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Cells of *Candida utilis* for *in vitro* (*R*)-phenylacetylcarbinol production in an aqueous/octanol two-phase reactor*

Bettina Rosche[1,*], Michael Breuer[2], Bernhard Hauer[2] & Peter L. Rogers[1]
[1]*School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney NSW 2052, Australia*
[2]*BASF-AG, Fine Chemicals and Biocatalysis Research, D-67056 Ludwigshafen, Germany*
**Author for correspondence (Fax: 61-2-9313-6710; E-mail: b.rosche@unsw.edu.au)*

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Abstract

(*R*)-Phenylacetylcarbinol (PAC), a pharmaceutical precursor, was produced from benzaldehyde and pyruvate by pyruvate decarboxylase (PDC) of *Candida utilis* in an aqueous/organic two-phase emulsion reactor. When the partially purified enzyme in this previously established *in vitro* process was replaced with *C. utilis* cells and the temperature was increased from 4 to 21 °C, a screen of several 1-alcohols (C4–C9) confirmed the suitability of 1-octanol as the organic phase. Benzyl alcohol, the major by-product in the commercial *in vivo* conversion of benzaldehyde and sugar to PAC by *Saccharomyces cerevisiae*, was not formed. With a phase volume ratio of 1:1 and 5.6 g *C. utilis* l^{-1} (PDC activity 2.5 U ml^{-1}), PAC levels of 103 g l^{-1} in the octanol phase and 12.8 g l^{-1} in the aqueous phase were produced in 15 h at 21 °C. In comparison to our previously published process with partially purified PDC in an aqueous/octanol emulsion at 4 °C, PAC was produced at a 4-times increased specific rate (1.54 versus 0.39 mg U^{-1} h^{-1}) with simplified catalyst production and reduced cooling cost. Compared to traditional *in vivo* whole cell PAC production, the yield on benzaldehyde was 26% higher, the product concentration increased 3.9-fold (or 6.9-fold based on the organic phase), the productivity improved 3.1-fold (3.9 g l^{-1} h^{-1}) and the catalyst was 6.9-fold more efficient (PAC/dry cell mass 10.3 g g^{-1}).

Introduction

(*R*)-Phenylacetylcarbinol (PAC) is a chiral precursor for the pharmaceuticals ephedrine and pseudoephedrine. PAC is commercially synthesized by pyruvate decarboxylase (PDC) through non-oxidative decarboxylation of pyruvate followed by carboligation to benzaldehyde (Figure 1). Benzyl alcohol is the major by-product in PAC production from benzaldehyde and sugar by whole yeast cells (approx. 30% of converted benzaldehyde). Acetaldehyde and acetoin are by-products of the enzymatic PAC production from benzaldehyde and pyruvate. *(R)*-PAC production with whole microorganisms has the advantage of pyruvate generation from glucose, while in cell-free reactions pyruvate has to be supplied.

Progress in enzymatic PAC production (Shin & Rogers 1996, Bringer-Meyer & Sahm 1988, Iwan *et al.* 2001, Leksawasdi *et al.* 2003, Rosche *et al.* 2003a,b–2005) has led to the development of an aqueous/octanol two-phase emulsion reactor operated at 4 °C with 80 g PAC l^{-1} produced in 49 h using an initial activity of 4.25 U ml^{-1} partially purified PDC and a 1:1 phase ratio (Rosche *et al.* 2002b, Sandford *et al.*

*Dedicated with gratitude to Prof. Dr. Franz Lingens – "Theo".

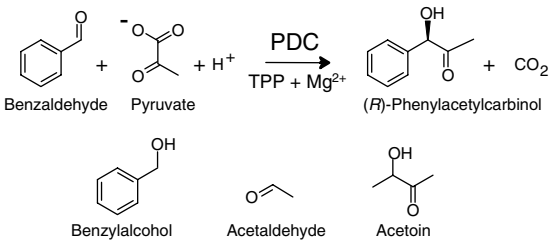

Fig. 1. Pyruvate decarboxylase (PDC) catalyzes the biotransformation of benzaldehyde and pyruvate into PAC, the chiral precursor for ephedrine and pseudoephedrine. Thiamine pyrophosphate (TPP) and Mg^{2+} are cofactors. Potential by-products are also shown.

2005). In the present study *Candida utilis* PDC is added in the form of resting cells as a less expensive catalyst and the temperature is raised from 4 to 21 °C in order to increase rates and to cut cooling cost. PAC production in aqueous–organic solvent two-phase systems using whole cells of *Saccharomyces cerevisiae* has been reported previously, but less than 1 g PAC l^{-1} was formed (Nikolova & Ward 1992, Kostraby *et al.* 2002).

Materials and methods

Catalyst preparation

The yeast *C. utilis* strain 70940 was obtained from the Culture Collection of the School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia (World Culture Collection Number 248). *Candida utilis* was grown at 30 °C either in shake-flasks in the presence of 200 mM MES buffer or in pH controlled bioreactors. The medium with an initial pH of 6 contained 90 g glucose l^{-1}, 10 g yeast extract l^{-1}, 3 g KH_2PO_4 l^{-1}, 2 g $Na_2HPO_4 \cdot 12H_2O$ l^{-1}, 10 g $(NH_4)_2SO_4$ l^{-1}, 1 g $MgSO_4 \cdot 7H_2O$ l^{-1}, and 0.05 g $CaCl_2 \cdot 2H_2O$ l^{-1}. Alternatively a minimal medium was used (100 g glucose l^{-1}, 10 g $(NH_4)_2SO_4$ l^{-1}, 1 g KH_2PO_4 l^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 0.02 g $CaCl_2 \cdot 2H_2O$ l^{-1}, 0.02 g $FeSO_4 \cdot 7H_2O$ l^{-1}, 0.01 mg $ZnSO_4 \cdot 7H_2O$ l^{-1}, 2 mg $MnCl_2 \cdot 4H_2O$ l^{-1}, 0.5 mg $CuSO_4 \cdot 5H_2O$ l^{-1}). Limited aeration allowed fermentative metabolism in order to produce the enzyme PDC. Cells were harvested by centrifugation, washed twice in water and stored as a pellet at −20 °C.

Analytical methods

PAC and benzaldehyde were quantified by HPLC with detection at 283 nm as described previously (Rosche *et al.* 2001). Benzyl alcohol was analyzed simultaneously with detection at 263 nm. Pyruvate and acetaldehyde were measured enzymatically and acetoin by gas chromatography (Rosche *et al.* 2002a). One unit (U) carboligase activity was defined as the amount of enzyme that produces 1 μmol PAC from pyruvate and benzaldehyde per min at pH 6.4 and 25 °C in a carboligase assay specified by Rosche *et al.* (2002a).

Test for effect of octanol on glucose uptake

A shake-flask culture of *C. utilis* grown in the yeast extract medium was divided into autoclaved and magnetically stirred 4 ml glass vials when the glucose concentration reached approximately 50 g l^{-1} (time zero). For each experiment three vials were incubated at 21 °C. The control vials contained 1.8 ml culture, while other vials contained 0.9 ml culture and 0.9 ml of either octanol or 1.5 M benzaldehyde in octanol. The mixtures were agitated just fast enough to maintain the organic phase in emulsion. Samples were withdrawn and after centrifugation the aqueous phase was diluted and analyzed for glucose levels by an enzyme based glucose analyser. The optical density (OD) was measured in the control at 600 nm using dilutions to give an OD value between 0.1 and 0.3.

Biotransformations

Biotransformations were carried out in magnetically stirred 4 ml screw-capped or 100 ml open glass vessels at 21 °C. The ratio of organic to aqueous phase was 1:1. The aqueous phase was placed into the vessel first and contained buffer, the substrate sodium pyruvate and the cofactors Mg^{2+} and thiamine pyrophosphate (TPP). The organic phase containing the substrate benzaldehyde was placed on top and the vessel was stirred slowly for equilibration (1 h) with phase separation maintained. For solvent screening, all organic phases were saturated with water prior to benzaldehyde addition in order to minimize subsequent phase ratio changes. Biotransforma-

tions were started by adding the cell suspension into the lower aqueous phase. The mixture was then stirred rapidly to maintain an emulsion. High MOPS buffer concentrations were applied because of rapid proton consumption during biotransformation and most efficient PDC carboligase activity occurring between pH 6.5 and 7 (Rosche *et al*. 2002a). For determining the time course of PAC production, acetic acid was added manually to maintain the pH in this range.

Samples (0.2–0.6 ml) were withdrawn at various times and the emulsion was centrifuged for 5 min in order to separate phases. A fraction of the top organic layer was extracted with a 200-fold volume of water for analysis. A portion of the lower aqueous phase was withdrawn also and protein was precipitated by the addition of 10% (w/v) trichloroacetic acid. Samples were centrifuged again to remove any solids.

Results

Effect of octanol on glucose uptake by Candida utilis

Candida. utilis was exposed to an octanol emulsion in order to evaluate its effect on the glycolytic conversion of glucose to the biotransformation substrate pyruvate. Since solvent effects on cells might be dependent on the growth phase, cells were exposed to octanol during growth as well as during the stationary phase. Figure 2 illustrates that in both cases glucose uptake stopped immediately. The same result occurred with 1.5 M benzaldehyde in the octanol phase and PAC was not formed (data not shown). However in the absence of solvent (control) *C. utilis* continued to consume glucose. The microscopic study of two-phase samples revealed that the cells stayed located in the aqueous phase. After the samples had been centrifuged for phase separation, there was a white layer between the upper octanol and the lower aqueous phase and a cell pellet in the bottom of the test tube. The thickness of this layer increased over time and it consisted of precipitated material as well as some cells and octanol droplets. Cells recovered from the two-phase system and plated on agar medium did not grow. Therefore in further experiments not glucose but pyruvate was provided as a substrate for biotransformation into PAC.

Solvent screen

Sandford *et al*. (2005) reported transformation results for partially purified *C. utilis* PDC with a

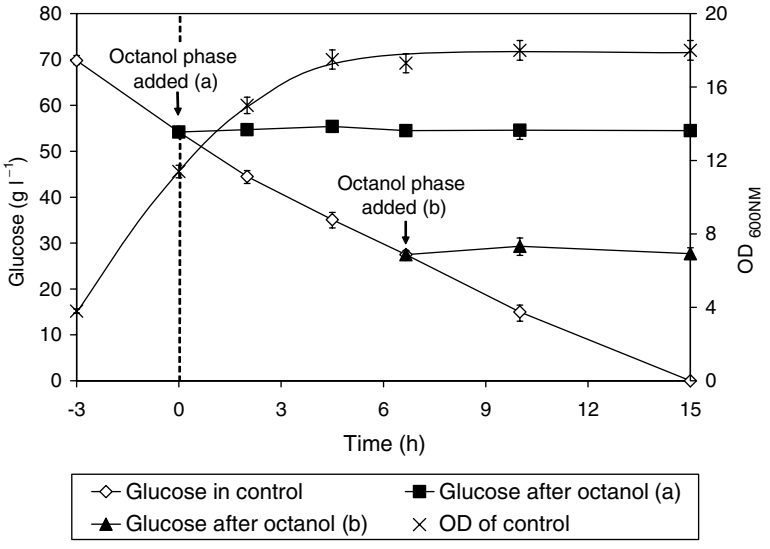

Fig. 2. Effect of an octanol emulsion on glucose uptake by *C. utilis* at 21 °C. The culture was divided into aliquots at time zero. The pH at this time was 5.5 and the dry cell mass 6.4 g l^{-1}. Octanol was added to culture aliquots during growth or in the stationary phase. Average data of three replicates are presented with the error bars indicating lowest and highest results.

range of solvents including 1-pentanol, 1-octanol and 1-nonanol and the latter two solvents supported excellent PAC production. Therefore 1-alcohols with 4–9 carbon atoms and two branched 1-alcohols were screened in the present study for two-phase PAC production from benzaldehyde and pyruvate using cells of *C. utilis*. The optimal chain length for PAC production was C7 and C8 as highest PAC concentrations were achieved with heptanol and octanol (Figure 3a). The branched alcohols isobutanol (C4) and isoamylalcohol (C5) resulted only in insignificant PAC concentrations. PAC partitioned strongly into the 1-alcohols C5–C9. Likewise benzaldehyde partitioned strongly into these organic phases with initially 20–30 mM in the aqueous phase (measured in a control without cells, data not shown). The putative by-product benzyl alcohol was not formed.

Heptanol versus octanol as organic phase

Heptanol and octanol were further evaluated for the aqueous/organic process at a 50 ml scale with pH control by addition of acetic acid. PAC formation in organic and aqueous phases is shown in Figure 3b. At a PDC concentration of 5 U ml^{-1}, PAC formation was to some extent faster in the heptanol than in the octanol system, but after 21 h similar PAC levels had been achieved with both solvents: 110–111 g l^{-1} in the organic phase and 12–14 g l^{-1} in the aqueous phase. With heptanol as the organic phase, slightly less by-product acetoin (2.6 g l^{-1}) was formed from pyruvate than in the octanol system (3.3 g l^{-1}). However, according to catalogue prices, heptanol costs approximately double that of octanol. Therefore further evaluation of the process proceeded with octanol as the organic phase. Figure 3b illustrates also that half of the initial activity (2.5 U ml^{-1}) resulted in a similar PAC production profile as observed with 5 U ml^{-1}, thus the biotransformation was not limited by the enzyme concentration in this range. Table 1 shows substrate, product and by-product concentrations for the 15 h time point. The molar balance at this time point indicates that 3% (v/v) benzaldehyde added initially was unaccounted for. A similar loss of benzaldehyde occurred in a control with no enzyme present. Including this loss, the molar yield of PAC on benzaldehyde

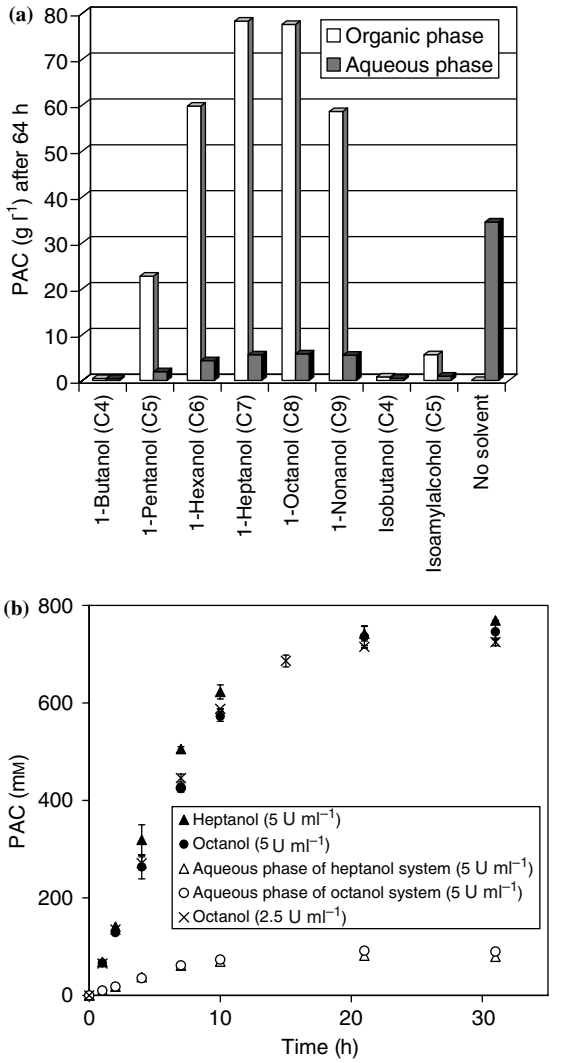

Fig. 3. PAC produced *in vitro* by non-viable cells of *C. utilis* from pyruvate and benzaldehyde in aqueous/organic two-phase emulsions at 21 °C. (a) Screening of alcohols as the organic phase (aqueous phase: 2 M MOPS/KOH initial pH 6.5, 5 mM $MgSO_4$, 1 mM TPP, 1.4 M pyruvate, *C. utilis* 18.5 g DCM l^{-1} equivalent to 3 U PDC ml^{-1}; organic phase: 1.6 M benzaldehyde in various alcohols; samples taken after 64 h). Highest and lowest values from two replicates were within 5% of the plotted average. (b) Comparison of an aqueous/octanol and an aqueous/heptanol system at a scale of 50 ml (aqueous phase: 2.5 M MOPS/KOH initial pH 6.5, 0.5 mM $MgSO_4$, 0.05 mM TPP, 1.43 M pyruvate, *C. utilis* 11.2 or 22.4 g DCM l^{-1} equivalent to 5 or 10 U PDC ml^{-1}, respectively. These activities corresponded to 2.5 and 5 U ml^{-1} total reaction volumes, respectively. Organic phase: 1.44 M benzaldehyde in octanol or heptanol). Error bars indicate highest and lowest values from two samples.

Table 1. Biotransformation summary for the 15 h sample from the aqueous/octanol *in vitro* system (see Figure 3b). Cells of *C. utilis* were added at a PDC carboligase activity of 2.5 U ml^{-1} total volume corresponding to a dry cell mass of 5.6 g l^{-1}.

	Aqueous phase	Octanol phase	In total reaction volume
Benzaldehyde (mM)	38	598	318
Pyruvate (mM)	360	0	180
PAC (mM)	85	686	386
Acetaldehyde (mM)	8.2	13	11
Acetoin (mM)	47	15	31
Molar yield PAC/bza (%)			96
Molar yield PAC/pyr (%)			72
Molar balance bza (%)			97
Molar balance pyr (%)			89

consumed was 96%. Some pyruvate was converted to acetaldehyde and acetoin in the biotransformation and the molar yield of PAC based on decrease of pyruvate was 72%. This calculation includes pyruvate which was unaccounted for in the molar balance (11% of the initial pyruvate), however, the loss of pyruvate in the enzyme free control was only 5%. The gap might be due to evaporative loss of the by-product acetaldehyde during biotransformation at 21 °C. The putative by-product benzyl alcohol was not formed.

Discussion

The selection of solvents for extractive biocatalysis and toxic solvent effects on cells, especially on

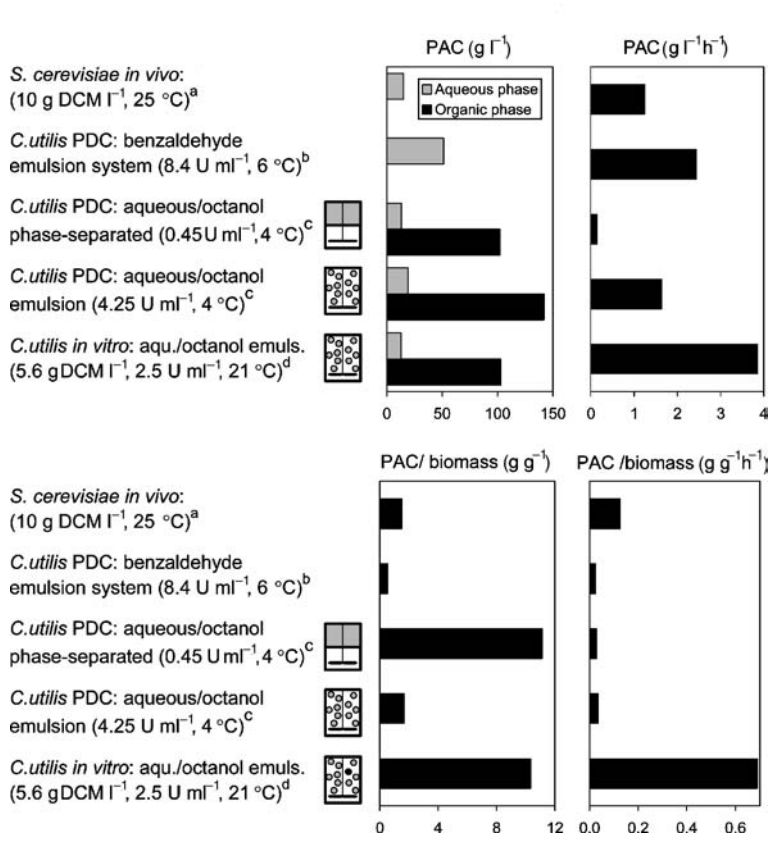

Fig. 4. Comparison of PAC production in various biotransformation processes: final PAC concentration and productivity (top), specific PAC production and specific productivity (bottom). Enzyme activities are given in U PDC carboligase per total reaction volume (unknown for *S. cerevisiae*), biomass in g dry cell mass (DCM). [a]*In vivo* analogue to commercial process; average values of 15 g PAC l^{-1} in 12 h with 10 g DCM l^{-1} were estimated from the literature; approx. 30% of benzaldehyde was converted into by-product benzylalcohol. [b]Rosche *et al*. (2003). [c]Sandford *et al*. (2005), Rosche *et al*. (2002b). [d]This study. Benzyl alcohol, the major by-product of the commercial fermentative process, was not formed.

the cell membrane, have been discussed in detail (e.g. Bruce & Dagulis 1991, León *et al.* 1998) and a successful example for whole cell *in vivo* biocatalysis in two-liquid phase systems is the pilot-scale production of (*S*)-styrene oxide by recombinant *E. coli* (Panke *et al.* 2002). In the present study it was shown that octanol, a solvent suitable for PAC production with partially purified PDC (Rosche *et al.* 2004, Sandford *et al.* 2005) was toxic to *C. utilis* cells. Therefore a catalyst in between *in vivo* whole cells and partially purified PDC was tested via adding whole cells of *C. utilis* for *in vitro* biotransformation of benzaldehyde and pyruvate.

Advantages of using whole cells instead of partially purified enzyme are the less expensive catalyst preparation and a possible protection of the enzyme inside the cells from deactivating substrates and products. Possible problems are substrate access and release of products (mass transfer limitation), putative protease activities and interferences by other enzymes (e.g. by-product formation) and/or cell components (e.g. product adsorption). Accordingly the formation of by-product benzylalcohol by oxidoreductases has been a disadvantage of using whole yeast cells for PAC formation from glucose and benzaldehyde. Furthermore Mochizuki *et al.* (1995) found that even without carbohydrate addition, whole cells of bakers' yeast formed benzylalcohol during the initial 2 h conversion of pyruvate and benzaldehyde. On the contrary the present method with *C. utilis* cells in a two-phase system prevented benzylalcohol formation. Furthermore only 0.047 g by-product acetoin was formed per g of PAC by *C. utilis* cells at 21 °C, which is a similar ratio as calculated from data for two-phase emulsion PAC production with partially purified PDC at 4 °C (Sandford *et al.* 2005). The maintenance of the acetoin to PAC ratio at the higher temperature was in contrast to the finding by Shin & Rogers (1996) that for partially purified *C. utilis* PDC a temperature increase from 4 to 25 °C caused a 3.2-fold increase of this ratio.

Figure 4 gives an overview of various PAC production processes with yeast. The previously published two-phase systems with partially purified PDC at 4 °C (Sandford *et al.* 2005) achieved high product levels and in the case of the phase separated system a high level of specific PAC production (g g^{-1}). However the productivity

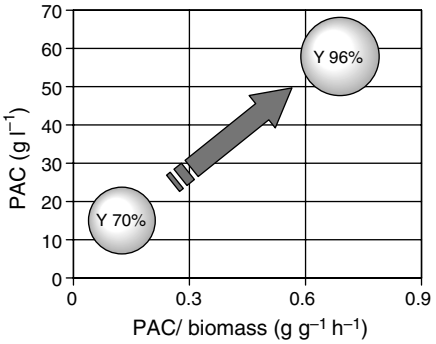

Fig. 5. Progress from *in vivo* whole cell PAC production (substrates glucose and benzaldehyde) to present *in vitro* two-phase system with *C. utilis* (substrates pyruvate and benzaldehyde). The bubble size represents the molar yield of PAC on consumed benzaldehyde. 26% higher yield, 3.9-fold PAC concentration (or 6.9-fold based on the organic phase), 3.1-fold productivity (3.9 g l^{-1} h^{-1}), 6.9-fold specific PAC production (PAC/dry cell mass 10.3 g g^{-1}) and 5.5-fold specific productivity (0.69 g g^{-1} h^{-1}) were achieved.

(g l^{-1} h^{-1}) was moderate and the specific productivity (g g^{-1} h^{-1}) was lower than that of the traditional *S. cerevisiae in vivo* process. In comparison the present *C. utilis in vitro* two-phase method at 21 °C achieved appreciable increases of productivity and specific productivity due to higher temperature and lower biomass requirement. Figure 5 demonstrates the progress from *in vivo* whole cell PAC production (*S. cerevisiae*, substrates glucose and benzaldehyde) to the present *in vitro* two-phase system with *C. utilis* (substrates pyruvate and benzaldehyde) with greatly increased product concentration, specific productivity and molar yield on benzaldehyde.

Acknowledgements

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