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# PHENYLACETALDEHYDE BY ACETIC ACID BACTERIA OXIDATION OF 2-PHENYLETHANOL

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## SUMMARY

This paper reports the production of 2-phenylacetaldehyde from 2-phenylethanol by acetic bacteria. Several strains of acetic bacteria were investigated and three were found to be effective for this bioconversion. Different conditions (different C source for the microorganisms, pH, substrate concentration, cell immobilization) were tested with yields ranging from 30 to 52.6%.

## *Introduction*

Amongst the products of interest as flavouring agents, aldehydes play an important role: their production by biotransformations, carried out by enzymes or microorganisms, allow them to be defined as "natural". Aldehydes are biologically produced by different microorganisms and acetaldehyde can be obtained from *Saccharomyces cerevisiae* by inhibiting reduction to ethanol during the alcoholic fermentation or from *Zymomonas mobilis* by using strains with low alcohol dehydrogenase activity (Wecker and Zall, 1987).

Several studies on oxidative aldehyde production were carried out using yeasts that can use methanol or ethanol as their sole carbon and energy source (Armstrong et al. 1984; Sakai and Tani, 1987; Duff and Murray, 1988; Williams et al., 1988; Harder and Veenhuis 1989; Murray and Duff, 1990 and refs. therein). An alternative microbial route to aldehyde production exploits organisms which degrade amino acids, aldehydes being formed as intermediates (Casey et al. 1992 and refs. therein).

Although the oxidation of several alcohols to acids by acetic bacteria has been extensively studied (Asai, 1968), their use to produce aldehydes has hardly been investigated. This microbial oxidation could be exploited as a versatile approach for the preparation of various aldehydes. The main problem in obtaining aldehydes by microbial whole cells is that of avoiding further oxidation to acids. This can be achieved exploiting different substrate specifity and/or low activity of the second oxidative enzyme.

In the present work different strains of acetic bacteria have been investigated to examine the feasibility of the oxidation of natural 2-phenylethanol, available from several yeasts by selective degradation of phenylalanine (Turner and Aldridge, 1983; Akita et al., 1990), to produce 2-phenylacetaldehyde, a component with a characteristic floral odor present in natural flavors and fragrances. The possibility of using calcium alginate-immobilized cells in this alcohol-to-aldehyde conversion process has also been verified.

## MATERIALS AND METHODS

Microorganisms and culture conditions

*Gluconobacter oxidans* N.C.I.M.B. 8035 and two newly isolated strains assigned to the genus *Acetobacter* (named *A* and $AB_2$) were used for aldehyde production and routinely maintained on GYC agar (De Ley et al. 1984). For submerged cultures three different cultural media have been used: GluY (glucose 25 g $l^{-1}$ and yeast extract 10 g $l^{-1}$), GlyY (glycerol 10 g $l^{-1}$ and yeast extract 10 g $l^{-1}$) and Frateur ethanol medium (Frateur 1950). The strains grown on GYC slants for 48 h at 28°C, were inoculated into 750 ml Erlenmeyer flasks containing 100 ml of each medium and incubated at the same temperature for 24 h on a reciprocal shaker (100 spm). Cellular dry weights ranged from 1.9 to 2.1 g $l^{-1}$.

Biotransformation conditions

Experiments were carried out directly by adding neat substrates onto submerged culture, after 24 h of growth. Bioconversion was also carried out in a bubble column (1 l, 28°C, aeration rate 1 vvm) equipped with an automatic pH controller, since in all experiments an acidic (pH≅3) was registered at the end of the growth phase and had to be brought to the desired value; in experiments performed in Erlenmeyer flasks the pH was corrected by $CaCO_3$ addition.

Immobilization of cells

Cells grown on GluY medium (200 ml in 1 l Erlenmeyer flasks) for 24 h at 28°C were separated by centrifugation (10000 rpm for 30 min), washed, resuspended in physiological solution (30 ml) and added to an equal volume of a sodium alginate solution (4% w/v). The resulting mixture was added dropwise to a stirred solution of $CaCl_2$ (0.1 M, 200 ml). To have small beads (diameter 0.8-1.5 mm) the mixture was extruded through a small diameter needle (0.42 x 12 mm) and the alginate droplets were blown off by a controlled stream of nitrogen into the $CaCl_2$ solution. The gel beads obtained were kept at 4°C for 1 h, washed with physiological solution and stored at 4°C.

Analytical methods

2-phenylethanol, phenylacetaldehyde and phenylacetic acid were determined by gaschromatographic analysis. Samples (500 μl) were added to an equal volume of a solution of internal standard (2g $l^{-1}$ of 1-phenylethanol in $CHCl_3$), pH was corrected below 2 with HCl 5 M; the organic extracts were treated with $CH_2N_2$ in order to detect phenylacetic acid as methyl ester. Substrate and product concentrations were determined on Carlo Erba Fractovap 2150 gas chromatograph equipped with a hydrogen flame ionization detector. The column (4 x 1500 mm) was packed with Carbowax 1500 (10% on Chromosorb W 80-100 mesh) with the column temperature kept at 150°C.

## RESULTS AND DISCUSSION

### *Phenylacetaldehyde production*

In an initial set of experiments we investigated the oxidation of 2-phenylethanol (2g $l^{-1}$) by twenty strains of acetic bacteria; 17 strains of *Acetobacter* and 3 strains of *Gluconobacter*, from official collections and new isolation, were tested.

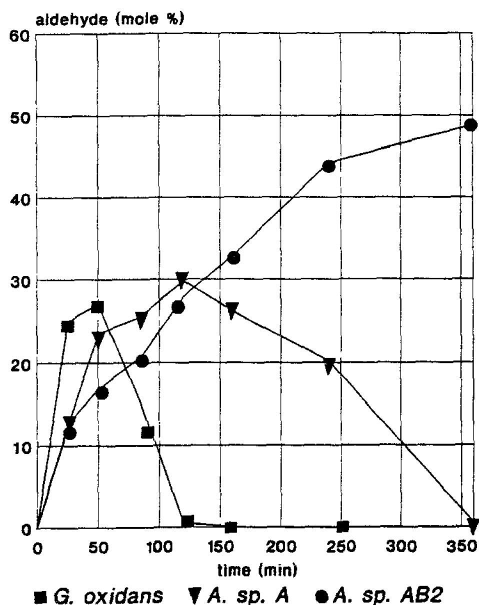


**Fig.1 - Phenylacetaldehyde production by *Gluconobacter oxidans* and *Acetobacter* sp. *A* and $AB_2$**

The screening showed that most of the strains, grown on glycerol (GlyY) medium, gave a fast and complete oxidation to phenylacetic acid. However three strains, namely *Gluconobacter oxidans* N.C.I.M.B. 8035 and the newly isolated *Acetobacter* sp. *A* and *Acetobacter* sp. $AB_2$ (from vinegar), produced phenylacetaldehyde in concentrations ranging from 0.54 to 0.98 g $l^{-1}$ (fig.1).

The first oxidation to aldehyde and the subsequent to acid are quite fast using *G. oxidans* and *A.* sp. *A*, while with *A.* sp. $AB_2$ the oxidative processes are slower and allow the accumulation of aldehyde.

To evaluate the influence of carbon sources other than glycerol on the microorganism oxidative activity, glucose (GluY) and ethanol (Frateur medium) were used as the alternative carbon source. Results showed that *A.* sp. *A* and *A.* sp. $AB_2$ produced and accumulated more aldehyde when grown on glucose (respectively 41%, 0.79 g $l^{-1}$ and 52.6%, 1.06 g $l^{-1}$ maximum yield). With *G. oxidans* no oxidation was observed when carbon sources different from glycerol were used. These observations led us to use *A.* sp. *A* and *A.* sp. $AB_2$, grown on GluY medium, for the next experiments.

### *Influence of 2-phenylethanol concentration*

The effect of the initial 2-phenylethanol concentration on aldehyde production with the two chosen strains was studied using substrate concentrations ranging from 1 to 16 g $l^{-1}$.

For both strains (fig. 2) higher initial aldehyde formation rate and maximum aldehyde concentrations were obtained with alcohol concentration of 4 g $l^{-1}$.

Lower aldehyde production with alcohol concentration of 8 g $l^{-1}$ and no aldehyde formation at higher substrate concentrations were observed, suggesting an inhibitory effect of the substrate if used at concentrations higher than 4 g $l^{-1}$.

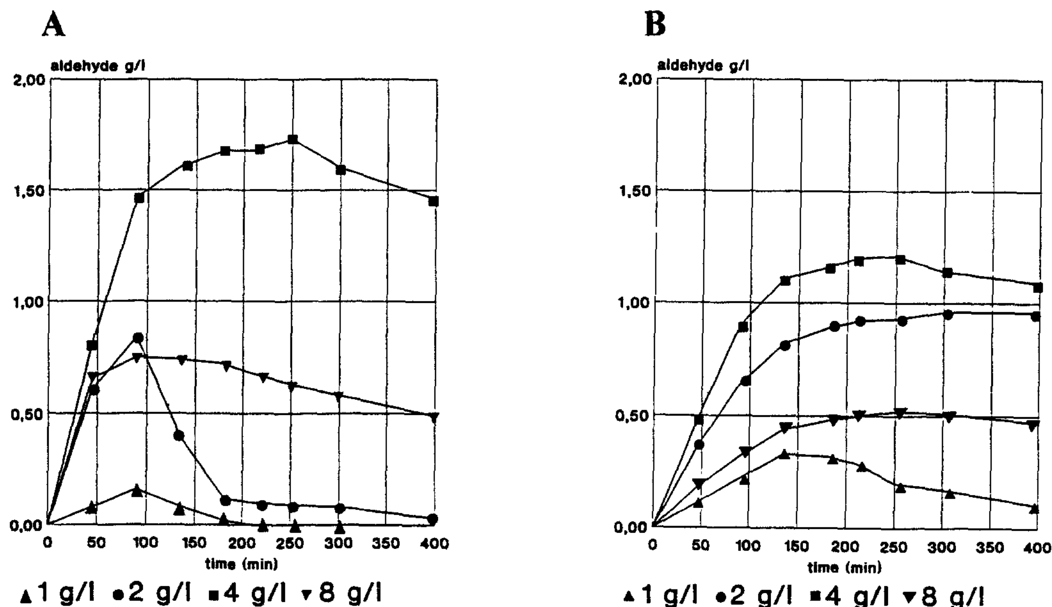


**Fig. 2 - Phenylacetaldehyde production at different substrate concentrations by *Acetobacter* sp. *A* (fig. 2a) and *A.* sp. $AB_2$ (fig. 2b).**

The time courses of product accumulation for the two strains are fairly similar for concentrations of 1, 4 and 8 g $l^{-1}$ but differ markedly with an initial concentration of 2 g $l^{-1}$. In the latter case, with *A.* sp. $AB_2$, aldehyde can be transiently accumulated whereas with *A.* sp. *A* aldehyde concentration decline rapidly after 90 minutes.

***Influence of aeration***

In order to obtain informations about the influence of oxygen on this bioconversion some exploratory experiments (4 g $l^{-1}$ initial alcohol concentration) were performed by comparing three different aeration conditions during the biotransformation phase, namely stationary and agitated flasks under normal or pure $O_2$ atmosphere.

In stationary conditions lower and delayed aldehyde maximum levels (0.26 g $l^{-1}$ for *A.* sp. *A* after 90 min and 0.64 g $l^{-1}$ for *A.* sp. $AB_2$ after 6 hours) were detected, while no significative differences were found in submerged cultures under air or under $O_2$.

These results, obtained in aerating systems with low $K_la$ values and with low cellular and substrate concentrations, support the hypothesis that in submerged cultures under air the $O_2$ concentration is not limiting for alcohol oxidation.

Moreover, differently form other neat biooxidations, in this case the availability of an $O_2$ amount larger than the necessary for the first oxidation is not warrant of a larger aldehyde production, since maximum concentration levels of aldehyde are dependent also by the ratio of the reaction rates of alcohol-to-aldehyde and aldehyde-to-acid conversions

### *pH effect*

pH effect was estimated in experiments carried out in a bubble column by using cultures at 24 h of growth and initial substrate concentration of 4 g $l^{-1}$.

Three different pH values (4, 6 and 7) were considered for both strains. At pH 6 the oxidation rate and aldehyde accumulation levels of the experiments performed in bubble column are a little lower than the ones already described in fig. 2 ( for *A.* sp. *A* in Erlenmeyer flasks maximum production 1.73 g $l^{-1}$, in bubble column 1.46 g $l^{-1}$ after 3-4 h)

At pH 4 *A.* sp. *A* accumulated aldehyde in 1.52 g $l^{-1}$ yield after 5 h, showing no significative differences compared to the results obtained at pH 6; *A.* sp. $AB_2$ appeared to be more sensitive to acidic conditions and produced only 0.24 g $l^{-1}$ as maximum yield.

It is worth noting that at pH 7 no conversion could be observed for either strain.

### *Immobilized cells*

On the basis of the performed experiments, *A.* sp. *A* was the best producer of aldehyde at higher substrate concentrations (4 g $l^{-1}$) at pH 6. Consequently whole cells of *A.* sp. *A* entrapped in calcium alginate beads were used in physiological solution to examine the feasibility of the biotransformation with immobilized systems.

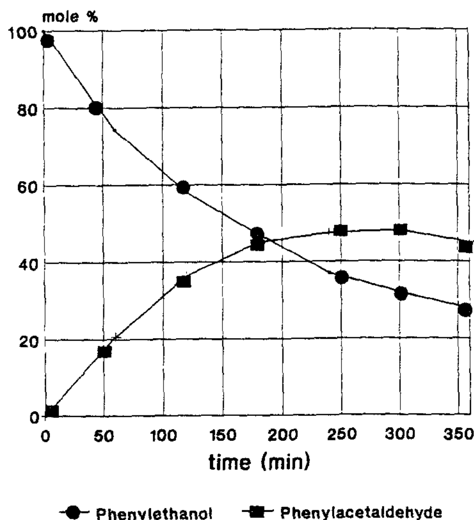


**Fig.3 - Phenylethanol consumption and phenylacetaldehyde production by *Acetobacter* sp. *A* immobilized cells.**

The process was performed in a bubble column mantained at 28°C, the solution was kept at pH 6 with constant aeration flux and 4 g $l^{-1}$ as initial alcohol concentration. Under these conditions a 48% maximum yield (1.92 g $l^{-1}$) was reached in 5 h as shown in fig. 3.

Immobilization increased the rate of conversion and yield (1.92 g $l^{-1}$ as maximum aldehyde concentration starting from 4 g $l^{-1}$ of alcohol). The higher

conversion obtained with immobilized cells can be ascribed to a protective effect of the alginate beads from toxic effects due to the substrate and/or the oxidation products.

### *Conclusions*

Continuous production of 2-phenylacetaldehyde using immobilized cells of *A.* sp. *A* is very promising, as shown by the good conversion obtained in this work, and this could lead to practical applications exploiting this acetic acid bacteria mediated bioconversion. The removal of the aldehyde by using some kind of continuous extraction could significantly increase the amount of aldehyde produced. In more effective systems (higher specific conversion rates) the role of $O_2$ should be reconsidered since the ORT/OUR ratio might be limiting under the used conditions.

The present work has demonstrated the possibility of obtaining 2-phenylacetaldehyde through oxidation of 2-phenylethanol by acetic bacteria. To the best of our knowledge this biochemical activity has been neglected but we are now working to extend this bioconversion process to different substrates.

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