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Optimisation of reaction conditions for production of *S*-(−)-2-hydroxypropiophenone by *Acinetobacter calcoaceticus*

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SUMMARY

Whole cells and cell-free extracts of *Acinetobacter calcoaceticus* containing benzoylformate decarboxylase efficiently condensed benzoylformate and acetaldehyde to produce the acyloin compound *S*-(−)-2-hydroxypropiophenone. Optimal concentrations of acetaldehyde cosubstrate for this reaction were found to be 1600 and 800 mM when whole cells and cell-free extracts were used respectively as biocatalysts. In both cases, optimal benzoylformate concentration was 100 mM. Temperature and pH optima for the biotransformation reaction were 30 °C and 6.0 respectively. Under optimised conditions, maximum production of 2-hydroxypropiophenone, amounting to 8.4 g L^{-1}, occurred after a 2-h incubation. Product formation equivalent to 6.95 g in 1 h corresponded to a productivity of 267 mg acyloin per g dry cells per h.

INTRODUCTION

Enzymes are attractive reagents in bioorganic synthesis, especially of bioactive molecules where enzyme stereospecificity can be exploited to produce the appropriate enantiomeric molecular form [7,17]. Enantiospecific production of L-phenylacetyl carbinol involving carbon–carbon bond formation, catalysed by pyruvate decarboxylase present in whole yeast cells, was one of the first industrial biocatalytic reaction processes to be commercialised [9]. While enzymes catalysing asymmetric carbon–carbon bond formation are of great interest, very few of these biocatalytic reactions are currently known [16].

The ability of pyruvate decarboxylase to produce acyloin compounds in the presence of an aldehyde cosubstrate led us to explore whether related enzymes, that is other non-oxidative α-ketoacid decarboxylases requiring thiamine pyrophosphate, could also catalyse these condensation reactions. One such enzyme, benzoylformate decarboxylase, participates in the catabolism of aromatic compounds as part of the mandelate pathway in bacteria such as *Acinetobacter calcoaceticus* and *Pseudomonas putida* that normally convert benzoylformate to benzaldehyde [1,2,5,6]. Benzoylformate decarboxylase manifests similarities with pyruvate decarboxylase in that it is a tetramer having similar subunit molecular weights. We have demonstrated the capacity of the benzoylformate decarboxylases from *P. putida* and *A. calcoaceticus* to produce 2-hydroxypropiophenone (HPP) when incubated with benzoylformate and acetaldehyde [15,19]. A comparison of the acyloin forming condensations catalysed by pyruvate decarboxylase and benzoylformate decarboxylase is illustrated in Fig. 1. The normal product of benzoylformate decarboxylase and trace amounts of benzyl alcohol are

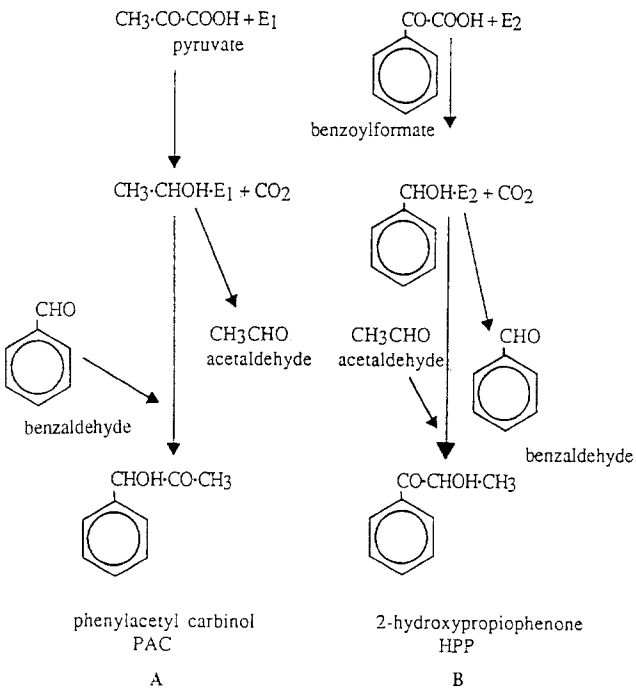

Fig. 1. Comparison of acyloin-forming condensation reactions catalysed by (A) pyruvate decarboxylase (E1) and (B) benzoyl formate decarboxylase (E2).

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produced as byproducts in this reaction. In the case of whole cell biocatalytic reactions involving *P. putida*, the optical purity of the HPP product was found to be 91–92%. In contrast, whole cell condensations using *A. calcoaceticus* produced *S*-(−)-2-hydroxypropiophenone, having an optical purity of >98%. The high enantioselectivity of this reaction makes a whole cell biocatalytic system involving *A. calcoaceticus* a potentially important system for use in enantioselective production of novel acyloin products. In this paper we describe factors affecting product formation using this reaction.

MATERIALS AND METHODS

Cultivation of Acinetobacter calcoaceticus

A. calcoaceticus NCIB 8250 was maintained on nutrient agar plates. Plates were incubated at 30 °C for 48 h and stored at 4 °C for up to one month. The liquid culture medium used for benzoylformate decarboxylase induction was a modification of the medium described by Barrowman and coworkers [1,2] and contained (g L^{-1}): KH_2PO_4, 4; $(NH_4)_2SO_4$, 2; L-glutamic acid, 0.9; $MgSO_4 \cdot 7H_2O$, 0.4; yeast extract, 3; and DL-mandelic acid (Sigma, St Louis, MO, USA), 3. This medium, 50 ml, contained in a 250-ml Erlenmeyer flask, was inoculated by loop from the agar stock cultures and incubated at 30 °C on an orbital shaker set at 200 r.p.m. for 16–20 h. This culture was serially transferred as a 10% inoculum through two further flasks containing the same medium and, in each case, was incubated under the same conditions. A 10% inoculum from the last culture was used to seed flask cultures for production of cells for synthesis. Flasks were incubated for 12 h at which point the cells exhibited maximum acyloin-forming activity.

Cell recovery and homogenisation

Bacterial cells were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C. The cells were washed with 50 mM potassium phosphate buffer, pH 7.0, recentrifuged and the pellets stored, frozen, until required.

To prepare cell-free extracts, centrifuged cells were resuspended in 100 mM sodium phosphate buffer, pH 6.0, containing 1.1 mM dithiothreitol, 0.1 mM thiamine pyrophosphate, 2 mM $MgSO_4$ and 1 mM EDTA to a final concentration of 0.3 g wet weight of cells per ml. Cells were sonicated with a Braun-sonic 2000 (B. Braun Biotech, Allentown, PA, USA) for 15 min, in 30-s bursts, separated by 30-s rests wherein the suspension was cooled by incubation in ice. The crude extract was recovered following removal of cell debris by centrifugation at 10 000 × *g* for 1 h at 4 °C.

Protein determination

Protein concentration was measured using the method of Bradford [3] with bovine serum albumen (Sigma, St Louis, MO, USA) as standard.

Enzyme reaction conditions

Unless otherwise stated, concentrations of constituents in the enzyme reactions in millimoles per litre were: sodium phosphate, 200; pH 6.0, benzoylformate, 100; acetaldehyde, 200; thiamine pyrophosphate, 1.5; magnesium chloride, 2.5 and 0.015 g (dry weight) cells ml^{-1} or the equivalent as cell-free extract or purified enzyme. Reactions were carried out at 30 °C and the activity was terminated by boiling for 5 min. Product formation was determined by gas chromatography.

Determination of 2-hydroxypropiophenone by GC analysis

For GC determination, 2 ml of rection mixture was thrice extracted with 2-ml volumes of glass-distilled ether. The ether extracts were pooled and concentrated under nitrogen to 1 ml. An internal standard of 0.5 ml of cyclohexanone was added to 2 ml of reaction mixture. This extract was analyzed in a Shimadzu GF, Model 14A equipped with an FID detector and a chromatopac C-R6A integrator. The GC contained a fused silica megabore column, 30 m × 0.52 mm (ID) coated with 1-μm thickness of 25% cyanopropyl, 25% phenyl, 50% methyl polysiloxane (Durabon 225, Chromatographic Specialities, Brockville, Ont., Canada). Operating conditions were: column and injector temperatures, 150 °C; detector temperature, 200 °C, with helium as carrier gas. Chemically-synthesised racemic 2-hydroxypropiophenone [14,19] was used as standard.

Biomass dry weight analysis

A known wet weight of cells was washed with distilled water on a preweighed 0.45-μm pore size MF Millipore filter paper (Bedford, Maine, USA). The cell sample was then dried on an aluminium foil dish in a 65 °C oven to constant weight. A 1-g quantity of cell wet weight was equal to 0.29 g cell dry weight.

RESULTS

The effect of *A. calcoaceticus* cell concentration on the production of 2-hydroxypropiophenone from benzoylformate (15 mg ml^{-1}, 100 mM) and acetaldehyde (8.8 mg ml^{-1}, 200 mM) was investigated in a 1-h reaction (Fig. 2). HPP production increased in an approximately linear manner with cell concentration in the range 0–0.09 g wet weight per ml. For studies on the effects of other parameters on the reaction, a cell concentration of 0.06 g wet weight per ml of whole cells or the equivalent amount of cell-free extract were used.

The time courses of HPP formation by whole cells and cell-free extract are illustrated in Fig. 3. Patterns of product formation were very similar for whole cells and cell-free extract. Maximum yield of product occurred in both systems after an incubation time of 150 min with higher yields in the presence of whole cells. The differences in product yields observed in Fig. 2 (cell concentration 0.6 g L^{-1}) and Fig. 3 after 1 h may be attributed to variations in the different cell batches used in these experiments.

The effect of temperature and pH on the reaction was determined by use of cell-free extracts. As illustrated in Figs 4 and 5, the reaction had an optimum temperature and pH of 30 °C and 6.0 respectively.

To determine the effects of substrate and cosubstrate

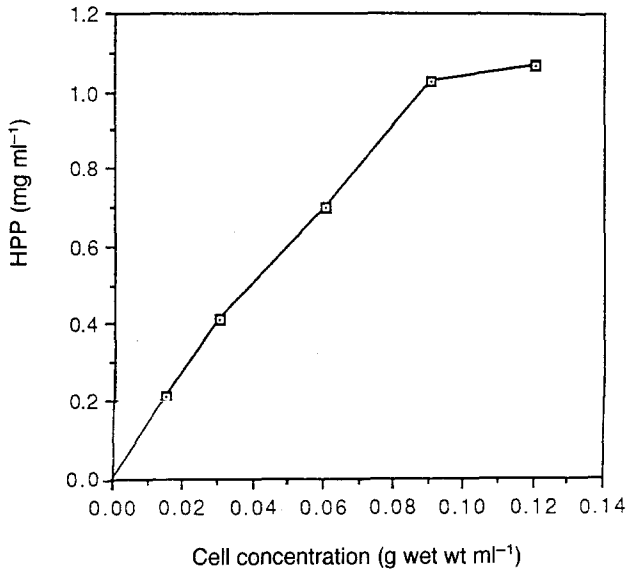

Fig. 2. Effect of whole cell concentration of HPP formation during biotransformation with benzoylformate 15 mg ml^{-1} (100 mM), and acetaldehyde 8.8 mg ml^{-1} (200 mM). Reaction time, 1 h.

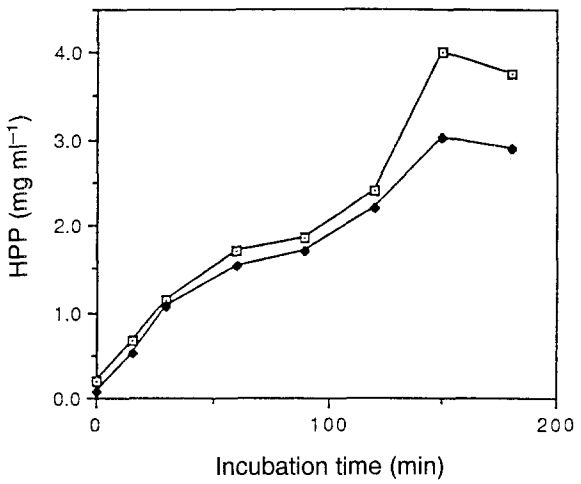

Fig. 3. Comparison of HPP formation with time between equivalent amounts of whole cells and cell-free extract. -⊡-, whole cells; -◆-, cell-free extract. Cell concentration, 0.06 g wet wt ml^{-1} reaction mixture. Benzoylformate and acetaldehyde concentrations as in Fig. 2.

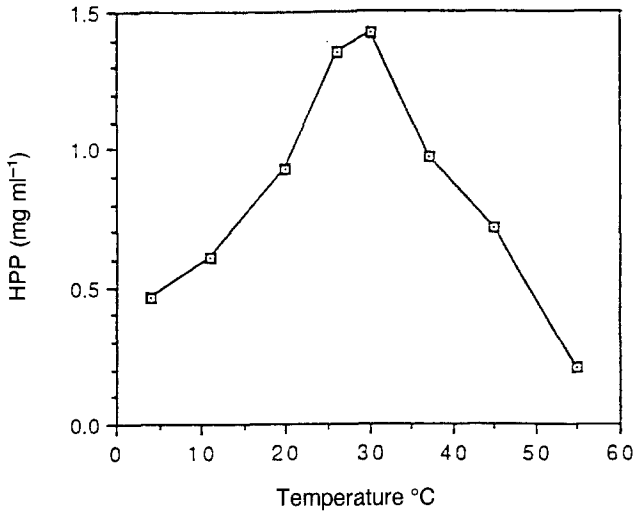

Fig. 4. Effect of temperature on HPP formation using a cell-free extract. Extract concentration equivalent, 0.06 g cell wet wt ml^{-1}. Benzoylformate and acetaldehyde concentrations and reaction time as in Fig. 2.

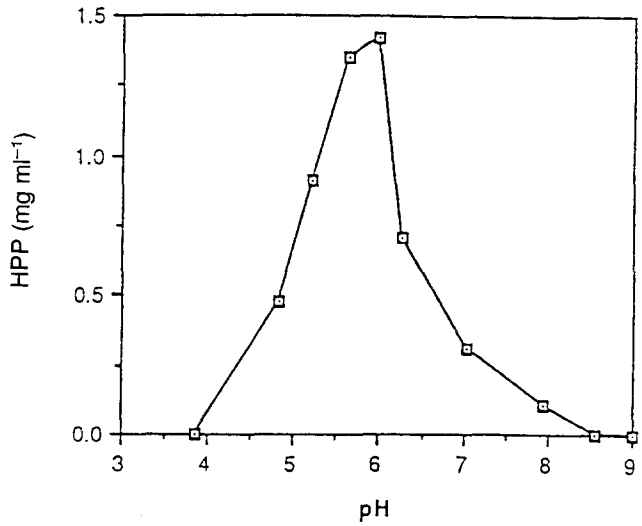

Fig. 5. Effect of pH on HPP formation using a cell-free extract. Extract concentration equivalent, 0.06 g cell wet wt ml^{-1}. Benzoylformate and acetaldehyde concentrations and reaction time as in Fig. 2.

concentrations on HPP production, the whole cell extract and whole cells were incubated with varying amounts of benzoylformate and acetaldehyde. With a cell-free extract, when benzoylformate concentration was varied in the range 0–112.5 mg ml^{-1} (0–750 mM), optimal HPP production was observed at a concentration of 15 mg ml^{-1} (100 mM) (Fig. 6). The effect of acetaldehyde concentration on HPP production by cell-free extract was investigated using a benzoylformate concentration of 100 mM. HPP formation increased with acetaldehyde concentration up to 35.2 mg ml^{-1} (800 mM) (Fig. 7). When the benzoylformate and acetaldehyde concentrations were varied in whole cell condensation reactions, an optimal benzoylformate concentration of 15 mg ml^{-1} (100 mM) was again observed (Fig. 8) while a higher acetaldehyde concentration of 70.4 mg ml^{-1} (1600 mM) produced maximum HPP production under the same conditions (Fig. 9). At high concentration, acetaldehyde can be expected to have an enzyme-denaturing effect. The higher acetaldehyde concentration observed to promote maximum bioconversion with whole cells compared to isolated enzymes may be attributed to the cells having a protective effect on enzyme activity.

The optimised results, with respect to concentration of whole cells (0.09 g wet weight per ml reaction mixture),

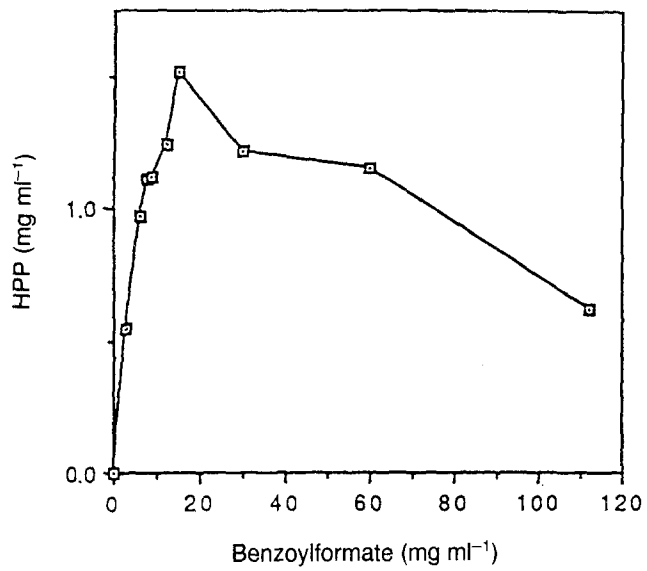

Fig. 6. Effect of benzoylformate concentration on HPP formation with a cell-free extract. Extract concentration equivalent, 0.06 g cell wet wt ml^{-1}. Acetaldehyde concentration and reaction time as in Fig. 2.

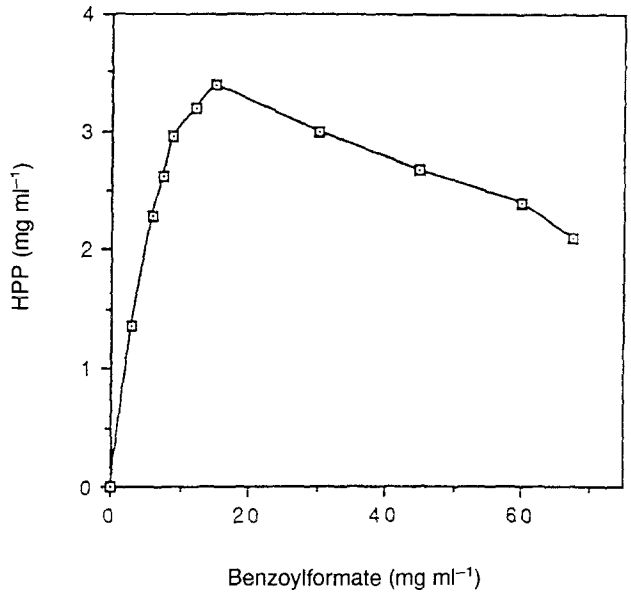

Fig. 8. Effect of benzoylformate concentration on HPP formation with whole cells. Cell concentration, 0.06 g ml^{-1}. Acetaldehyde concentration and reaction time as in Fig. 2.

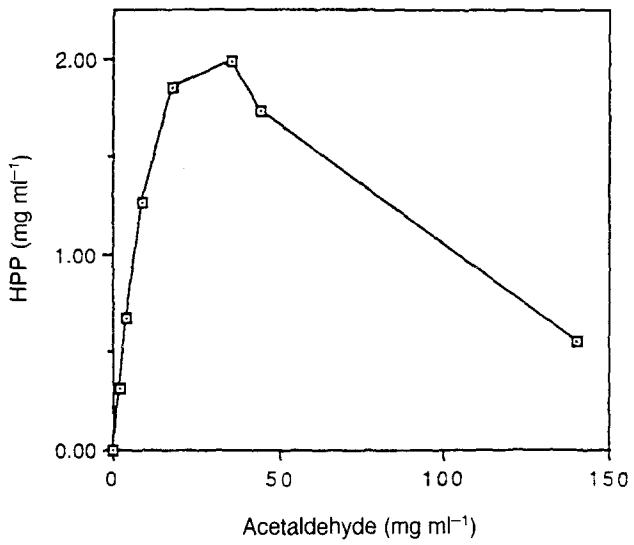

Fig. 7. Effect of acetaldehyde concentration on HPP formation with a cell-free extract. Extract concentration equivalent, 0.06 g cell wet wt ml^{-1}. Benzoylformate concentration and reaction time as in Fig. 2.

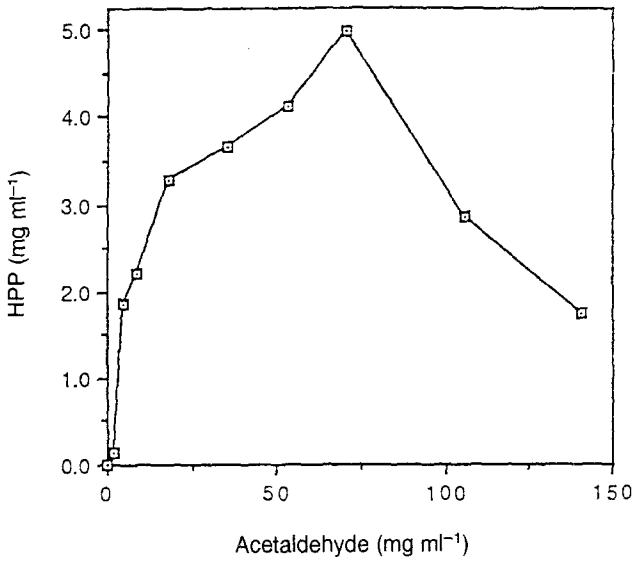

Fig. 9. Effect of acetaldehyde concentration on HPP formation with whole cells. Cell concentration, 0.06 g ml^{-1}. Benzoylformate concentration and reaction time as in Fig. 2.

benzoylformate 15 mg ml^{-1} (100 mM) and acetaldehyde 70.4 mg ml^{-1} (1600 mM), were combined with a view to producing optimal reaction conditions. A time course for HPP production using these conditions is illustrated in Fig. 10. Maximum HPP formation occurred at 120 min and resulted in production of 8.4 g L^{-1} 2-hydroxypropiophenone with 56% conversion of benzoylformate to HPP.

DISCUSSION

Reaction conditions have been optimised for enantioselective production of 2-hydroxypropiophenone resulting in formation of 8.4 g L^{-1} of product in a 2-h incubation, with 56% conversion of benzoylformate to product. An optimal yield of 6.95 g of product per litre per hour was observed which corresponds to a productivity of 267 mg of acyloin product per gram dry weight of cells per hour. The latter

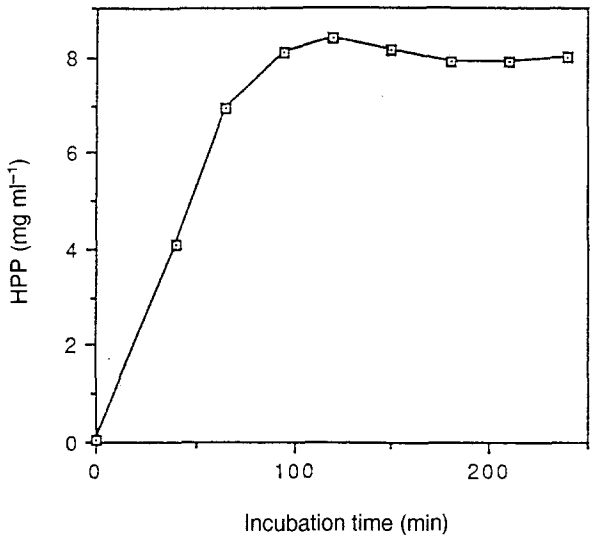

Fig. 10. Optimisation of biotransformation by whole cells using benzoylformate (15 mg ml^{-1}, 100 mM) acetaldehyde (70.4 mg ml^{-1}, 1600 mM) and 0.09 g cell wet wt ml^{-1}.

value was much higher than the optimum productivity observed in the less enantiospecific reaction of benzoylformate to HPP by *P. putida* [18]. This productivity compares very favourably with yields of the acyloin product of yeast pyruvate decarboxylase where optimal product formation in 1-h incubation periods amounted to 0.2 g per gram dry weight of yeast [11,12].

Pyruvate decarboxylase manifests relatively high specificity towards the natural and unnatural α-keto acid substrate and other nonoxidative thiamine pyrophosphate-linked decarboxylases also appear to exhibit this high substrate specificity [1,4,8]. However, in the conversion of pyruvate and benzaldehyde to L-phenylacetyl carbinol, commercial and highly purified preparations of pyruvate decarboxylase accept a variety of related aromatic cosubstrates in place of benzaldehyde to form a family of corresponding acyloin products [10,13]. Benzoylformate decarboxylase and pyruvate decarboxylase may complement each other as catalysts for bioorganic synthesis. Pyruvate decarboxylase may be used to produce acyloin compounds containing a fixed methyl group and a variable aromatic group whereas benzoylformate decarboxylase may product acyloin compounds having a fixed aromatic group and variable aliphatic moieties.

ACKNOWLEDGEMENTS

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REFERENCES

1 Barrowman, M.M. and C.A. Fewson. 1985. Phenylglyoxylate decarboxylase and phenylpyruvate decarboxylase from *Acinetobacter calcoaceticus*. Curr. Microbiol. 12: 235–240.
2 Barrowman, M.M., W. Harnett, A.J. Scott, C.A. Fewson and J.R. Kusel. 1986. Immunological comparison of microbial TPP-dependent non-oxidative α-keto acid decarboxylases. FEMS Microbiol. Letts 34: 57–60.
3 Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72: 248–254.
4 Fuganti, C., P. Oraselli, G. Poli, S. Servi and A. Zorzella. 1988. Decarboxylative incorporation of α-oxobutyrate and α-oxovalerate into (*R*)-α-hydroxyethyl and *n*-propyl ketones on reaction with aromative and α,β-unsaturated aldehydes in baker's yeast. J. Chem. Soc. Chem. Commun. 619–621.
5 Hegeman, G.D. 1966. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. J. Bacteriol. 91: 1140–1159.
6 Hegeman, G.D., E.Y. Rosenberg and G.L. Kenyon. 1970. Mandelic acid racemase from *Pseudomonas putida*: purification and properties of the enzyme. Biochem. 9: 4029–4036.
7 Jones, N.B. 1986. Enzymes in organic synthesis. Tetrahedron Letts 42: 3351–3403.
8 Juni, E. 1961. Evidence for a two-site mechanism for decarboxylation of α-carboxylase. J. Biol. Chem. 236: 2302–2308.
9 Kieslich, K. 1984. Biotransformations. In: Biotechnology (Rehm, H.J. and G. Reed, eds), Vol. 6a, pp. 1–4, Verlag Chemie, Weinheim.
10 Křen, V., D.H.G. Crout, H. Dalton, D.W. Hutchinson, W. König, M.M. Turner, G. Dean and N. Thomson. 1993. Pyruvate decarboxylase: a new enzyme for the production of acyloins by biotransformation. J. Chem. Soc. Chem. Commun. 341–343.
11 Long, A. and O.P. Ward. 1989. Biotransformation of benzaldehyde by *Saccharomyces cerevisiae*: characterisation of the fermentation and toxicity effects of substrates and products. Biotechnol. Bioeng. 34: 933–941.
12 Long, A. and O.P. Ward. 1989. Biotransformation of aromatic aldehydes by *Saccharomyces cerevisiae*: investigation of reaction rates. J. Ind. Microbiol. 4: 49–53.
13 Long, A., P. James and O.P. Ward. 1989. Aromatic aldehydes as substrates for yeast and yeast alcohol dehydrogenase. Biotechnol. Bioeng. 33: 657–660.
14 McCormick, J.P., W. Tomasik and M.W. Johnson. 1981. α-Hydroxylation of ketones: osmium tetroxide/*N*-methylmorpholine-*N*-oxide oxidation of silyl enol ethers. Tetrahedron Letts 22: 607–610.
15 Prosen, E., O.P. Ward, S. Collins, N.J. Dewdney, Y. Hong and R. Wilcocks. 1993. Enantiospecific production of *S*-(−)-2-hydroxypropiophenone mediated by benzoylformate decarboxylase from *Acinetobacter calcoaceticus*. Biocatalysis 8: 21–29.
16 Servi, S. 1990. Baker's yeast as a reagent in organic synthesis. Synthesis: 1–25.
17 Ward, O.P. 1991. Bioprocessing. Open University Press, UK.
18 Wilcocks, R. and O.P. Ward. 1992. Factors affecting 2-hydroxypropiophenone formation by benzoylformate decarboxylases from *Pseudomonas putida*. Biotechnol. Bioeng. 39: 1058–1063.
19 Wilcocks, R., O.P. Ward, S. Collins, N.J. Dewdney, Y. Hong and E. Prosen. 1992. Acyloin formation by benzoylformate decarboxylase from *Pseudomonas putida*: a novel biotransformation. Appl. Environ. Microbiol. 58: 1699–1704.