acid production.

3.3. Oxidation in two-liquid phase system

The oxidation was carried out in a two-liquid phase system composed of water and isooctane (v/v l l^{-1}), since it was previously observed that under these conditions acetic acid bacteria may accumulate the intermediate aldehyde in good yields. Free and immobilized cells were used for the oxidation of two selected substrates, observing that the aldehyde accumulated only on 2-phenylethanol oxidation (Fig. 3).

Acetobacter aceti 2000/28 MIM oxidized 2-phenylethanol with higher rates than observed in monophasic aqueous medium. This may be due to an increased solubility of oxygen in water caused by the effect of 'oxygen vector' of

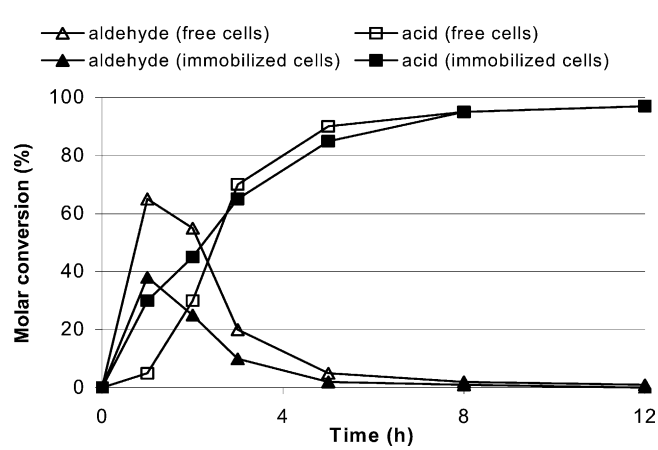

Fig. 3. Time course of the oxidation of 2-phenylethanol with free and immobilized cells in water/isooctane (l l^{-1} v/v).

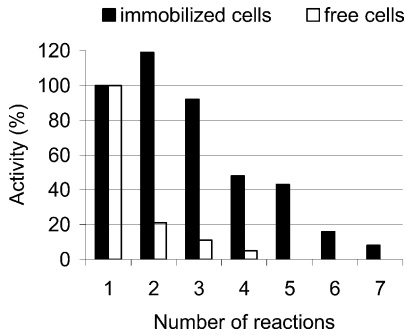

Fig. 4. Reusability of free or immobilized cells for the oxidation of 2-phenyl-1-ethanol. The relative activity is calculated by comparing specific activity, expressed as mole of phenylacetic acid formed in 1 h by 1 g of biocatalyst.

the organic solvent [17]. Aldehyde can be accumulated in two-liquid phase systems in good molar conversion during the first hours of the reaction; less aldehyde was produced by the immobilized cells. The alginate gel with its high content of water (<95%) represents an additional phase boundary which is stabilized by the polymer network and, therefore, diffusion to the organic extracting phase is partially hampered and the aldehyde is further oxidized by the immobilized cells giving the corresponding carboxylic acid.

3.4. Reusability of the biocatalysts

Free and immobilized cells were re-used in successive batch to check the operational stability of the biocatalysts (Fig. 4). Free cells were centrifuged and re-suspended in the biotransformation medium, while immobilized cells were filtered and re-used.

Immobilization in calcium alginate resulted in a notable stabilization of enzymatic activity, since reusability of the biocatalyst was extended to seven consecutive runs with conversion rates still significant. These results indicate a higher tolerance towards the organic substrate by the immobilized whole cell compared to free cells. The high activity observed in the second cycle could be due to a hyperactivation of the biocatalyst, as previously observed by Henley and Sadana [18] and by Moreno et al. [19].

3.5. Biotransformation in different reactors and fed-batch operation

A conventional STR and an ALR were tested as bioreactor for the biotransformation of 2-phenylethanol with immobilized cells. In experiments carried out in the STR, both agitation and aeration were varied, while in ALR the only variable was the aeration rate (Table 2).

The differences observed among the two bioreactors clearly indicate that the ALR seems the most suited for the biotransformation, probably by avoiding shear stress while ensuring both efficient aeration and agitation.

The bioconversion was then carried out with fed-batch addition of the substrate to the ALR, by adding 2.5 g of the substrate every 24 h. The bioprocess was performed with the pH maintained in the range of 6.5–6.8, since the acidity of the medium is enhanced by phenylacetic acid production.

Fed-batch operation was maintained for 9 days, reaching 23 g l^{-1} of phenylacetic acid. Parallel experiments with free cells (data not shown) indicated that the activity of the biocatalyst dramatically decreased after 2 days. It should be pointed out that no antifoaming agent needed to be used in experiments with immobilized cells, while massive foaming occurred with freely suspended cells in ALR (Fig. 5).

Table 2
Activity of immobilized cells of *Acetobacter aceti* MIM 2000/28 in a STR and an ALR

Reactor	Aeration (vvm)	Agitation (rpm)	Specific activity (mmol (g h)$^{-1}$)
STR	1.5	150	1.8
STR	1.5	300	2.1
STR	2.0	150	1.9
STR	2.0	300	2.3
ALR	1.0	–	3.3
ALR	1.5	–	3.6
ALR	2.0	–	3.5

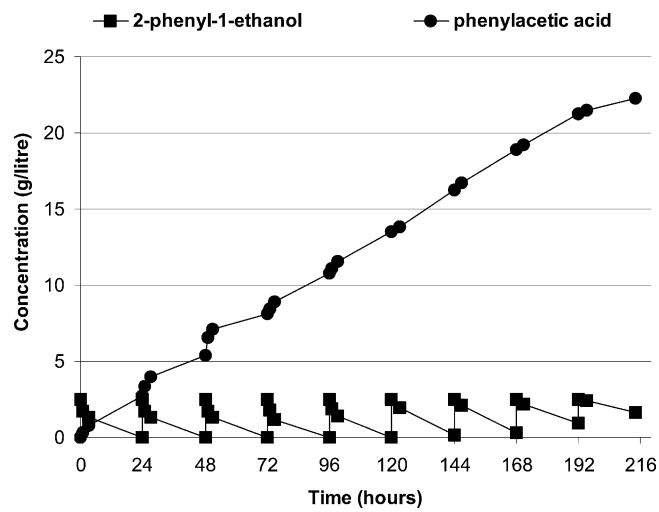

Fig. 5. Fed-batch oxidation of 2-phenyl-1-ethanol to phenylacetic acid with immobilized cells of *Acetobacter aceti* MIM 2000/28.

4. Conclusions

Acetobacter aceti MIM 2000/28 appears to be a suitable biocatalyst for the selective oxidation of 2-phenylethanol. Both phenylacetaldehyde and phenylacetic acid can be obtained by simple manipulation of the bioconversion medium, such as the addition of an organic solvent. Entrapment in alginate gel beads offers a stable immobilized biocatalyst which can be easily used in ALRs ensuring satisfactory productivity and easy operational features since no foam is formed and recovery of the product is simplified.

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