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# Whole cell yeast biotransformations in two-phase systems: effect of solvent on product formation and cell structure

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## SUMMARY

Biotransformation of benzaldehyde and pyruvate to (R)-phenylacetyl carbinol by *Saccharomyces cerevisiae* was investigated in two-phase aqueous-organic reaction media. With hexane as organic solvent, maximum biotransformation activity was observed with a moisture content of 10%. Of the organic solvents tested, highest biotransformation activities were observed with hexane and hexadecane, and lowest activities occurred with chloroform and toluene. Biocatalyst samples from biphasic media containing hexane, decane and toluene manifested no apparent cell structural damage when examined using scanning electron microscopy. In contrast, cellular biocatalyst recovered from two-phase systems containing chloroform, butylacetate and ethylacetate exhibited damage in the form of cell puncturing after different incubation periods. Phospholipids were detected in reaction media from biocatalytic systems which exhibited cell damage in electron micrographs. Phospholipid release was much lower in the two-phase systems containing toluene or hexane or in 100% aqueous biocatalytic system.

## INTRODUCTION

Baker's yeast has attracted substantial attention as a catalyst for biotransformation processes, especially involving carbon–carbon bond formation and oxidation reduction reactions, because yeast is so inexpensive and easy to obtain [24]. Yeast induced carbon–carbon condensation of benzaldehyde to acetaldehyde in the production of (R)-phenylacetyl carbinol (PAC), a precursor of ephedrine, was one of the first microbial biotransformation processes to be commercialised [10]. During production of PAC, some of the benzaldehyde is also reduced to benzyl alcohol [17]. In our laboratory, biotransformation processes for production of PAC and benzyl alcohol have been investigated in detail in aqueous systems with a view to characterising factors influencing these reactions [16,17]. We have demonstrated that a wide range of substituted aromatic aldehydes can be converted to the corresponding substituted carbinol products and indeed substituted aromatic alcohol by-products [18]. While the conversion of benzaldehyde to benzyl alcohol can be catalysed by yeast alcohol dehydrogenase, we have shown that mutant strains lacking the main ADH isoenzymes manifest similar capacity to wild-type strains to produce benzyl alcohol [19]. The capacity of baker's yeast to carry out asymmetric reductive biotransformations of carbonyl compounds in aqueous media has been widely recognised.

Thus, conventionally, yeast has been used as a biocatalyst for organic synthesis in aqueous conditions [24,27]. However, the majority of organic chemicals are poorly water soluble but highly soluble in organic solvents and the implementation of biotransformation systems in organic solvent-containing media offers potential to increase the solubility of poorly water-soluble substrates [28]. While some investigations of biotransformations using isolated yeast enzymes in micro-aqueous solvent systems and two-phase systems have been documented [4,5,23,32], little emphasis has been placed on whole cell systems. Whole cell biotransformations have advantages over isolated enzymes in that costs due to catalyst extraction and purification are avoided, an opportunity to recycle the enzyme-containing cells is provided, and the biotransforming enzyme may be stabilised in the intra-cellular milieu [16,19,31].

Only a small amount of water is required by enzymes to maintain catalytic activity [11]. Where enzyme reactions have been carried out in organic solvents having a very low water content (<0.02%) the system has been described as 'micro-aqueous' [23,29]. Where organic and aqueous phases are present in excess of mutual saturation levels, the reaction medium is described as 'biphasic' [12,14]. Whole cell biocatalysts tend to require more water than isolated enzymes and appear to generally require

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biphasic systems for biotransformation of poorly water soluble substrates [6].

We have recently reported the capacity of wild-type and mutant strains of *Saccharomyces cerevisiae* to carry out reductive biotransformations in aqueous-organic solvent biphasic systems [20]. We now report a study of the biotransformation of benzaldehyde and pyruvate to PAC in two-phase aqueous-organic solvent systems with emphasis on the effect of solvent on product formation and cell structure.

## MATERIALS AND METHODS

*Yeast culture.* Fresh, pressed commercial baker's yeast (30% dry weight) was obtained from Fleischmanns Inc., Kitchener, Ontario, Canada.

*Preparation of biocatalysts for biotransformation.* Fresh pressed commercial baker's yeast (50 g) was suspended in 50 ml 0.05 M sodium citrate buffer (pH 6.0), and lyophilised using a Labconco (Kansas, MI, USA), lyophiliser, Lock 4,5. The lyophilised cells, 300 mg, were mixed with 1 g of celite (for gas chromatography, particle size 30–80 mesh, British Drug House, Poole, UK) and the mixture was resuspended in 0.05 M sodium citrate buffer (pH 6.0). The suspension was lyophilised again and stored at 4 °C for use in biotransformation experiments.

*Preparation of organic solvents.* All organic solvents were obtained in anhydrous form or with the lowest water content possible. Prior to use the solvents were saturated with 0.05 M sodium citrate buffer (pH 6.0). To prepare two-phase systems containing different moisture levels, different proportions of pre-saturated solvents and 0.05 M sodium citrate buffer (pH 6.0) were mixed. For example, to prepare a 10% moisture level, 10 ml of buffer was mixed with 90 ml of presaturated solvent.

*Biotransformation conditions.* Lyophilised biocatalysts, 1.3 g (1 g support material and 300 mg dried cells) was added to a 250-ml Erlenmeyer flask containing 30 ml of biotransformation medium: aqueous or corresponding aqueous-organic solvent two-phase system. Sodium pyruvate (50 g/l) was used as substrate. The reaction was initiated by the addition of the cosubstrate benzaldehyde (6 g/l). Biotransformations were conducted on an orbital shaker set at 28 °C and 300 rpm. The progress of product formation was monitored using gas chromatography. Samples were also taken at 0, 2, 6 and 26 h to study the effect of the organic solvents on the cell structure by electron scanning microscopy.

*Gas chromatography (GC).* PAC, benzaldehyde and benzyl alcohol concentrations were determined by GC analysis using a Shimadzu GC 14A gas chromatograph equipped with a flame ionization detector and connected to a Shimadzu Chromatopac CR6A integrator. The column was a fused silica megabore 30 m long and 0.52 mm internal diameter coated with 1 $\mu$m thickness of 25% cyanopropyl, 25% phenyl, 50% methyl polysiloxane (Durabon 225; Chromatographic Specialities, Brockville, Ontario, Canada). Analytical conditions were: injection and column temperature 150 °C and detector temperature 200 °C. Helium gas was used as the carrier and cyclohexanone was used as internal standard. Aqueous phase samples were extracted twice with an equal volume of diethyl ether. PAC (Knoll, AG, Ludwigshafen, Germany), benzyl alcohol and benzaldehyde (Sigma, St. Louis, MO, USA) were used as standards.

*Scanning electron microscopy (SEM).* Yeast cells were air dried and stored in a desiccator containing silica gel (self-indicating about 6–20 mesh, British Drug House Inc., Toronto, Canada). Sample preparation: the cells were gold coated (200–300 Å) with SEM Coating Unit PS3. Accelerating voltage of 15 kV was used in a Hitachi S-570 Scanning Electron Microscope.

*Thin-layer chromatography (TLC).* Cells, incubated in the aqueous phase and two-phase systems for 26 h, were separated from the medium. The supernatants from the two-phase systems were evaporated using a rotary vacuum evaporator and the residues were kept for TLC analysis. The aqueous phase sample was freeze dried. The residue was dissolved in a 6 ml mixture of chloroform/methanol/water (1:2:0.8), vortexed for 1 min and centrifuged at $5000 \times g$ for 5 min and the supernatant was separated. The extraction procedure was repeated and the two supernatants were combined. To the extract, 8 ml of water and 3 ml of chloroform was added and the mixture vortexed for 1 min and centrifuged at $5000 \times g$ for 5 min. The bottom (chloroform) layer was dried at 36 °C using a heating block under nitrogen stream for TLC analysis.

The residues were dissolved in 50 $\mu$l of chloroform and used for TLC. TLC plates were Si 250, precoated glass plates (silica gel, 250 $\mu$m, hard-surfaced analytical layer from J.T. Baker Inc., NJ, USA). The standards (phosphatidylethanolamine, phosphatidylcholine, phosphatidic acid, phosphatidylinositol, phosphatidylserine and phosphatidylglycerol) were purchased from Matreya Inc., PA, USA. Samples (10 $\mu$l) were applied to the TLC plate (20 cm × 20 cm) and developed in a mixture of chloroform/methanol/water (65:25:4). Dittmer and Lester spray reagent [9], which is specific for phospholipids was used for detection. The $R_F$ values are presented in Table 1.

## RESULTS

Production of PAC from benzaldehyde and pyruvate was chosen as a model system in these investigations. The catalyst consisted of lyophilized yeast cells adsorbed on

TABLE 1

$R_F$ values of phospholipids separated by thin-layer chromatography

| Phospholipids | $R_F$ |
|---|---|
| Phosphatidylethanolamine (PE) | 0.58 |
| Phosphatidylinositol (PI) | 0.22 |
| Phosphatidylcholine (PC) | 0.26 |
| Phosphatidic acid (PA) | 0.37 |
| Phosphatidyl serine (PS) | 0.25 |
| Phosphatidylglycerol (PG) | 0.40 |

TABLE 2

The effect of moisture content on PAC production by whole yeast cells

| % moisture[a] | Activity (mmol/h/g) |
|---|---|
| 0.5 | $3.7 \times 10^{-2}$ |
| 2.0 | $3.5 \times 10^{-2}$ |
| 10.0 | $5.4 \times 10^{-2}$ |
| 20.0 | $4.2 \times 10^{-2}$ |
| 40.0 | $4.4 \times 10^{-2}$ |

[a] See procedure for preparation of organic solvents in Materials and Methods.

celite. Scanning electron micrographs of the carrier celite and yeast cells lyophilised on celite are presented in Fig. 1.

The effect of moisture content on production of PAC by cells immobilised on celite was investigated using hexane as organic solvent (Table 2). Maximum biotransformation activity was observed with a moisture level of 10%. The effect of solvent type on rate of production of PAC was investigated in two-phase systems containing 10%

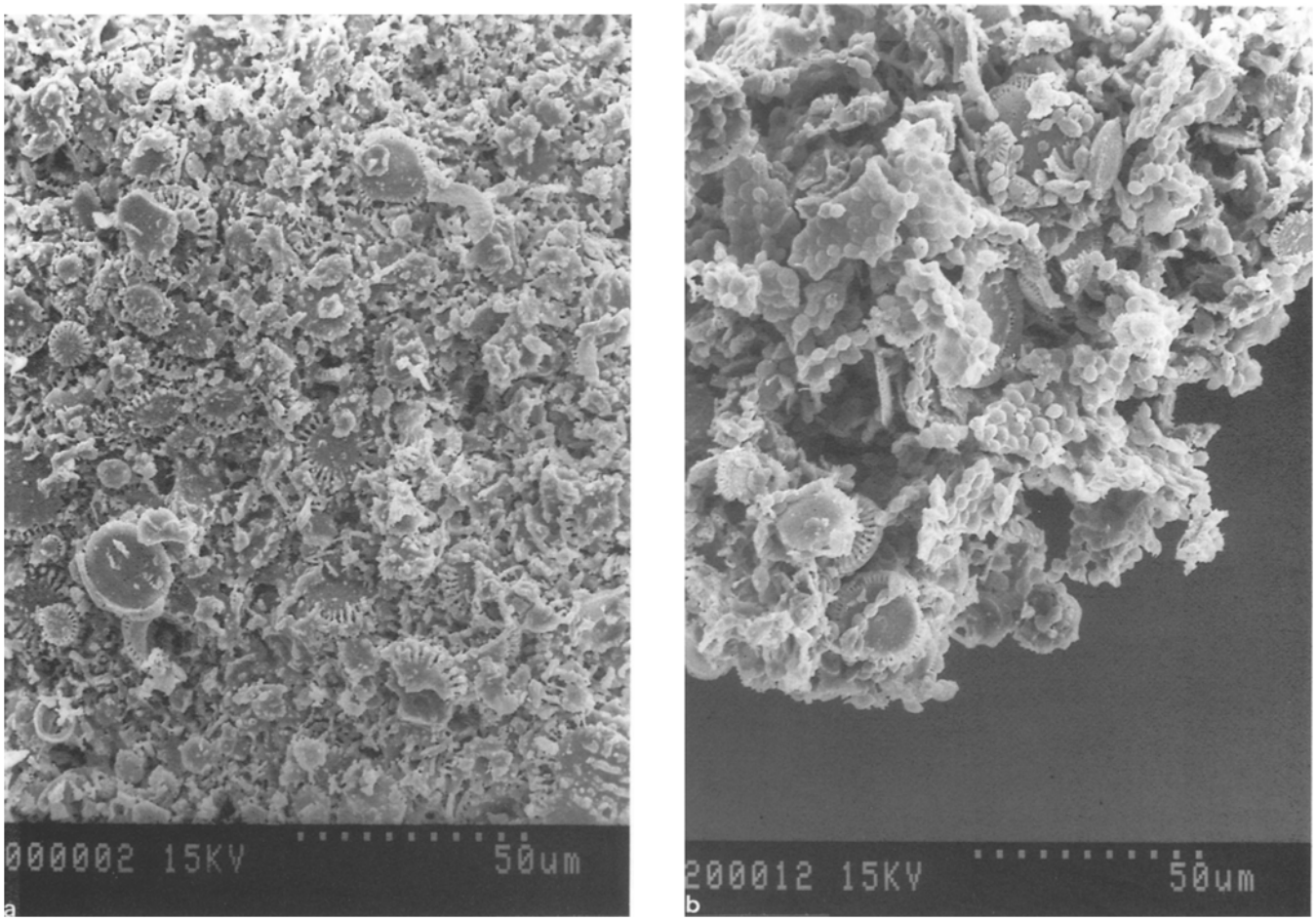


Fig. 1. Scanning electron micrograph of (a) the celite carrier and (b) yeast cells adsorbed on celite.

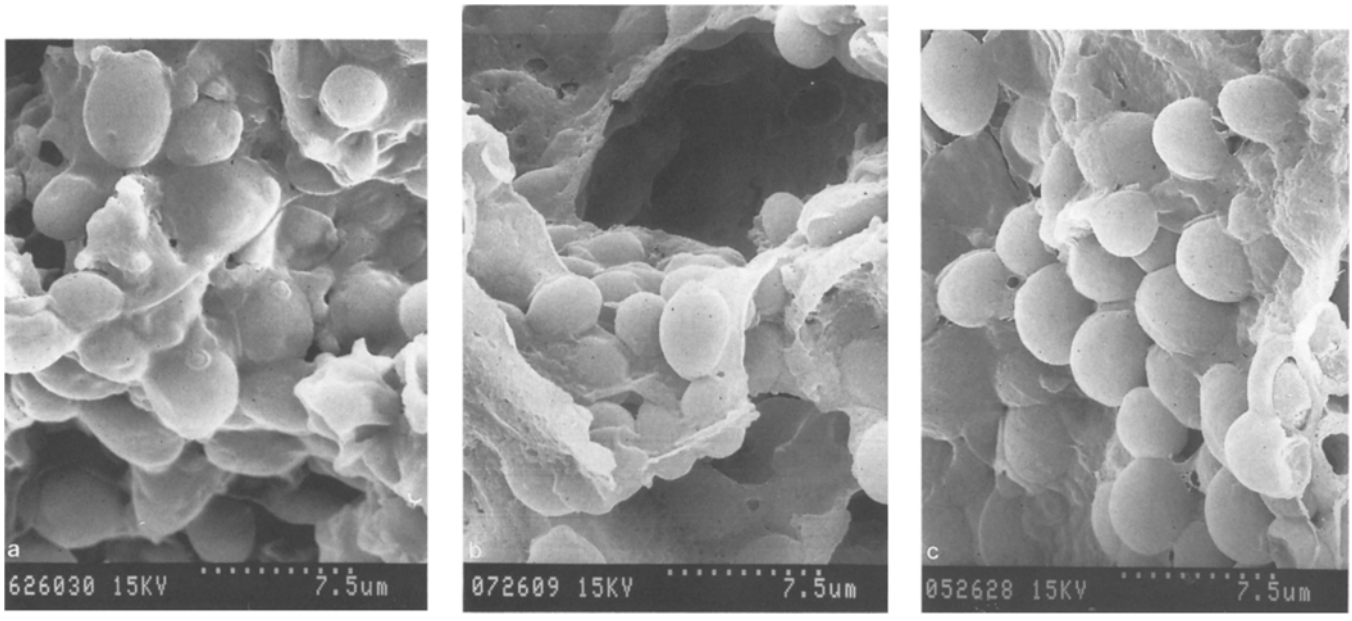


Fig. 2. Scanning electron micrographs of yeast cells from biphasic media containing (a) hexane, (b) decane, and (c) toluene after a 26-h biotransformation.

moisture and related to log P. The results are presented in Table 3. Highest biotransformation activities were observed with hexane and hexadecane and lowest activities were noted with chloroform and toluene.

Samples of the biocatalyst were withdrawn from the reaction mixture at 0, 2, 6, and 26 h, and were examined using scanning electron microscopy. Biocatalyst samples from biphasic media containing hexane, decane and toluene manifested no apparent damage after 26 h, as illustrated in Fig. 2. An electron scanning micrograph of a biocatalyst suspended only in aqueous phase for 26 h is illustrated in Fig. 3, again manifesting no apparent dam-

TABLE 3

Effect of organic solvent on PAC production by whole yeast cells

| Solvent[a] | Log P | Activity (mmol/h/g) |
|---|---|---|
| Ethylacetate | 0.68 | $2.84 \times 10^{-2}$ |
| Butylacetate | 1.70 | $3.10 \times 10^{-2}$ |
| Chloroform | 2.00 | $1.00 \times 10^{-2}$ |
| Toluene | 2.50 | $0.70 \times 10^{-2}$ |
| Hexane | 3.50 | $6.00 \times 10^{-2}$ |
| Dodecane | 6.60 | $4.00 \times 10^{-2}$ |
| Hexadecane | 8.80 | $5.60 \times 10^{-2}$ |

[a] The biotransformation medium consisted of each organic solvent containing 10% moisture.

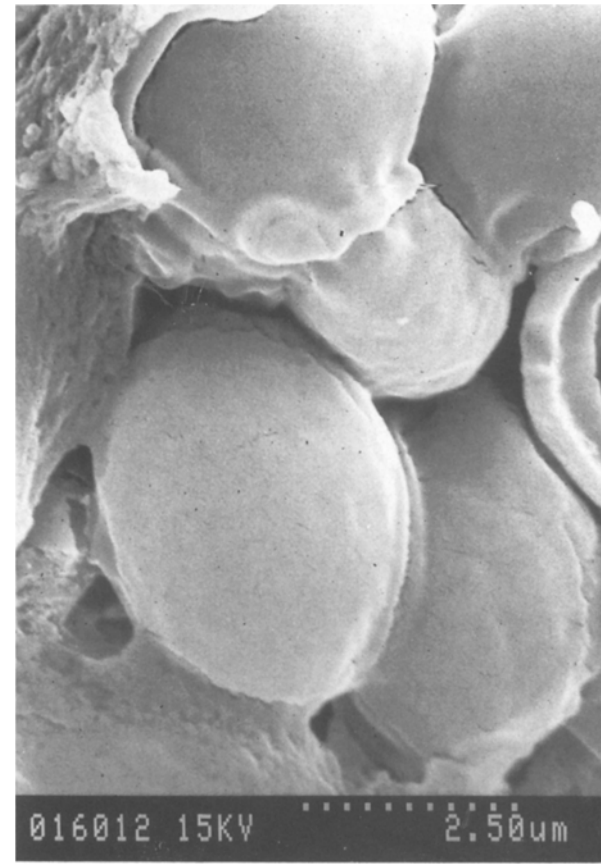


Fig. 3. Scanning electron micrograph of biocatalyst in aqueous phase after a 26-h biotransformation.

age. Biocatalyst recovered from two-phase systems containing chloroform at zero time and after a 2-h incubation are illustrated in Fig. 4. Note that the cells from the 2-h sample appear to be punctured. In contrast, biocatalyst recovered from butyl acetate containing media appeared intact at 2 h but damaged at 6 h (Fig. 5), while samples from ethylacetate containing media appeared intact after 6 h but damaged at 26 h (Fig. 6).

The effect of solvent on release of phospholipids from the cell was also investigated after 26 h incubation using TLC (Fig. 7). The biocatalyst was recovered by centrifugation from the reaction mixture and the latter was concentrated for TLC analysis. When the samples were subjected to TLC and tested for phospholipids, little or no phospholipid was detected from cells incubated in toluene, hexane or in the 100% aqueous system. Note that samples from decane, dodecane and hexadecane were not analyzed as these solvents could not be evaporated because of their high boiling points. On the other hand, samples from reaction mixtures containing ethylacetate, butylacetate, and chloroform manifested the presence of phospholipids, as is illustrated in Fig. 7. Thus, phospholipids were detected in samples from biocatalytic systems which exhibited cell damage in electron micrographs.

## DISCUSSION

In this study, the ability of baker's yeast to produce PAC by biotransformation of benzaldehyde and pyruvic acid in biphasic systems was demonstrated. Very little attention has been paid to implementation of whole cell biotransformations in aqueous-organic media. Where whole cell biotransformations in biphasic media have been investigated, studies, in general, focused on reactions involving steroids, lipids and esters. However, yeast cells having esterase activity stereoselectively hydrolysed methyl esters in organic solvents [22]. We have previously demonstrated the reduction of benzaldehyde to benzyl alcohol by baker's yeast in aqueous-organic biphasic systems [20]. As was observed above for PAC production,

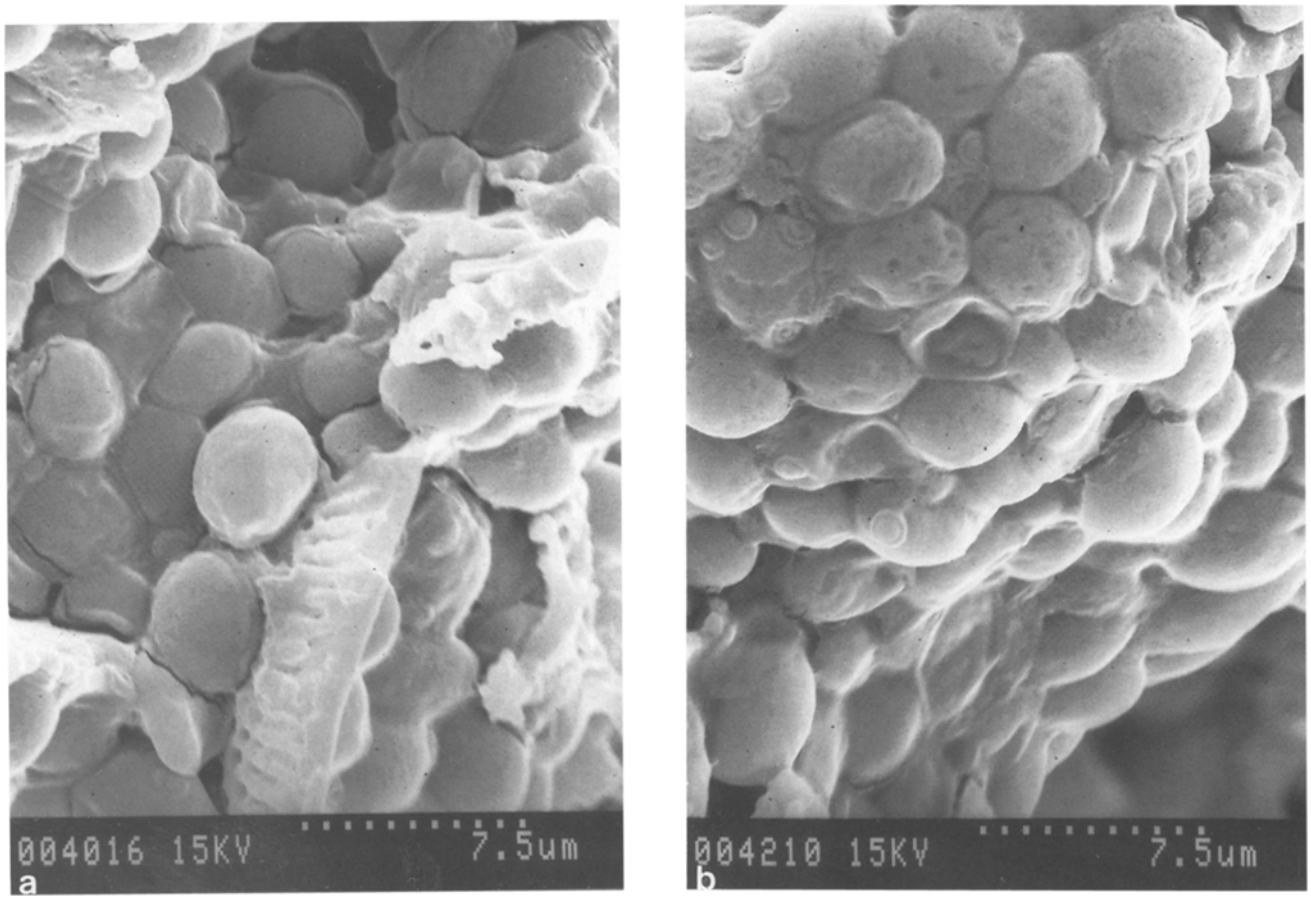


Fig. 4. Scanning electron micrographs of biocatalyst recovered from two-phase systems containing chloroform (a) at 0 time, and (b) after a 2-h biotransformation.

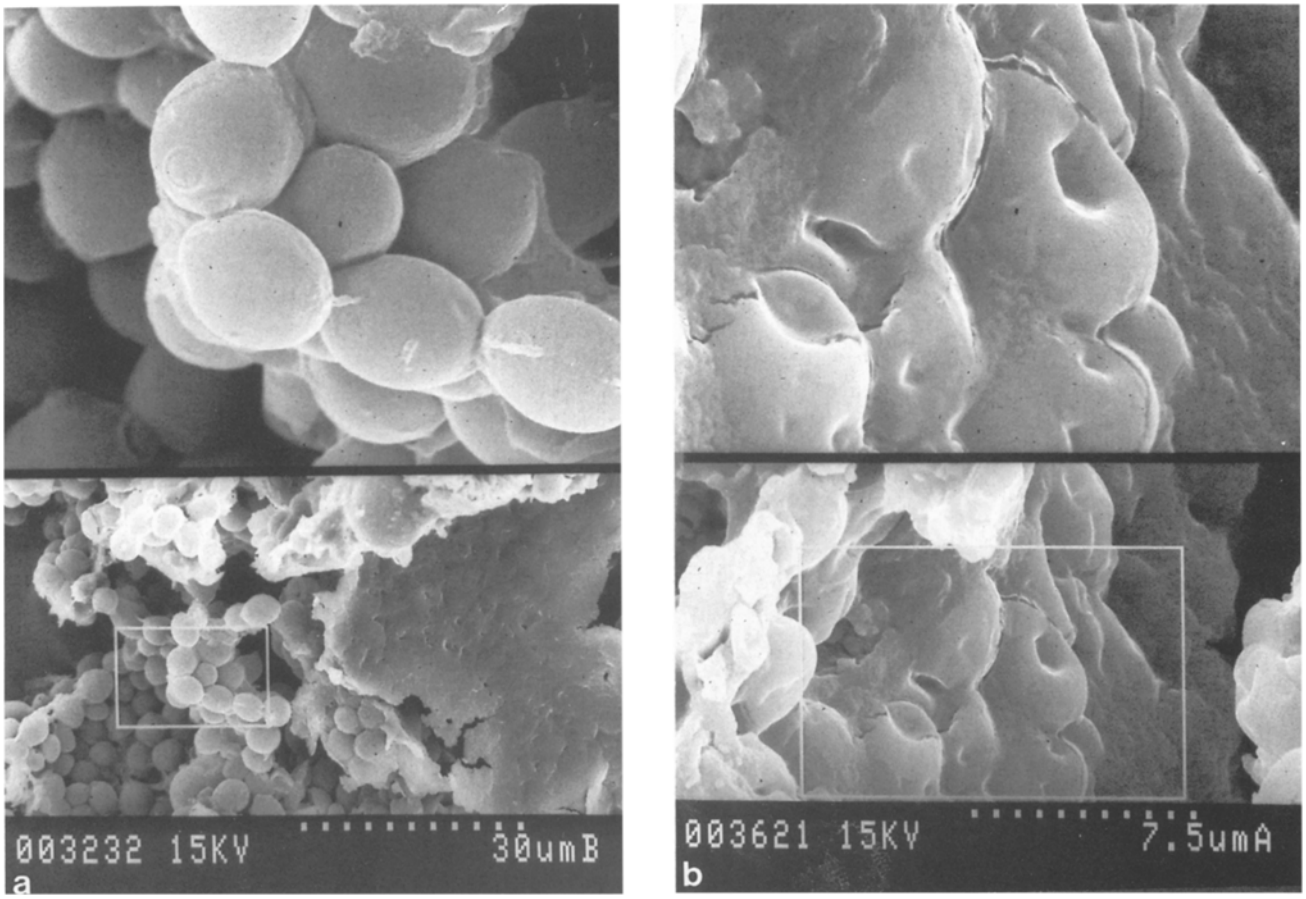


Fig. 5. Scanning electron micrographs of biocatalyst recovered from two-phase systems containing butyl acetate after a biotransformation period of (a) 2 h, and (b) 6 h.

biotransformation activity for conversion of benzaldehyde to benzyl alcohol increased with moisture content up to a value of 10% v/v and, in both cases, the organic solvent hexane manifested higher biotransformation activity [20]. Deetz and Rozzell [4] found an increase in activity of isolated alcohol dehydrogenase in acetonitrile up to a moisture content of 10%. In steroid transformations using *Nocardia* species, provided enough aqueous phase was present to completely swell the cells, changes in the ratios of aqueous-organic phases had little effect on reaction rate [2,30]. An important extension of our work will be to investigate the yeast-mediated biotransformation of less water-soluble substrate and cosubstrate analogues of pyruvate and benzaldehyde to produce analogues of PAC having potential biological activity.

It was noted that the cell surfaces of yeast biocatalyst samples recovered from biphasic media containing hexane, decane and toluene after a 26-h biotransformation reaction manifested no apparent damage, while cell puncturing was observed after shorter biotransformation periods with the more hydrophilic solvents (having log $P \leq 2.0$), chloroform, ethylacetate and butylacetate. Furthermore, we observed that the cell damaging solvents, ethylacetate, butylacetate, and chloroform, resulted in the extraction of phospholipids from the cells into the biphasic medium, whereas little or no phospholipids were detected from cells incubated in toluene or hexane biphasic media. Phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol constitute 80–90% of total whole cell and cell membrane phospholipids in *S. cerevisiae* [21]. Phospholipids are recognised as very important components of cell membranes which influence membrane permeability and elasticity [12]. Certain organic solvents have been shown to cause rapid breakdown of the permeability barrier function of cell membranes [3,7,8,25,26]. While the extent of membrane damage to bacterial suspensions appears to be related to the amount of solvent used [3,25], a relationship to solvent properties such as hydrophobicity has also been suggested [7]. Lilly et al. [15] have summarised the potential effects of organic solvents on cell morphology of

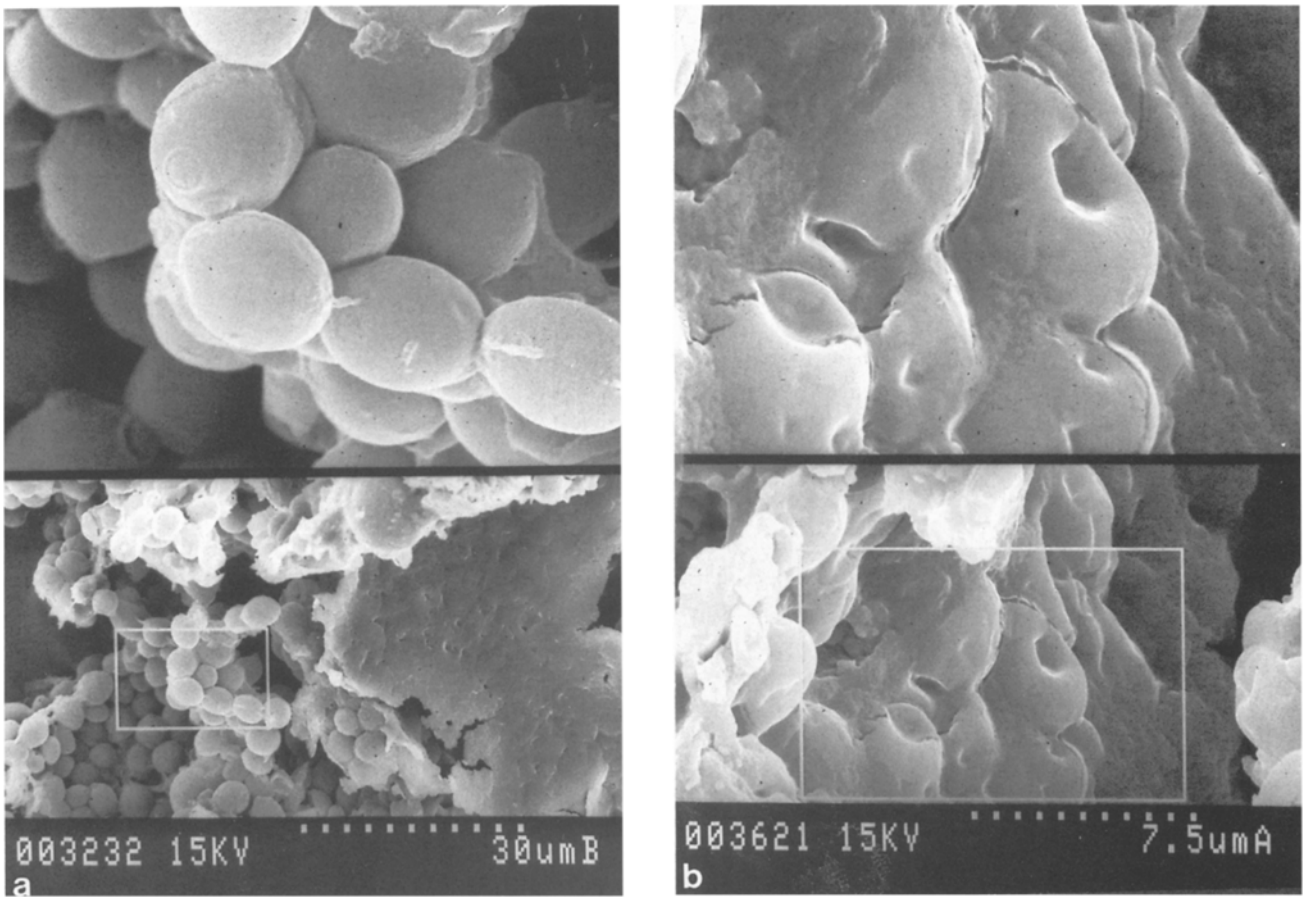


Fig. 6. Electron micrographs of biocatalyst recovered from biotransformation media containing ethyl acetate after (a) 6 h, and (b) 26 h.

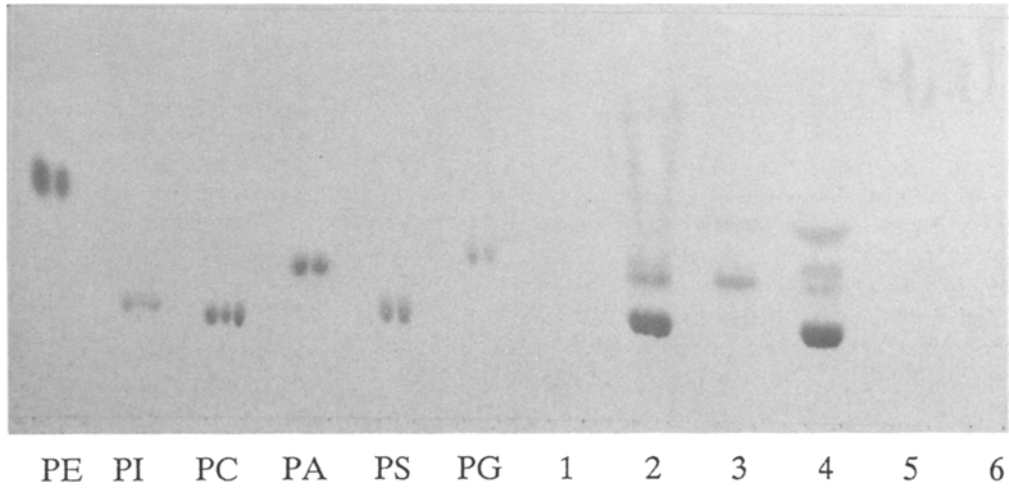


Fig. 7. Thin-layer chromatography of the effect of solvent release of phospholipids from the cell. Incubation time was 26 h. PE, PI, PC, PA, PS, PG: phospholipid standards see Table 1 for explanation of abbreviations. 1–6: phospholipids released during biotransformations. 1, aqueous phase; 2–6, two-phase systems containing: 2, ethylacetate; 3, butylacetate; 4, chloroform; 5, toluene; 6, hexane.

micro-organisms in terms of cytoplasmic shrinkage, loss of membrane organisation and ultrastructural changes. In maintaining the integrity of the biocatalyst in whole cell biotransformations carried out in aqueous-solvent two-phase systems, clearly selection of solvents which minimise cell damage requires careful consideration and experimentation [1].

Biocatalyst yeast cells taken from biotransformation reactions containing solvents with log P values of $\geq 2.5$ manifested no surface damage in scanning electron micrographs. Although two of the latter three solvents, namely hexane and dodecane, produced high reaction rates in biotransformations, toluene did not. Therefore, there was not a perfect correlation between apparent resistance of yeast cells to solvent damage and biocatalytic activity. Nevertheless, the results indicate that hexane, which manifested optimal rates of biocatalytic activity in these biotransformations, did not appear to damage cell structure, as judged from scanning electron micrographs.

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