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NOTE

Could Organic Solvents Be Used for the Alteration of Flux of Hydrophobic Intermediates through a Metabolic Pathway in Microorganisms?

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Abstract The addition of decane to biotransformation media containing *Yarrowia lipolytica* led to the accumulation of intermediate L-phenylacetaldehyde and L-phenethyl acetate during bioconversion of L-phenylalanine, whilst none of these products were obtained in conventional aqueous fermentations. The results obtained support an earlier hypothesis (Spinnler *et al.* 1996. *Proc. Natl. Acad. Sci. USA* **93:** 3373–3376) that organic solvents, acting as "thermodynamic traps" for hydrophobic intermediates, can substantially alter metabolic fluxes.

Key words: Microorganism, metabolic pathways, hydrophobic intermediates, organic solvents, phenethyl alcohol, phenethyl acetate, phenylacetaldehyde, yeast, solvent engineering

The use of whole cells as biocatalysts for the manufacture of industrially important chemicals is now well established [3, 6, 7, 9, 13]. The main attraction of this approach is the ability to produce rather complex compounds in effectively one step from simple and inexpensive starting materials. However, the selection of suitable microorganism strains still remains a challenging problem. Typically, this is achieved by extensive screening programmes, where thousands of microorganisms are tested to assess the accumulation of the desired product in the reaction mixture. Such protocols often discriminate against the strains which do not secrete the target compound in the media even if it is produced intracellularly in reasonable quantities but are further metabolized.

Recently, we have shown that microorganisms can be "forced" to secrete hydrophobic intermediates into the media when they are grown in the presence of non-toxic organic solvents and small quantities of Tween-80, which is added to facilitate the transfer of these metabolites from the aqueous interior of cells into the solvent [10]. As a result of this thermodynamically driven efflux, the intermediates of interest accumulate in the organic phase in sufficient quantities for their analysis and identification. The application of this simple principle to the analysis of the bioconversion of (R)-ricinoleic acid to (R)-γ-decalactone catalyzed by the yeast *Sporidiobolus ruinenii* enabled us to detect every intermediate hydroxy acid in the β-oxidation pathway, leading to the formation of the lactone in a single GC chromatogram. Effectively, we "sequenced" the whole metabolic pathway with no requirement for the laborious isolation of each individual metabolite from cell free extracts [10]. Significantly, all the intermediate hydroxy acids and (R)-γ-decalactone, the final product of this biotransformation, accumulated in the organic phase in similar quantities, whilst in a conventional aqueous fermentation the lactone was present as the sole product. This suggested that the presence of organic solvent, which acted as a thermodynamic "trap" for the escaped intermediates, might have altered the metabolic flux through the pathway, as schematically illustrated in Fig. 1 (pathway A).

The aim of this communication was to verify this hypothesis by showing that the metabolic flux through a pathway can indeed be altered to enable the accumulation of products/intermediates which are not detected in the media under conventional conditions. The bioconversion of L-phenylalanine **1** to phenethyl alcohol **4** (Fig. 1, pathway B) was chosen as an appropriate model because numerous microorganisms have been shown to catalyze this sequence of reactions but none of them produced phenylacetaldehyde **3**, a useful natural flavour compound [2, 4].

Yarrowia lipolytica (KCCM 50506, Korean Culture Centre of Microorganisms, Yonsei University, Seoul, Korea) and *Hansenula anomala* (CBS 110, Centraalbureau Voor Schimmelcultures Baarn, The Netherlands) were maintained on malt agar (MA) at 4~6°C and grown on a slant of MYA (containing in g/l; malt extract, 10; yeast extract, 4; glucose, 4; agar, 15; adjusted to pH 6.0) for 48 h

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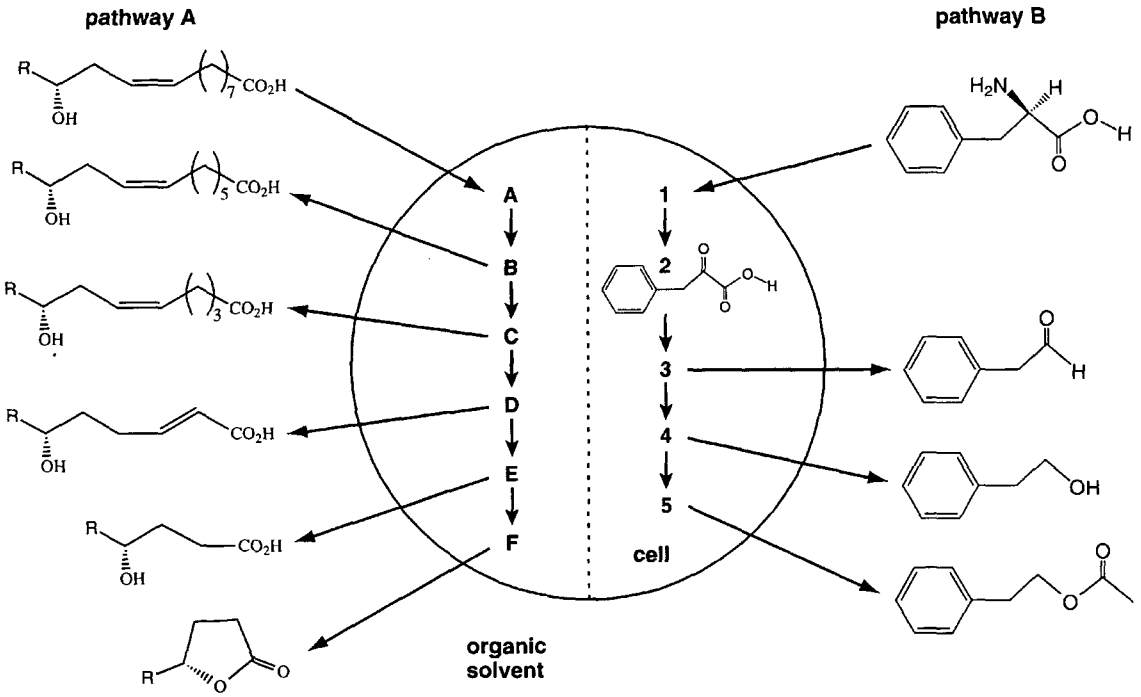

Fig. 1. The use of organic solvents as a thermodynamic trap for the analysis of the bioconversion of (R)-ricinoleic acid (R)-γ-decalactone (pathway A) and alteration of the metabolic flux in a cell during the biotransformation of L-phenyalanine **1** to phenylacetaldehyde **3**, phenethyl alcohol **4**, and phenethyl acetate **5** (pathway B).

at 30°C prior to culturing. The microorganisms grown on MYA were suspended in 1 ml of distilled water and used to innoculate 20 ml of MYB culture medium in a 100-ml Erlenmeyer flask (containing in g/l; malt extract, 10; yeast extract, 4; glucose, 4; adjusted to pH 6.5) which was subsequently incubated at 30°C with orbital shaking (Luckham, 300 rpm) for 24 h. Cells from this broth (OD=1.7) were harvested by centrifugation of a 5 ml aliquot (3600 rpm for 5 min) followed by rinsing of the pellet with two 5-ml aliquots of buffer (50 mM MES, pH 6.6). These cells were then resuspended in 5 ml of GF medium (containing in g/l; glucose, 20; L-phenylalanine, 2; Tween-80, 1; dissolved in 50 mM MES buffer, adjusted to pH 6.6) in 50-ml Duran bottles. The medium was purged with oxygen, the bottles sealed, and the biotransformations were performed at 30°C with agitation in an Luckham orbital shaker-incubator (250 rpm). In the two-phase experiments, decane (5 ml) was added to this biotransformation medium.

Analysis of the products present in the biotransformation broth was performed after the addition of internal GC standard to the aqueous layer (1-phenylpropanol or phenylethyl propionate in 100 µl of ethanol to a final concentration of 1 mg/ml), centrifugation of the sample (3000 rpm for 3 min), and separation of the supernatant prior to extractive workup. In the aqueous biotransformations, the reaction mixture was extracted with decane (2×5 ml) and 10 µl of the organic layer was taken for GC chromatographic analysis. In the two-phase experiments, 5 ml of decane was added to the supernatant obtained from the reaction mixture and 10 µl of this organic layer was injected directly for GC analysis. GC analysis was performed using an HP 5890 GC (Split) System with a capillary column, Phenyl Methyl Siloxane 5% crosslinked, Hp-Ultra 2 (25 m × 0.2 min; film thickness=0.33 µm) connected to a FID detector, and using helium as a carrier gas at a flow rate of 0.7 ml/min. The injection temperature was set to 300°C and the detector was at 340°C. The oven temperature was programmed from 70°C to 300°C at 10°C/min. Samples were injected using a split/splitless injector, split ratio 1/100.

As a result of intensive screening, a number of microorganisms, e.g. *Saccharomyces cerevisiae, Kloeckera saturnus, Hansenula anomala, Pichia etchelsii*, and *Yarrowia lipolytica*, were found to produce phenethyl alcohol from L-phenylalanine in a conventional aqueous fermentation [1, 5]. The amount of product obtained varied depending on the microorganism and reaction conditions used but, crucially for the purpose of this investigation, there was no evidence for the production of the intermediate phenylacetaldehyde in these experiments [1]. We reasoned that if these microorganisms are capable of producing phenethyl alcohol in suitable quantities, there should be a relatively high flux through the corresponding pathways. Hence, the addition of Tween-80 to facilitate the penetration of phenylacetaldehyde

(the immediate precursor of phenethyl alcohol) through cell membranes and an organic solvent to trap it irreversibly may well alter this flux, thus leading to the accumulation of this compound in the organic phase. In order to test the feasibility of such a "solvent engineering" we performed the biotransformation using two microorganisms, *Hansenula anomala* and *Yarrowia lipolytica*, in a standard aqueous medium supplemented with an equal volume of decane. The latter was selected as a suitable non-toxic organic solvent for the extraction of phenylacetaldehyde, since in our previous work [10] decane was shown to have minimum deleterious effects on the viability of yeast. Preliminary tests confirmed that both *Hansenula anomala* and *Yarrowia lipolytica* remained viable in the presence of decane and, more significantly, were able to catalyze the transformation of L-phenylalanine to phenethyl alcohol under these conditions (Table 1).

We then proceeded to investigate the composition of the organic phase and to compare the product(s) formed with those obtained in an aqueous biotransformation of L-phenylalanine (Table 1). As expected, both microorganisms produced significant amounts of phenethyl alcohol in the standard aqueous media and addition of 0.1% Tween-80 had virtually no effect on the amount of product formed. However, when the same reaction was carried out in the presence of decane, a rather different pattern was observed with the two microorganisms studied. The addition of the organic solvent had a pronounced inhibitory effect on the ability of *Hansenula anomala* to synthesize phenethyl alcohol with approximately four times less product formed during a 48 h incubation period. Also, no phenylethyl acetate and phenylacetaldehyde were found in the decane. In contrast, the presence of decane led to a slightly improved production of phenethyl alcohol by *Yarrowia lipolytica* and, more importantly, phenylacetaldehyde was found in the organic phase, too.

Another interesting feature of the latter reaction was the formation of phenethyl acetate **5**. This finding was unexpected but hardly surprising since the production of **5** from L-phenylalanine by yeast strains had been previously documented [1]. It is generally assumed that phenethyl alcohol **4** is readily excreted into the media by some microorganisms (and hence it is the major product of the biotransformation), whilst, in others, **4** is further esterified to give phenylethyl acetate. For example, *Kloeckera saturnus* was shown to produce **5** in large excess over **4** [1]. It seems clear therefore that the enzymes involved in the esterification of phenethyl alcohol are present (or can be induced) in most, if not all, yeast strains, although their activity relative to the rate of phenethyl alcohol secretion may differ widely depending on the strain and fermentation conditions used [1]. Presumably, it is the ratio between the rate of esterification/degradation and excretion that determines which product is found in the media, and in what quantity. The addition of organic solvent therefore may be expected to alter this balance. Thus, the formation of phenylethyl acetate as well as phenylacetaldehyde in the presence of decane was interpreted as further evidence of solvent-induced alteration of the metabolic flux.

Encouraged by these results, we performed another series of experiments aimed at increasing the concentration of phenylacetaldehyde in the organic phase to a level attractive for preparative synthesis. To this end, the reaction was followed over a period of 96 h to assess the kinetics of product accumulation. It was found that the phenylacetaldehyde concentration in the reaction media reached a maximum of about 24 mg/l within 48 h and then declined over longer incubation periods (not shown). The addition of glucose had a marginal effect on the product accumulation but a five fold increase in the initial concentration of L-phenylalanine to 10 g/l did lead to a noticable increase in the concentration of **3** in the organic phase. Unfortunately, even at a final aldehyde concentration of 47 mg/l after 48 h, the productivity of the system was judged insufficient to be attractive for practical applications.

In conclusion, we have shown that the addition of organic solvents to the biotransformation media can cause a significant alteration to the intracellular flux of hydrophobic

Table 1. The bioconversion of L-phenylalanine **1** by *Hansenula anomala* and *Yarrowia lipolytica*.

Microorganism	% Concentration of Tween-80 (v/v)	Reaction media	Concentration of phenethyl alcohol **4** (mg/l)	Concentration of phenylacetaldehyde **3** (mg/l)	Concentration of phenethyl acetate **5** (mg/l)
Y. lipolytica	0	buffer[1]	312	-	-
Y. lipolytica	0.1	buffer[1]	393	-	-
Y. lipolytica	0.1	buffer/decane[2]	552	24	38
Y. lipolytica	0.1[3]	buffer/decane[2,3]	662[3]	-	-
H. anomala	-	buffer[1]	1481	-	-
H. anomala	0.1	buffer[1]	1300	-	-
H. anomala	0.1	buffer/decane[2]	344	-	-

[1]Aqueous reactions were performed at 30°C over 48 h in 50 mM MES buffer, pH 6.6 (5 ml), containing glucose (2% w/v) and L-phenylalanine (0.2% w/v).
[2]In the two-phase reactions, decane (5 ml) was added to the aqueous reaction mixture.
[3]This reaction was performed at a concentration of 1% (w/v) L-phenylalanine.

intermediates leading to their "escape" from the cell. The feasibility of such a solvent engineering was demonstrated by the formation of phenylacetaldehyde **3**, a product which had not been previously observed in aqueous reaction mixtures, and phenethyl acetate **5**. This phenomenon is clearly different from the well documented used of solvents in aqueous-organic two phase fermentations [8, 11, 12], where the function of added solvent is to facilitate the use of high substrate concentrations and/or extraction of poorly water soluble products from the fermentation broth, rather than to produce intermediates that are not easily obtained under conventional conditions. We can envisage two possible applications of the solvent engineering methodology proposed in this work. Firstly, it may aid the screening of microorganisms for the production of hydrophobic metabolites. Currently, strains that do not secrete the target compound in the media are often discarded from further analysis. It is evident from the results obtained in this study, that re-testing strains which are capable of forming products downstream in the metabolic pathway in the presence of organic solvents is a sensible strategy. Secondly, this approach may lead to the preparation of new products by conventional two-phase fermentations. Although we found the concentration of phenylacetaldehyde in the reaction mixture to be insufficient for practical exploitation, an extensive optimization of the reaction conditions or the use of other strains in place of *Yarrowia lipolytica* may well result in dramatic improvements. In this context, a combination of genetic and solvent engineering seems especially attractive, as the former enables the reduction of flux through the unwanted part of the pathway whilst the latter should enhance the extraction of the desired product from the cell.

It is not yet clear why the response of *Hansenula anomala* and *Yarrowia lipolytica* to the exposure of decane should be so dissimilar. Several explanations can be advanced, e.g. different sensitivity to the organic solvent (as the name suggests *Yarrowia lipolytica* would be expected to tolerate hydrocarbons better) or the presence of very different intracellular levels of phenylacetaldehyde in the two microorganisms (the rate of accumulation of any intermediate in the organic phase should be dependent on its concentration in the cytoplasm). Further experimentation will be required to discriminate between these and other possibilities. The important point, however, is that one out of two microorganisms tested in this work showed the production of the desired product and positive results were obtained previously during the bioconversion of (R)-ricinoleic acid to (R)-γ-decalactone catalyzed by the yeast *Sporidiobolus ruinenii* [10]. These observations suggest that, although one cannot yet rationally predict the outcome of screening, there is a reasonable chance of finding a suitable microorganism using this methodology.

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REFERENCES

1. Albertazzi, E., R. Cardillo, S. Servi, and G. Zucchi. 1994. Biogeneration of 2-phenylethanol and 2-phenylethylacetate: Important aroma compounds. *Biotechnol. Lett.* **16:** 491–496.
2. Cheetham, P. S. J. 1993. The use of biotransformations for the production of flavours and fragrances. *TIBTECH.* **11:** 478–488.
3. Faber, K. and M. C. R. Franssen. 1993. Prospects for the increased application of biocatalysts in organic transformations. *Trends Biotechnol.* **11:** 461–470.
4. Fuganti, C., S. Servi, M. Barbeni, and P. Cabella. 1993. New avenues in natural products: Strategies in the biogeneration of flavours, pp. 295–345. *In*: A.-U. Raman and F. Z. Basha (eds.), *Studies in Natural Products Chemistry*, vol. 13A, Elsevier.
5. Fukuda, K., M. A. Watanabe, and K. Asano. 1990. Altered regulation of aromatic amino acid biosynthesis in β-phenethyl-alcohol-overproducing mutants of *sake* yeast *Saccharomyces cerevisiae. Agric. Biol. Chem.* **54:** 3151–3156.
6. Holland, H. L. 1998. Microbial transformations. *Curr. Opi. Chem. Biol.* **2:** 77–84.
7. Janssens, L., H. L. DePooter, N. M. Schamp, and E. J. Vandamme. 1992. Production of flavours by microrganisms. *Process Biochem.* **27:** 195–215.
8. Lilly, M. D. and J. M. Woodley. 1996. A structured approach to design and operating of biotransformation processes. *J. Ind. Microbiol.* **17:** 24–29.
9. Roberts, S. M. 1998. Preparative biotransformations: The employment of enzymes and whole-cells in synthetic organic chemistry. *J. Chem. Soc. Perkin Trans. 1* **1:** 157–169.
10. Spinnler, H. E., C. Ginies, J. A. Khan, and E. N. Vulfson. 1996. Analysis of metabolic pathways by the growth of yeast cells in the presence of organic solvents. *Proc. Natl. Acad. Sci. USA* **93:** 3373–3376.
11. Van Sonsbeek, H. M., H. H. Beeftink, and J. Tramper. 1993. Two-liquid-phase bioreactors. *Enzyme Microb. Technol.* **15:** 722–729.
12. Woodley, J. M., P. J. Cunnah, and M. D. Lilly. 1991. Stirred tank 2-liquid phase biocatalytic reactor studies — kinetics, evaluation and modelling of substrate mass transfer. *Biocatalysis* **5:** 1–12.
13. Zaks, A. and D. R. Dodds. 1997. Application of biocatalysis and biotransformations to the synthesis of pharmaceuticals. *Drug Discovery Today* **2:** 513–531.