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Biotransformations in two-liquid-phase systems Production of phenylacetaldehyde by oxidation of 2-phenylethanol with acetic acid bacteria

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Abstract

Phenylacetaldehyde can be obtained by oxidation of 2-phenylethanol with acetic acid bacteria in two-liquid-phase systems where the aldehyde is removed into the organic phase before its further conversion to acid. Two *Acetobacter* strains (ALEF and ALEG) were able to accumulate aldehyde when aliphatic hydrocarbons were used. A two-liquid-phase system, composed of water and isooctane (v/v, 1/1), was particularly suited for a significant accumulation of the aldehyde: *Acetobacter* sp. ALEG furnished 9 g/l of phenylacetaldehyde within 4 h starting from 10 g/l of alcohol and still 8 g/l were recovered after 24 h in the organic phase, whereas strain ALEF gave 3.5 g/l of aldehyde from 5.0 g/l of substrate. *Acetobacter* sp. ALEG also showed satisfactory long-term stability, being able to perform the transformation with 80% of the original activity after 3 days of contact with the solvent.

Keywords: Two-liquid-phase system; Biotransformation; Aldehyde production; Alcohol oxidation; Acetic acid bacteria

1. Introduction

Many aldehydes are important ingredients of natural flavors, and their production by biocatalytical means starting from natural substrates is very attractive [1]. They can be obtained by enzymatic oxidation of primary alcohols, but the use of isolated alcohol dehydrogenases or oxidases is complicated by the requirement of cofactors and systems for their regeneration [2]. The use of whole microbial cells may be an alternative, but further oxidation of the aldehyde to the corresponding acid must be avoided to accumulate the desired product. It was reported previously that strains of acetic acid bacteria can produce various aldehydes by oxidation of the respective alcohol only if the aldehyde dehydrogenases are absent or have very low oxidation rates [3–4]. The use of water-immiscible organic solvents in whole-cell biocatalysis has been mainly exploited for biotransformations involving sparingly water-soluble or toxic compounds [5], but it also has been observed that the addition of organic solvents may alter the metabolic flux of microorganisms by extracting hydrophobic intermediates [6]. The use of two-liquid-phase systems, where hydrophobic aldehydes can be extracted in situ from the aqueous phase, may therefore provide a more general method for the production of aldehydes with whole cells[7–9].

In this work we focused our attention on the production of phenylacetaldehyde by oxidation of the 2-phenylethanol using acetic acid bacteria in two-liquid-phase systems.

2. Materials and methods

2.1. Microorganisms and culture conditions

Strains from an official collection (National Collection of Industrial and Marine Bacteria, Aberdeen, UK) or from our collections (Microbiologia Industriale Milano; Microbiologia Agraria Alimentare Ecologica Milano) were routinely

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Table 1
Oxidation of 2-phenylethanol (2.5 g/l) with different acetic acid bacteria. Molar conversion (%) after 1 and 3 h

Micro-organism	1 h		3 h	
	Aldehyde	Acid	Aldehyde	Acid
Acetobacter mesoxydans MAAEM	—	15	<5	25
Acetobacter pasteunianus NCIMB 8618	—	30	<5	50
Acetobacter xylinum MAAEM	—	25	<5	55
Acetobacter sp. A MIM	25	15	30	35
Acetobacter sp. AB2 MIM	15	5	25	10
Acetobacter sp. ALED MIM	—	20	<5	35
Acetobacter sp. ALEE MIM	10	60	<5	85
Acetobacter sp. ALEF MIM	25	30	15	80
Acetobacter sp. ALEG MIM	10	65	<5	95
Acetobacter sp. B MIM	25	10	25	30
Acetobacter sp. CB MIM	—	25	<5	40
Acetobacter sp. CC MIM	—	20	<5	30
Acetobacter sp. CH MIM	—	25	<5	50
Gluconobacter oxydans NCIMB 8035	25	45	5	80

maintained on GYC solid medium (glucose 50 g/l, yeast extract 10 g/l, $CaCO_3$ 30 g/l, agar 15 g/l, pH 6.3) at 28°C. Submerged cultures were carried out in a GlyY medium (glycerol 25 g/l, yeast extract 10 g/l, pH 5) into 1-l Erlenmeyer flasks containing 150 ml of medium on a reciprocal shaker (100 spm).

2.2. Determination of dry weight

After centrifugation of 100 ml of submerged cultures, cells were washed with distilled water and dried at 110°C for 24 h. After 24 h of growth, *Acetobacter* sp. ALEF gave an average 2.5 ± 0.3 g/l dry weight corresponding to an OD of 20.0 ± 0.3; *Acetobacter* sp. ALEG gave 4.5 ± 0.3 g/l dry weight corresponding to an OD of 25.0 ± 0.2.

2.3. Determination of partition coefficients

The partition coefficients of 2-phenylethanol and phenylacetaldehyde between water (5 ml) and isooctane (5 ml) were measured in 10-ml vials by dissolving the solute (2.5 g/l) into the organic phase, adding the aqueous phase, and

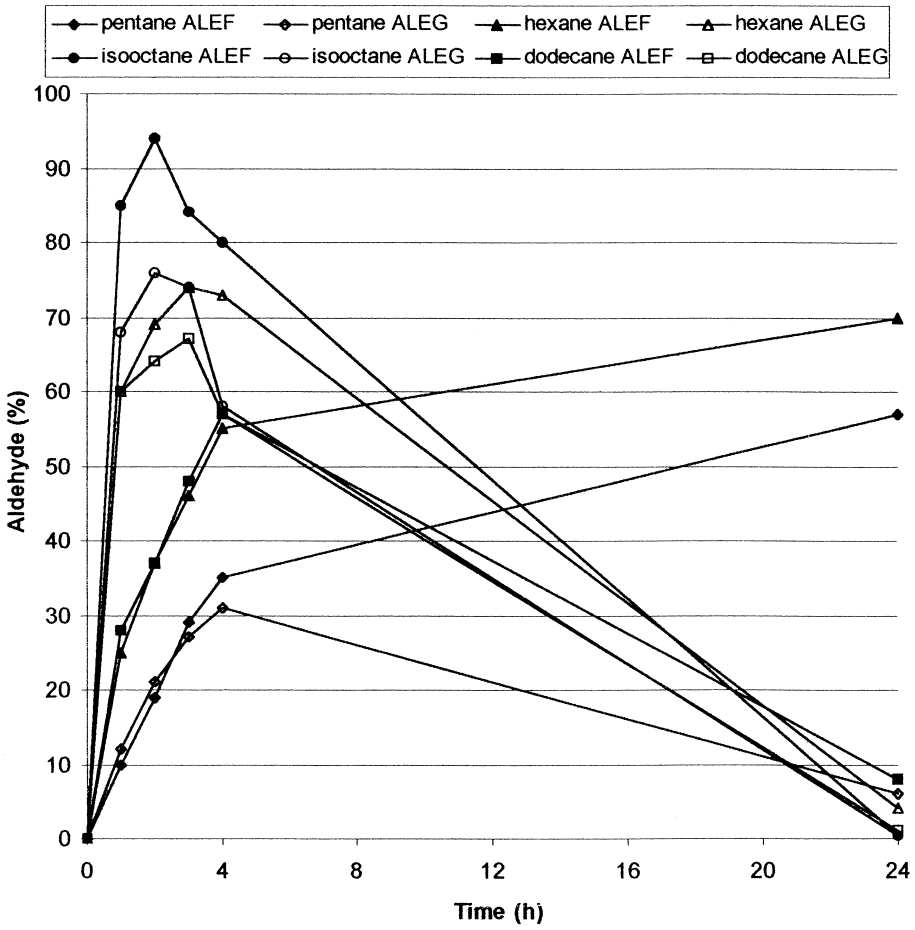

Fig. 1. Production of phenylacetaldehyde by oxidation of 2-phenylethanol (2.5 g/l) with *Acetobacter* sp. ALEF (DO = 15) and *Acetobacter* sp. ALEG (D.O. (optical density) = 20) in different two-phase systems.

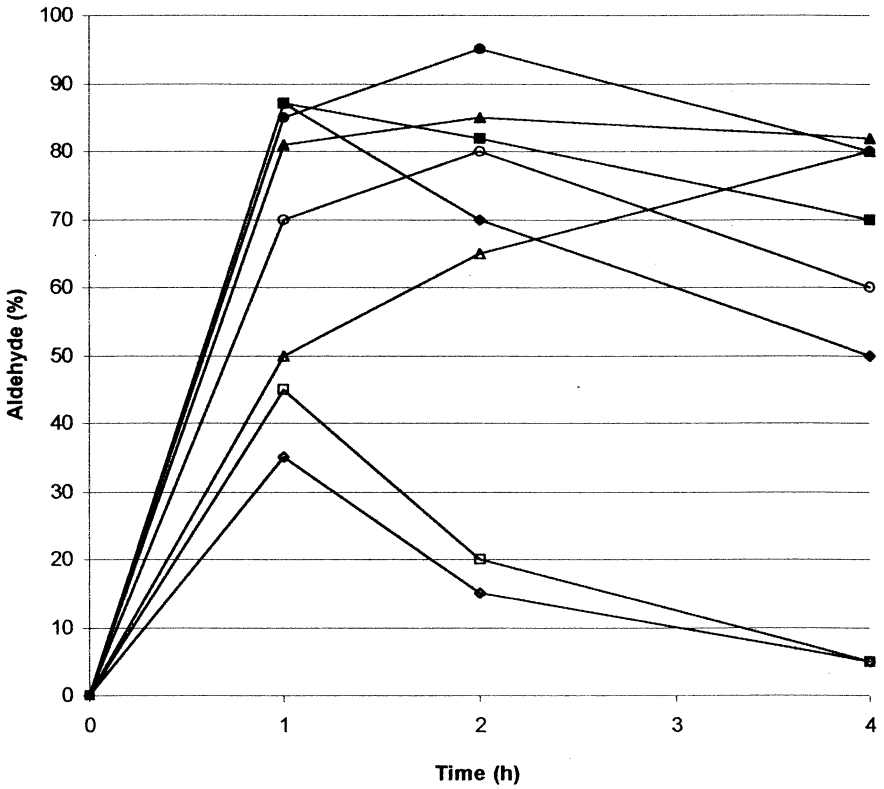

Fig. 2. Production of phenylacetaldehyde by oxidation of 2-phenylethanol (2.5 g/l) with *Acetobacter* sp. ALEF (DO = 15) and ALEG sp. (DO = 20) at different Φ. *Acetobacter* sp. ALEF: Φ = 3 (♦); 1.5 (■); 1 (●); 0.75 (▲). *Acetobacter* sp. ALEG: Φ = 3 (◇); 1.5 (□); 1 (○); 0.75 (△).

shaking the vials on a reciprocal shaker (100 spm) for 1 h. The concentrations of solutes were then determined in each phase. Partition coefficients (K_p) were expressed as the ratio of the concentration of the solute in the organic to that in the aqueous phase.

2.4. Biotransformation conditions

Experiments were carried out with 24-h submerged cultures. In experiments with two-liquid-phase systems, solvents were added to reach the desired volumes. Neat 2-phenylethanol (1–10 g/l) was directly added to the suspensions and flasks were shaken on a reciprocal shaker (100 spm).

2.5. Catalytic stability assays

Cells grown for 24 h were maintained under agitation with or without isooctane (10% v/v) at 28°C. Aliquots (15 ml) of the aqueous cell suspension were periodically withdrawn and used to test the oxidation of 2.5 g/l of 2-phenylethanol under standard conditions. Biocatalytic activity was expressed as alcohol consumed (μmol/ml) in 1 h.

2.6. Analytical methods

Substrate and product concentrations were determined by gas-liquid chromatography on a Carlo Erba Fractovap G1 gas chromatograph equipped with a hydrogen flame ionization detector. The column was packed with Carbowax 1500 (Costantino & C., Fauria, Torino, Italy) (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 HCl 5 M and extracted with an equal volume of a $CHCl_3$ solution of an internal standard (1-phenylethanol). The organic extracts were directly analyzed to determine 2-phenylethanol and phenylacetaldehyde, whereas the determination of phenylacetic acid was performed after treatment with diazomethane [3].

3. Results

The oxidation of 2-phenylethanol was tested with 24-h grown acetic acid bacteria (Table 1) by directly adding 2.5 g/l of 2-phenylethanol to the culture medium.

The tested strains showed marked production of the acid, although only a few strains furnished only transient production of phenylacetaldehyde (Table 1).

Among the more active strains, *Acetobacter* sp. ALEF and *Acetobacter* sp. ALEG were selected for further experiments where two-liquid-phase systems were used to check if the extraction of the aldehyde to an immiscible organic solvent could allow for its accumulation. Two-liquid-phase

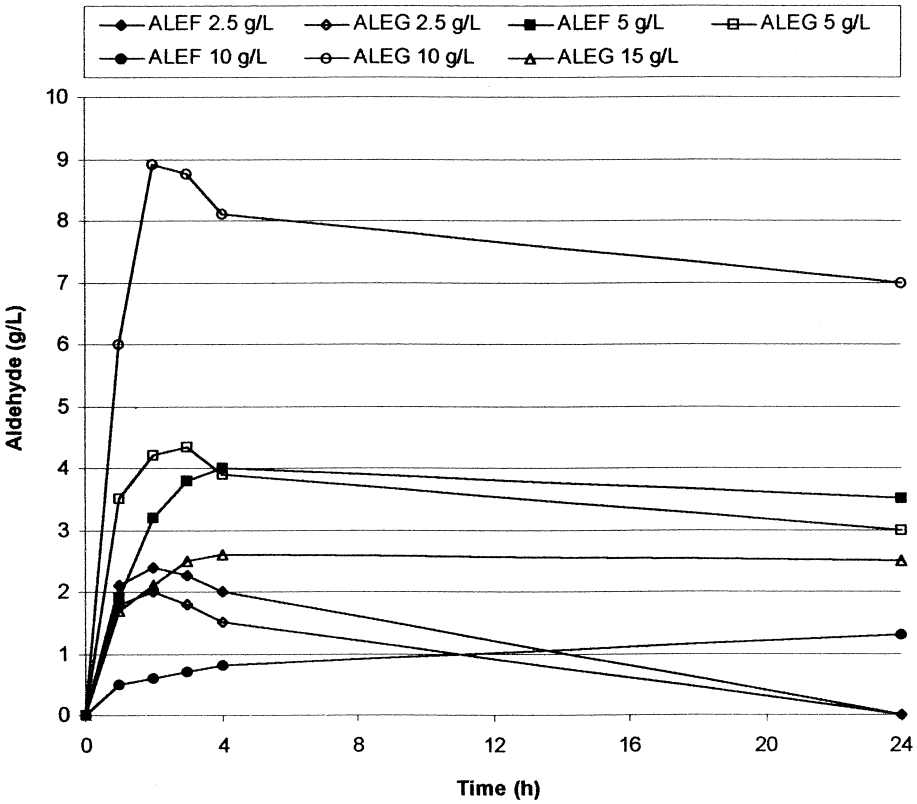

Fig. 3. Production of phenylacetaldehyde by oxidation of 2-phenylethanol with *Acetobacter* sp. ALEF (OD = 15) and *Acetobacter* sp. ALEG (O.D. (optical density) = 20) at different substrate concentration in a two-phase system ($\Phi = 1$).

systems were composed by the growth medium and organic solvents with different polarity (ethyl acetate, benzene, pentane, hexane, isooctane, dodecane; 1/1 v/v).

Notable accumulation of the aldehyde was obtained only in the presence of hydrocarbons (Fig. 1), whereas no oxidative activity was detected when ethyl acetate and benzene were employed. The concentration of phenylacetaldehyde reported is the sum of the products detected in the aqueous and organic phase.

Acetobacter sp. ALEG gave similar reaction profiles with the four solvents, the aldehyde being produced in the first 2 to 4 h before its oxidation to acid at longer times. The oxidation with *Acetobacter* sp. ALEF showed strong differences depending on the solvent used: the aldehyde could be obtained with good yields within 3 to 4 h in the presence of isooctane and dodecane, but it was oxidized completely at 24 h. With hexane and pentane, no further oxidation of the produced aldehyde was observed even after prolonged times. The highest rates of aldehyde production were always obtained with isooctane; this two-liquid-phase system was, therefore, chosen for further experiments.

The partition coefficients of the alcohol and the aldehyde between water and isooctane were measured ($K_{p\ 2\text{-phenylethanol}} \cong 0.5$; $K_{p\ \text{phenylacetaldehyde}} \cong 8.7$). These K_p indicate that the water/isooctane system is highly selective (selectivity $\alpha \cong 17$–18), with the aldehyde partitioning mostly to the hydrophobic solvent, whereas the more polar substrate remains available for oxidation in the aqueous phase in significant amounts.

The biotransformation was carried out in two-liquid-phase systems with different phase ratios (Φ: vw/vi), as shown in Fig. 2.

Acetobacter sp. ALEF was not particularly affected by the use of different phase ratios, whereas the oxidation rates of *Acetobacter* sp. ALEG decreased at higher Φ, where less alcohol was available for the biocatalyst in the aqueous phase. The highest accumulation of the aldehyde always was encountered with $\Phi = 1$.

The biotransformation was then carried out with higher substrate concentrations in the water/isooctane medium with $\Phi = 1$ (Fig. 3).

The oxidation of 2-phenylethanol by *Acetobacter* sp. ALEF was severely inhibited by substrate concentrations higher than 5 g/l, with the highest rate observed starting from 2.5 g/l of 2-phenylethanol. Strain ALEG was still able to produce large amounts of the aldehyde at 10 g/l of alcohol without significant oxidation to acid even after 24 h.

Phenylacetaldehyde (2.5 g/l) also was used as a substrate to check possible inhibitory effects due to its presence at relatively high concentrations. The oxidation of the aldehyde was carried out both in water and in water/isooctane media to check if it was available for biotransformation in the two-phase system (Fig. 4).

Only 40 to 60% molar conversions were achieved in the

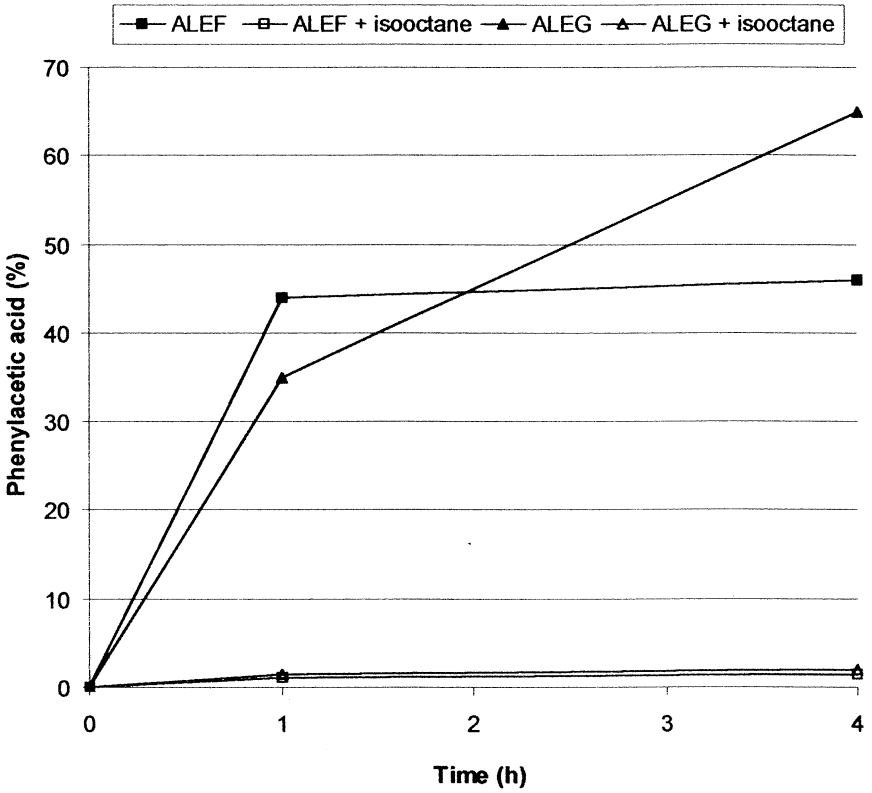

Fig. 4. Oxidation of 2-phenylacetaldehyde (2.5 g/l) with *Acetobacter* sp. ALEF and ALEG in water and in a two-phase system (water/isooctane 1/1).

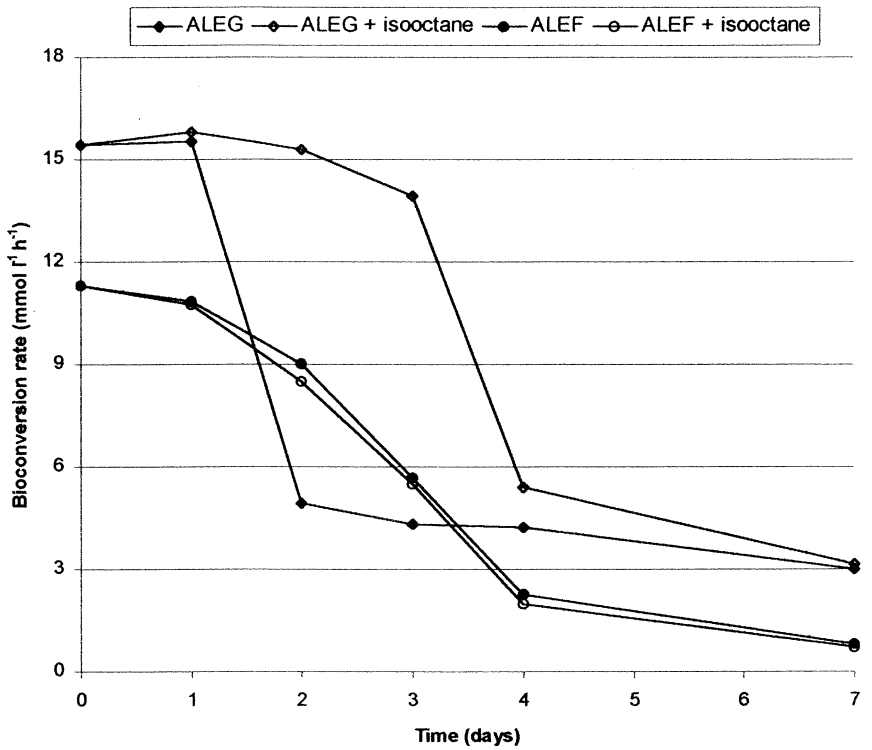

Fig. 5. Rates of 2-phenylethanol oxidation with 24-h grown cells maintained at 28°C in the culture medium under agitation with or without the contact with isooctane (10% v/v).

aqueous medium, indicating a partial inhibition of the oxidative systems of the bacteria. In the water/isooctane two-phase system, the aldehyde was not notably oxidized within the first 4 h.

The two strains were studied to determine the molecular toxicity [10–12] of isooctane on their biocatalytic activity. Cells grown for 24 h were maintained in their growth medium with and without contact with isooctane and periodically checked for their oxidative activity on 2-phenylethanol (Fig. 5).

The activity of the strain ALEG was stabilized by the contact with the organic solvent within 3 days and then a fast decrease of the oxidative ability was observed, whereas the ability of *Acetobacter* sp. ALEF to oxidize 2-phenylethanol decreased in an almost linear fashion with no significant differences in the behavior of the solvent-contacted cells.

4. Discussion

The oxidation of primary alcohols by acetic acid bacteria to the respective acids is a widespread transformation that proceeds first through the action of alcohol dehydrogenases and second through aldehyde dehydrogenase. The overall dehydrogenase activities are generally not very specific and aldehydes are not normally accumulated. This is the case of the oxidation of 2-phenylethanol, where phenylacetaldehyde could be only transiently produced before its further oxidation to acid by most of the strains tested. Two strains (*Acetobacter* sp. ALEG and ALEF), selected from among the more oxidative acetic bacteria, were able to carry out the biotransformation in two-liquid-phase systems by using different aliphatic hydrocarbons (pentane, hexane, isooctane, dodecane). These media allowed for accumulation of phenylacetaldehyde within 3 to 4 h, markedly delaying the action of the aldehyde dehydrogenases. In these two-liquid-phase systems, substrate (2-phenylethanol) can be transferred to the biocatalyst from the apolar phase across phase boundaries at a sufficiently high rate to fully exploit the cell-bound activity, whereas the more hydrophobic aldehyde is removed in situ from the aqueous phase before further enzymatic reactions occur.

The highest overall dehydrogenase activity was observed in both cases in water/isooctane, with 75 to 95% formation of the aldehyde within the first 2 h, the acid being produced only after 3 to 4 h. Isooctane furnishes favorable partitioning coefficients, providing an advantageous distribution for the selective and effective removal of the aldehyde from the aqueous phase. The choice of the organic solvent must take into account not only the partition effects, but also the stability of the enzymatic activity, as resulted by the different behavior of the two strains toward the effects of hexane and pentane.

The two types of dehydrogenases of *Acetobacter* sp. ALEF were influenced differently by the organic solvent. The alcohol dehydrogenase(s) were inhibited only partially (55–60% molar conversion into aldehyde with hexane and 35–40% with pentane), whereas the action of the aldehyde dehydrogenase(s) were severely lowered, allowing for the accumulation of the aldehyde.

The concentration of the substrate also played an important role, because alcohol concentrations above 2.5 g/l selectively inhibited the oxidation of the aldehyde with the two microorganisms. *Acetobacter* ALEG in the presence of isooctane furnished 8.0 g/l of phenylacetaldehyde after 24 h, starting from 10 g/l of 2-phenylethanol.

Finally, it was observed that contact with isooctane did not negatively affect the ability of the bacteria to catalyze 2-phenylethanol oxidation. In the case of *Acetobacter* sp. ALEG, the presence of the solvent even stabilized the oxidative activity, and still 80 to 85% of the initial performance was observed after 3 days in solvent-contacted cells, suggesting that long-term processes may be feasible.

5. Conclusions

The use of two-liquid-phase systems has proven to be suited for the production of phenylacetaldehyde by oxidation of 2-phenylethanol with acetic acid bacteria. This microbial biotransformation seems to be a promising and possibly general method to furnish aldehydes, provided that bacteria are not inhibited by contact with solvents. Work also is in progress to improve this extractive bioconversion by using membrane reactors that may allow for the extraction, avoiding direct contact between the biocatalyst and the extracting phases.

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