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Enhanced production of *R*-phenylacetylcarbinol (*R*-PAC) through enzymatic biotransformation

Bettina Rosche [a,*], Vanessa Sandford [a], Michael Breuer [b], Bernhard Hauer [b], Peter L. Rogers [a]

[a] *School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia*
[b] *BASF-AG, 67056 Ludwigshafen, Germany*

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Abstract

Enzymatic biotransformations offer the potential for improved concentrations, yields and productivities in the production of chiral precursors for the pharmaceutical industry. The present review describes new developments for the production of *R*-phenylacetylcarbinol (*R*-PAC) from substrates pyruvate and benzaldehyde using an enzymatic process, and compares the results with those for traditional fermentative yeast biotransformations. With batch processes, using pyruvate decarboxylase (PDC) from yeast and filamentous fungi, 50 g/l *R*-PAC was achieved in benzaldehyde emulsions, while concentrations in excess of 100 g/l were determined in the organic phase for a two-phase octanol/aqueous process. Higher yields and productivities were found also for the enzymatic biotransformations.

Keywords: Biotransformation; Pyruvate decarboxylase; *R*-phenylacetylcarbinol; Ephedrine/pseudoephedrine; Two-phase process

1. Introduction

R-phenylacetylcarbinol (*R*-PAC) is the chiral precursor for the production of the pharmaceuticals ephedrine and pseudoephedrine. It is currently produced via the biotransformation of benzaldehyde and pyruvate by fermenting baker's yeast. As shown in Fig. 1, the biotransformation is catalysed by a side-reaction of the enzyme pyruvate decarboxylase (PDC), which transfers enzyme-bound "active acetaldehyde" onto benzaldehyde to form *R*-PAC via a nucleophilic addition.

Maximal literature values for *R*-PAC production for various strains of yeasts are reported in the range 12–20 g/l *R*-PAC, with 65–70% yields of *R*-PAC based on benzaldehyde consumed in a 10–14 h biotransformation process [1–5]. The yeast-based process is limited by loss of PDC activity due to the toxic effects of benzaldehyde, insufficient accumulation of pyruvate by the yeast, and by *R*-PAC yield reduction resulting from conversion of benzaldehyde to by-product benzylalcohol due to alcohol dehydrogenase (ADH) and/or other oxidoreductase activity. The problem of pyruvate limitation has been addressed recently in a Japanese patent [6] in which the authors use a strain of *Torulopsis glabrata* to produce up to 30 g/l *R*-PAC with a yield of 70% based on benzaldehyde added.

An enzyme-based process using partially purified PDC with substrates pyruvate and benzaldehyde has a

* Corresponding author. Tel.: +61-2-9385-3895; fax: +61-2-9313-6710.
E-mail address: b.rosche@unsw.edu.au (B. Rosche).

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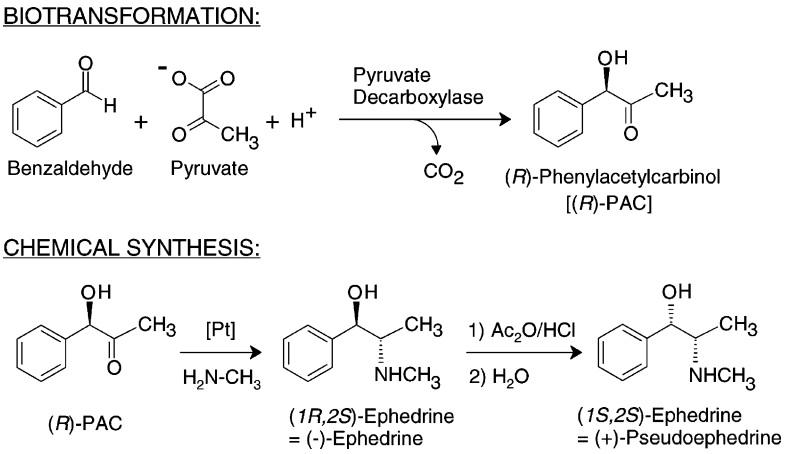

Fig. 1. Production of *R*-PAC, ephedrine and pseudoephedrine: Ac_2O, acetic anhydride.

number of potential advantages including higher rates of *R*-PAC production, higher final *R*-PAC concentrations and enhanced yields based on benzaldehyde. In this regard, Shin and Rogers [7] have reported final *R*-PAC concentrations of 28 g/l in 8–10 h with *R*-PAC yields up to 96% theoretical (based on consumed benzaldehyde) using partially purified PDC from *Candida utilis*. Higher levels of *R*-PAC were not achieved despite increasing substrate and enzyme concentrations. Recent work has reported the use of a potent mutant of PDC from *Zymomonas mobilis* and its application for the continuous production of *R*-PAC from substrates acetaldehyde and benzaldehyde using an enzyme–membrane bioreactor [8,9]. High space-time productivities were achieved although *R*-PAC concentration was low (3.3 g/l).

Further evaluation of enzyme-based processes using PDC from yeast and fungal sources is reported in the present review with particular focus on a two-phase biotransformation process in which the inactivating effects of substrate benzaldehyde and product *R*-PAC are reduced significantly through their partitioning into an organic phase, away from the aqueous phase containing the enzyme and substrate pyruvate.

2. Enzymatic *R*-PAC production in benzaldehyde emulsions

Following screening studies of both yeast [10] and fungal sources [11] for enhanced PDC activities and *R*-PAC production, detailed kinetic investigations have been carried out using partially purified PDC from *Rhizopus javanicus* in order to increase *R*-PAC levels in a basic batch biotransformation [12]. The effects of environmental conditions (*T*, pH), toxic substrate (benzaldehyde) and PDC concentrations as well as the effect of additives (enzyme stabilisers or ethanol) on *R*-PAC production were investigated.

It was established that lowering the temperature from 23 to 4 °C decreased initial rates, however the final *R*-PAC concentration was increased. The activity optimum for PDC was at pH = 6.5 and the best *R*-PAC yields were achieved at pH = 6.5–7.0, with a dramatic decrease at a pH higher than 7.0. For this reason, it is critical to prevent pH increases above 7.0 caused by proton consumption during the biotransformation as shown in Fig. 1.

A small-scale process for kinetic studies was designed with the pH maintained by means of high MOPS buffer concentration [12]. The stability of PDC was shown to be a function of the MOPS buffer concentration, with an 8–10-fold improvement in enzyme stability in 2 M MOPS buffer. This stabilising effect was not specific to the addition of MOPS buffer and could be achieved also by alcohols (2 M glycerol, 0.75 M sorbitol or 10% (w/v) polyethylene glycol 6000) or by salt (1 M KCl). It has been reported that certain salts and other osmolytes like polyols, mono and polysaccharides or polyethylene glycol are widely used to prevent unfolding of proteins [13]. Other enzyme stabilisers like protease inhibitors and

sulfhydryl group protecting agents did not increase PDC stability. Ethanol addition could increase benzaldehyde solubility but had no beneficial effect on *R*-PAC production.

A detailed batch biotransformation profile in a benzaldehyde emulsion under optimised conditions showed that *R. javanicus* PDC produced 50.6 g/l (337 mM) *R*-PAC from 400 mM benzaldehyde and 600 mM pyruvate in 29 h [12]. The enantiomeric excess of *R*-PAC was 99% and the molar yields on consumed benzaldehyde and pyruvate were 97 and 59%, respectively. In a control without enzyme 17% of pyruvate was lost, indicating chemical instability of this substrate under reaction conditions possibly due to polymerisation, which is reported in the literature [14]. Some of the initial pyruvate (24%) was converted into by-products acetaldehyde and acetoin. Benzylalcohol was not produced. The remaining carboligase activity of 43% at a final pH of 7.3 indicated reasonable stability of the enzyme in the benzaldehyde emulsion and at high product concentrations.

The improved biotransformation conditions were successfully applied to *C. utilis* PDC. A detailed kinetic comparison of *R*-PAC formation by *R. javanicus* and *C. utilis* PDC at 8.4 U/ml revealed similar product levels (49.3–51.2 g/l, respectively) in 21 h at 6 °C. The improvement over previously published results (28 g/l) [7] was most probably due to the enhanced pH control and enzyme stabilisation by high concentrations of MOPS buffer.

For an industrial application pH could be controlled at low buffer concentration via the addition of acid, e.g. substrate pyruvic acid. Enzyme stabilisation could be achieved, e.g. by the addition of salts or other osmolytes. However, a small-scale batch system with pH control by high buffer concentration was an appropriate method for further process development into a two-phase system.

3. Development of aqueous/organic two-phase biotransformation process

Limitations of the benzaldehyde emulsion process were investigated using *C. utilis* PDC. It was found that the enzyme was inactivated by substrate benzaldehyde, product *R*-PAC and by-product acetoin. The substrate benzaldehyde is sparingly soluble in the aqueous reaction medium, forming an emulsion above 50 mM. Enzyme inactivation was significantly stronger at increased benzaldehyde levels. For this reason, a two-phase batch reactor was evaluated for increased *R*-PAC production (Fig. 2) by facilitating higher initial benzaldehyde and final *R*-PAC levels partitioned away from the enzyme in an organic phase and thereby reducing enzyme deactivation [15].

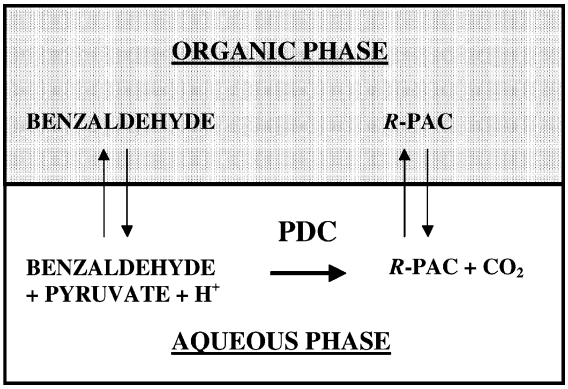

Fig. 2. Organic/aqueous two-phase system for *R*-PAC production.

Various organic solvents have been evaluated for the effect of the solvent on *C. utilis* PDC stability in the aqueous phase and for their effect on *R*-PAC production determined in both the aqueous and organic phases [15]. More than 60% PDC activity could be maintained for at least 7 days at 4 °C in the absence of substrates, with the following solvents (ordered by descending PDC stability): methyl *tert*-butyl ether (MTBE), 1-nonanol, dodecane, heptane, 1-octanol, hexane, octane, 1-pentanol. Less than 50% PDC activity remained with nonane, toluene, methylcyclohexane and 1-butanol. In a 72 h biotransformation with these 12 solvents, less *R*-PAC was formed than in a benzaldehyde emulsion reactor, except for the alcohols octanol and nonanol which resulted in *R*-PAC concentrations of approximately 100 g/l in the organic phase with additional 20 g/l in the aqueous phase. For all solvents benzaldehyde partitioned strongly into the organic phase. *R*-PAC also partitioned strongly into the organic phases pentanol, octanol and nonanol, while the other solvents left *R*-PAC mainly in the aqueous phase.

The production of *R*-PAC in a two-phase octanol/water system was evaluated in detail [15], with

the potential advantages of such a system being that both the inactivating benzaldehyde and *R*-PAC would be partitioned at relatively high concentrations in the octanol phase, while the pyruvate and PDC were contained in the aqueous phase and in contact with relatively low concentrations of benzaldehyde and *R*-PAC.

Kinetic evaluations were carried out with both rapidly stirred (organic phase as emulsion) and phase-separated systems with high substrate concentrations (initial benzaldehyde 160 g/l in organic phase; initial pyruvate 123 g/l in aqueous phase 2.5 M MOPS buffer) at pH = 6.5, $T = 4\,^{\circ}C$ over a range of carboligase activities (0.4–8.5 U/ml). In the various two-phase reactor experiments, it was found that the benzaldehyde levels in the aqueous phase remained at less than 50 mM.

In the rapidly stirred system, *R*-PAC production increased with enzyme level, reaching a maximum at 8.5 U/ml. In a detailed evaluation, it was found that the carboligase activity in the aqueous phase was fully maintained for the first 20 h and then declined, with this decline corresponding to an increase in pH above 7.0. The *R*-PAC formed was extracted into the upper octanol phase and away from the PDC, thereby reducing the effects of enzyme deactivation. After 49 h, all of the pyruvate was utilised and further *R*-PAC production ceased, with 23% of initial activity remaining. The final *R*-PAC concentration in the octanol phase was 140 g/l with 21 g/l in the aqueous phase. Molar yields based on the substrates utilised were: *R*-PAC/pyruvate = 75%; *R*-PAC/benzaldehyde = 91%. When *C. utilis* PDC was replaced with *R. javanicus* PDC, only 67% of above *R*-PAC concentration was achieved, indicating that the latter enzyme is less suitable for a two-phase process.

With phase separation maintained under similar initial conditions using *C. utilis* PDC, *R*-PAC production was achievable at lower enzyme levels, indicating that deactivation of PDC occurred more slowly than in the emulsion system. The maximum final *R*-PAC concentration was reached at 3.8 U/ml, while the optimum amount of product per amount of catalyst was achieved at 0.9 U/ml. At enzyme levels higher than 3.8 U/ml, less *R*-PAC was produced, with some pyruvate converted to by-product acetoin instead of *R*-PAC. The phase-separated system carried out with 3.8 U/ml carboligase activity resulted in a final *R*-PAC concentration of 167 g/l in the organic phase and 28 g/l in the aqueous phase, with molar yields of *R*-PAC/pyruvate = 93% and *R*-PAC/benzaldehyde = 98%. An extended time period of 395 h was required for completion of the biotransformations at the lower stirring rate with phase separation maintained. At 0.9 U/ml, 112 g/l *R*-PAC was achieved in the organic phase with an additional 16 g/l in the aqueous phase. With both rapidly stirred and phase-separated operations, the enantiomeric excess of *R*-PAC produced was >99%.

4. Comparison of enzymatic biotransformations

For a comparison of the various enzymatic processes for *R*-PAC production reported herein, the following values have been determined.

(a) *R*-PAC concentrations (in both phases for the two-phase systems) as shown in Fig. 3.
(b) Molar *R*-PAC yields (%) based on both benzaldehyde and pyruvate consumed, as shown in Fig. 4.
(c) *R*-PAC productivities (g/l/day) as shown in Fig. 5.
(d) Specific *R*-PAC production (mg *R*-PAC/U PDC) as shown in Fig. 6.

Where appropriate, typical values for the fermentation-based biotransformation process reported previously in the literature [1–5] are included for comparison based on: 15 g *R*-PAC/l in 12 h with 70% molar *R*-PAC yield on benzaldehyde.

From the data shown in Fig. 3, it is evident that significant increases in final *R*-PAC concentrations could be achieved with an enzymatic biotransformation when compared to a whole cell yeast-based process. With the enzymatic process not being limited by pyruvate availability and giving free access of substrates to the enzyme, a concentration of 50 g/l *R*-PAC was achieved with PDC from both yeast and filamentous fungi. No by-product benzylalcohol was formed. With the partitioning of the inactivating substrate benzaldehyde away from the PDC into the organic phase (octanol) in a two-phase system, even higher *R*-PAC concentrations were achieved. With both two-phase-separated and two-phase emulsion systems, *R*-PAC concentrations greater than 100 g/l were determined, even at a low initial enzyme activity (0.9 U/ml) in the phase-separated process.

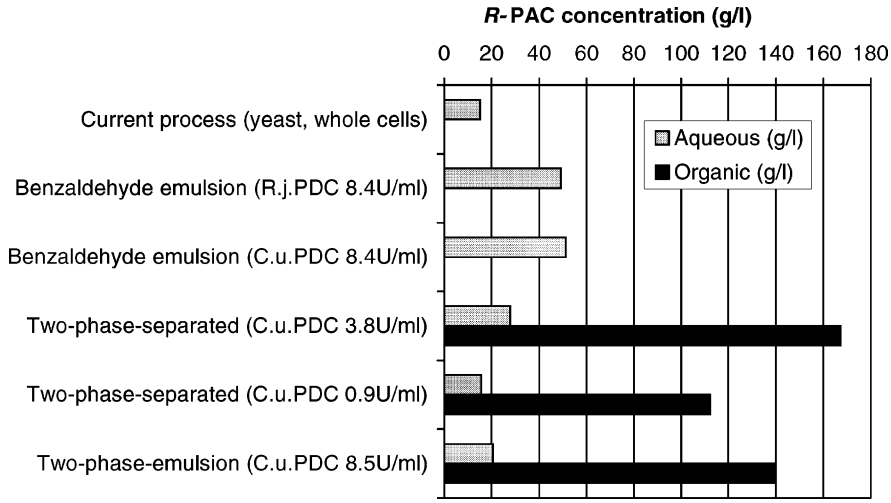

Fig. 3. *R*-PAC concentrations of various processes (in both phases for two-phase systems). PDC concentrations refer to the aqueous phase only: R.j., *Rhizopus javanicus*; C.u., *Candida utilis*.

An enzyme-based process also has the potential to achieve high molar conversion yields (see Fig. 4) and in all cases yields of *R*-PAC based on benzaldehyde consumed were much greater than that for the whole cell yeast-based process (estimated at 70%). In most cases, the yields based on benzaldehyde were greater than 95% for the enzyme-based processes. These lower yields for the whole-cell-process are not unexpected, as the presence of alcohol dehydrogenases and/or other oxidoreductases in the yeast will result in conversion of a significant amount of the benzaldehyde to benzyl alcohol. The yields based on pyruvate

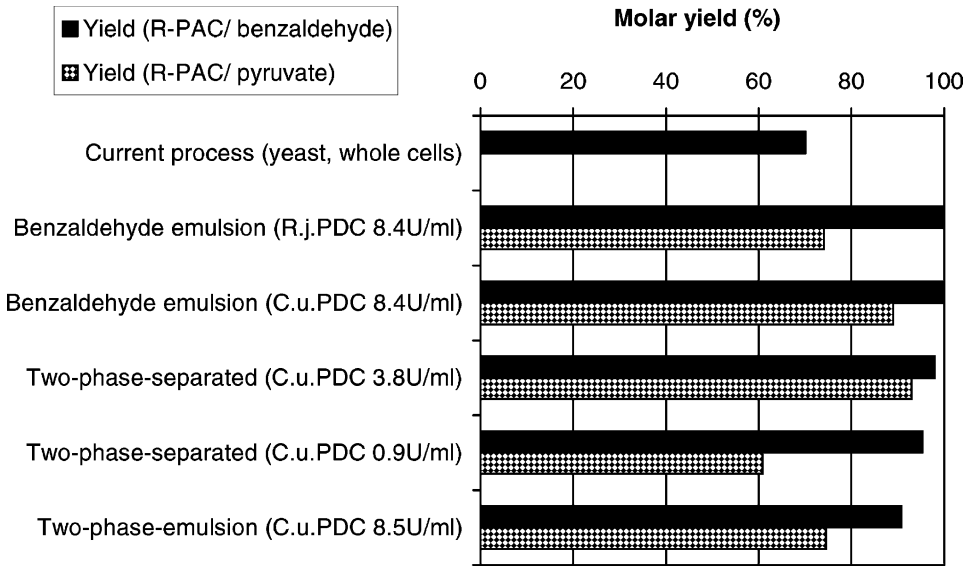

Fig. 4. Molar *R*-PAC yields based on substrates benzaldehyde and pyruvate consumed. No yield on pyruvate can be given for the whole-cell-process where sugar is used as substrate (for abbreviations, see Fig. 3).

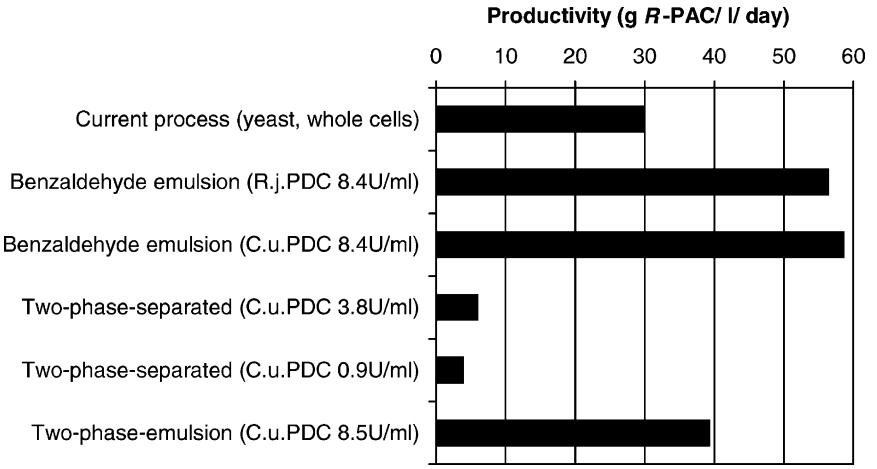

Fig. 5. *R*-PAC productivities (based on total reaction volume) (for abbreviations, see Fig. 3).

consumed were variable for the enzyme-based processes (Fig. 4), ranging from 60 to 93% (the latter for the two-phase-separated process with 3.8 U/ml initial PDC activity). Some loss in yield was attributed to pyruvate loss, which was also observed in the absence of enzyme. Pyruvate can be converted by PDC also into by-products acetoin and acetaldehyde and further studies are needed to minimise the formation of these by-products by optimising initial PDC activities and pyruvate concentrations (e.g. by programmed feeding of pyruvate).

Comparative data for volumetric productivities are shown in Fig. 5. Both the benzaldehyde emulsion process with PDC from *R. javanicus* and the two-phase emulsion system with PDC from *C. utilis* show volumetric productivities in excess of that estimated for a whole cell yeast-based process. The lower productivities evident for the two-phase-separated system may result from the lower PDC concentration and the lower ratio of interfacial area to volume, which restricts benzaldehyde transfer from the octanol to the aqueous phase, and the *R*-PAC transfer from the aqueous to the organic phase. When specific *R*-PAC production is compared (Fig. 6), the highest values were attained with two-phase systems—illustrating the advantages of substrate and product partitioning. A relative

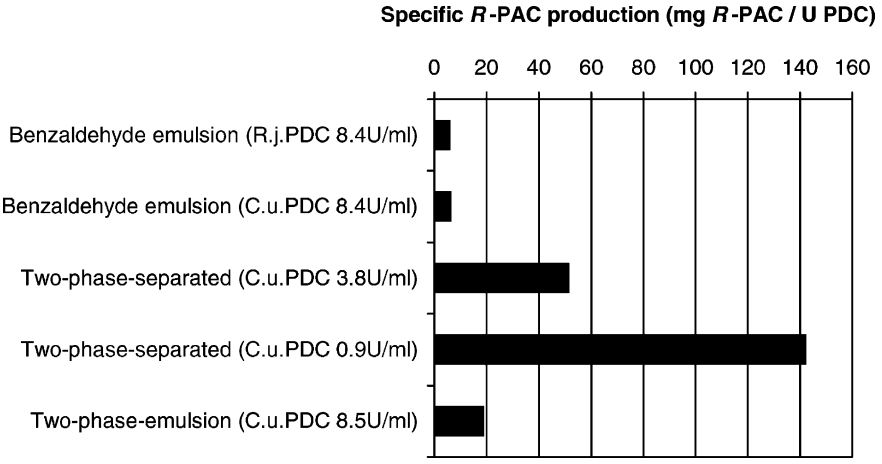

Fig. 6. Specific *R*-PAC productivities based on added PDC (for abbreviations, see Fig. 3).

comparison of phase-separated operation at 0.9 U/ml and two-phase emulsion operation at 8.5 U/ml illustrates that the former resulted in low volumetric productivity (Fig. 5), but very high specific *R*-PAC production (Fig. 6), while the situation is reversed for the latter. This results in similar specific productivities for both systems (8.6 and 9.2 mg *R*-PAC/U/day, respectively). In comparison, the benzaldehyde emulsion system produced 7.0 mg *R*-PAC/U/day. Optimisation of the two-phase systems is likely to involve a balance between the degree of agitation, as well as the interface: volume ratio, in order to maximise *R*-PAC production rates while minimising PDC deactivation. Control of pH at an optimum in the range 6.5–7.0 will also be critical to this process optimisation.

Aqueous/organic two-phase systems have been evaluated previously for *R*-PAC production using whole cells of baker's yeast adsorbed onto celite [16]. However, the maximum *R*-PAC concentration achievable was 0.45 g/l *R*-PAC in a 6-h reaction using hexadecane with 10% aqueous phase. Kostraby et al. [17] also demonstrated that *R*-PAC could be synthesised from benzaldehyde and pyruvic acid in organic solvent by non-fermenting cells of *Saccharomyces cerevisiae*. The aqueous buffer was fully absorbed by the dry yeast so that the reaction system was composed of hydrated yeast in petroleum spirit as the organic phase. However less than 1 g/l *R*-PAC was produced.

The present results for an enzyme-based process can be compared also with the recent published data of Goetz et al. [8] and Iwan et al. [9], in which *R*-PAC was produced in an enzyme–membrane bioreactor using a potent mutant of PDC from *Z. mobilis*. This process has the advantage of using acetaldehyde rather than pyruvate in the biotransformation, and acetaldehyde is currently a cheaper substrate than pyruvate. A high space-time yield (volumentric productivity) of 81 g *R*-PAC/l/day was reported for a continuous process and this is significantly greater than the values reported for the present two-phase systems (Fig. 5). However, for the process based on PDC from *Z. mobilis*, although enzyme activity was maintained for at least 40 h, the *R*-PAC concentrations were low (3.3 g/l) and only 45% conversion of 50 mM concentrations of both aldehydes was achieved. Our group is currently investigating also the possibility of combining the advantages of the different processes in a two-phase biotransformation with benzaldehyde and acetaldehyde using *Z. mobilis* PDC.

5. Conclusions

From a review of all the present investigations, it is clear that the results achieved with enhancing *R*-PAC production using an enzymatic biotransformation provide an interesting comparison with the traditional yeast-based fermentation and offer the potential for significant cost reductions. Further improvements in the enzymatic process are likely to come from process optimisation, reduced pyruvate cost or use of acetaldehyde, as well as cost-effective methods for enzyme production and stabilisation.

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