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Alcohol-Oxidation Activity of Whole Cells of *Pichia pastoris* Entrapped in Hybrid Gels Composed of Ca-Alginate and Organic Silicate

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ABSTRACT

Formation of hybrid gels from an aqueous mixture of alginate and alkoxysilanes has been applied to immobilization of whole cells of *Pichia pastoris* catalyzing oxidation of benzyl alcohol in organic solvent. The amount of benzaldehyde produced after a prolonged reaction period was 1.2 and 1.8 times greater with the hybrid gels of alginate + silicate and alginate + methyl-substituted silicate, respectively, than with the alginate single gel. This was ascribed to a facilitated release rate of aldehyde, which acted as a strong inhibitor against the enzyme alcohol oxidase, from the inside of the cells to organic medium through hydrophobic gel matrix.

Index Entries: Sol-gel entrapment; alginate; silicate; hybrid gel; immobilized whole cells; *Pichia pastoris*; oxidation; benzyl alcohol; organic solvent.

INTRODUCTION

Ca-alginate gel is widely used for immobilization by entrapment of microbial cells, but is rarely employed in organic media owing to its hydrophilic nature. In previous reports *(1,2)*, we have demonstrated that a mixed matrix composed of alginate gel and silicone polymer could be effectively used for immobilization of biocatalysts functioning in organic media.

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Recently, inorganic silicate is often used as an immobilization material by hybridizing it with the alginate. Fukushima et al. *(3)* reported that *Saccharomyces cerevisiae* immobilized in a mixed gel composed of colloidal silica and alginate, provided a higher production rate of ethanol than that in the alginate single gel. Alkaline protease and β-galactosidase immobilized in this composite gel had improved thermal and operational stabilities. More recently, Heichal-Segal et al. *(4)* developed a novel immobilization technique forcing the sol-gel reaction of tetramethoxysilane as the silicate precursor within the Ca-alginate gel beads. The resultant alginate-silicate composite gel-entrapped β-glucosidase preserved an increased stability against both denaturing agents and heat inactivation.

In our recent publications *(5,6)*, we have explained that the organically modified silicates containing alkyl groups could be successfully used for immobilization of lipase-catalyzing esterification in organic solvent. In this study, we attempted to entrap whole cells of *Pichia pastoris* in a hybrid gel composed of alginate and organic silicate, and examined their catalytic activity for the oxidation of benzyl alcohol in organic solvent *(2)*.

MATERIALS AND METHODS

Benzyl alcohol and benzaldehyde were obtained from Ishizu Pharmaceutical (Osaka, Japan) and xylene from Kanto Chemical (Tokyo, Japan). Tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTrMOS) were of reagent grade from Tokyo Chemical Industry (Tokyo, Japan) and sodium alginate (NSPM) from Kibun Food Chemifa, Kamogawa, Japan. Colloidal silica (Snowtex 30; suspension of 30 wt% ultrafine silica particles with diameter of 10 ± 0.5 nm) was kindly donated by Nissan Chemical Industries (Tokyo, Japan).

The microorganism used was *Pichia pastoris* IFO 1013 obtained from the Institute for Fermentation (Osaka, Japan). Cell cultivation and induction of peroxisomal alcohol oxidase were conducted according to the same procedure as described elsewhere *(2)*. Immobilization of harvested cells was performed as follows: 1 mL of cell paste (0.1 g dry-cell mass) was mixed with an aqueous Na-alginate solution (pH 8.5; final alginate concentration, 2.3 wt% on the basis of the aqueous mixture except for the silicate precursor). In a separate tube, a prescribed amount of either TMOS or TMOS + MTrMOS was mixed with 1 mL distilled water, and then 10 μL of 0.04 N-HCl to make a homogeneous sol. The hydrolyzed alkoxysilane was then mixed vigorously with the alginate solution. In part of the experiments, colloidal silica was used in the place of either TMOS or TMOS + MTrMOS. Table 1 lists the compositions of each reagent added to make various mixtures of alginate-silicate sol. The resulting mixture (approx 10 mL) was poured into a 0.5 M Tris buffer solution (pH 8.5,

Table 1
Compositions of Starting Solutions Used for the Sol-Gel Entrapment of Whole Cells of *Pichia pastoris*

No.	Precursor[a] [mL]	Water [mL]	0.04 N-HCl [μL]	Na-alginate [mg]	Tris buffer [mL]	Cell paste[b] [mL]	Aqueous phase[c] [mL]
1[d]	0	0	0	233	9	1	10
2	1	1	10	210	7	1	9
3	2	1	10	187	6	1	8
4	3	1	10	163	5	1	7
5[e]	3	1	10	0	5	1	7
6[f]	5	1	10	117	3	1	5

[a] Volume of TMOS; Nos. 2–5: volume of colloidal silica; Nos. 3, 4, and 6: volume of TMOS + MTrMOS; No. 3.

[b] Concentrated cells (100 mg-dry-cell mass) in 0.5 MTris buffer (pH 8.5).

[c] Total volume of water, Tris buffer and cell paste added.

[d] Composition used for preparation of alginate-single gel.

[e] Composition used for preparation of silicate-single gel.

[f] Composition used only for preparation of colloidal silica-containing alginate.

100–400 mL) involving 0.1 M $CaCl_2$, and allowed to stand overnight at an ambient temperature to obtain the hybrid gel-entrapped cells. The resultant gel block was recovered, cut into cubes (2–3 mm in side length), and a small amount of moisture attached on the gel surface was wiped off with filter paper.

The reacting mixture contained 4.5–7 g of the cell-entrapping gels and 19 mL organic phase containing 27.6 g/L of benzyl alcohol in xylene as the solvent. The batch reactions were carried out at 30°C in a 500-mL shake flask on a reciprocal shaker (140/min). The concentration of benzaldehyde was quantified by gas chromatography under the same conditions as described elsewhere (*2*).

The equilibrium partitioning of benzyl alcohol and benzaldehyde between the gel phase and the organic phase was measured at 30°C by incubating for more than 12 h a mixture of 5 g gel and 10 mL xylene involving both the solutes at the concentration of 1 g/L-organic liquid. The volume of the gels was determined from the volume increment by adding the prescribed amount of gels to xylene in a graduated cylinder. The equilibrium concentrations of solutes in the gel phase were calculated by using

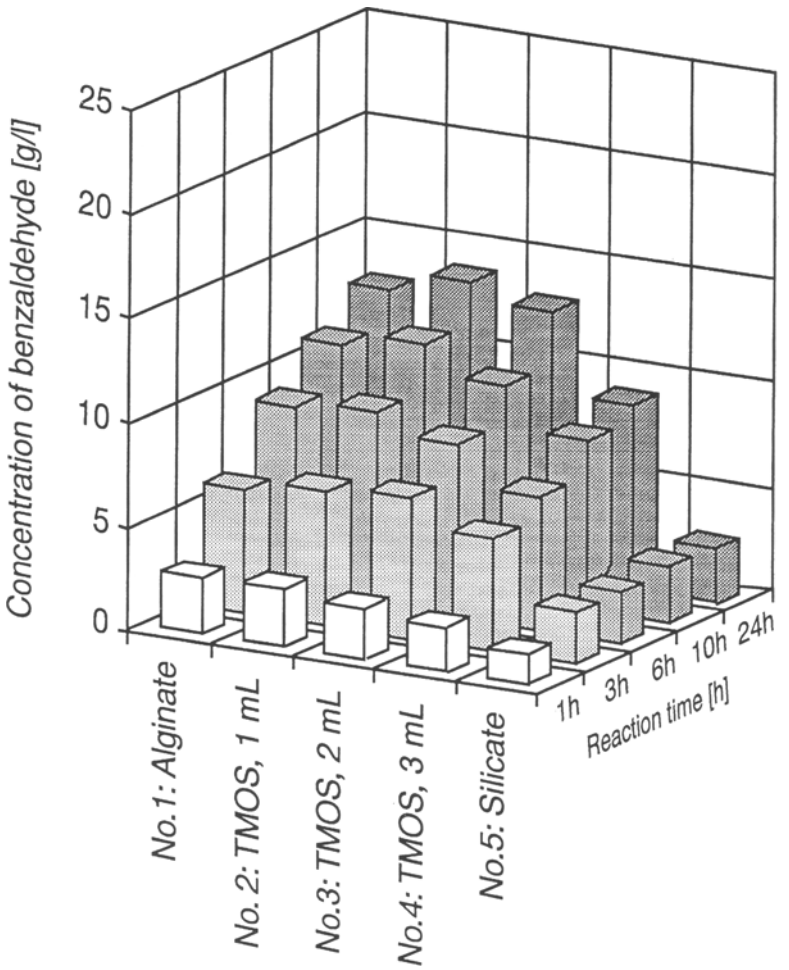

Fig. 1. Production pattern of benzaldehyde by whole cells of *Pichia pastoris* entrapped in hybrid gels composed of Ca-alginate and silicate; effect of amount of TMOS added. Compositions of starting solutions; Nos. 1–5 in Table 1.

a mass balance equation from those in the organic phase. The partition coefficients were expressed as the ratios of the solute concentrations in the gel phase to those in the organic phase.

RESULTS AND DISCUSSION

Figure 1 shows the effect of silica content on the production of benzaldehyde by the whole cells of *Pichia pastoris* entrapped in the alginate-silicate hybrid gels. Although 1 mL TMOS-derived hybrid gel (No. 2 in Table 1) exhibited somewhat higher aldehyde yield than the alginate single gel, a further addition of TMOS caused a decrease in the aldehyde produc-

tion. The oxidation activity of the silica single gel-entrapped cells was significantly low. These results suggest that the apparent activity of the entrapped cells might be influenced not only by a favorable effect of the coexistence of silica, but also by inactivation of the cells by methanol which is a by-product during the hydrolysis of TMOS. To eliminate the latter unfavorable effect, we attempted to dilute the methanol by relatively increasing the volume of an aqueous $CaCl_2$ solution as compared to that of TMOS added. As the result, 2 mL TMOS-derived hybrid gel (No. 3) prepared in 400 mL diluent was optimum, giving approx 20% higher activity than the above 1 mL TMOS-derived gel (No. 2) in 100 mL-diluent. To confirm this finding, similar hybrid gel-entrapped cells were prepared from a mixture of Na-alginate and colloidal silica which is a suspension of ultrafine silica particles. In this case, it is expected that a true effect of the presence of silica can be determined, because no methanol is formed during preparation. Figure 2 compares the aldehyde productions by the entrapped cells containing different amounts of silica. The hybrid gel prepared with 2 mL colloidal silica (No. 3) which involved 0.75 g of silica, displayed a maximum productivity of aldehyde. This amount of silica is equivalent to 0.8 g of silica produced from 2 mL TMOS. It is likely that excess silica in the hybrid gels might have hindered the internal diffusion of substrate and product.

Figure 3 represents the aldehyde productions by the whole cells entrapped in the alginate-organic silicate hybrid gels, prepared at different volume ratios of MTrMOS to TMOS (No. 3; total volume of the silanes, 2 mL). Although little difference was observed in the aldehyde production after the first hour, the amount of aldehyde accumulated with the reaction progress was greater with the alginate-organic silicate hybrid gel than with the alginate-silicate hybrid gel. As to the amount of aldehyde produced after 24 h, the hybrid gel derived from a 1:2 mixture of MTrMOS and TMOS was optimum, giving 1.5 times higher production of aldehyde than the alginate-silicate hybrid gel.

Figure 4 shows scanning electron micrographs of immobilized cells in the hybrid gels of alginate + silicate and alginate + methyl-substituted silicate. In the case of the alginate-silicate hybrid gel, the sample was shrunk during lyophilization, and lumps of silica gel segregated from the alginate matrix were observed. It seemed that most of the cells were entrapped in the alginate. In contrast, gel shrinkage did not occur with the alginate-organic silicate hybrid gel, and the cells seemed to be dispersed in the mixed gel matrix.

For bioconversions in organic media, it is well known that the hydrophobicity-hydrophilicity balance of the support materials in which biocatalysts are entrapped, is one of the important factors *(1,2,7)*. In Table 2, the partition coefficients of benzyl alcohol and benzaldehyde, defined as the ratio of the concentration of solutes in the gels to that in the xylene phase, are summarized for three types of the gels. Although both the

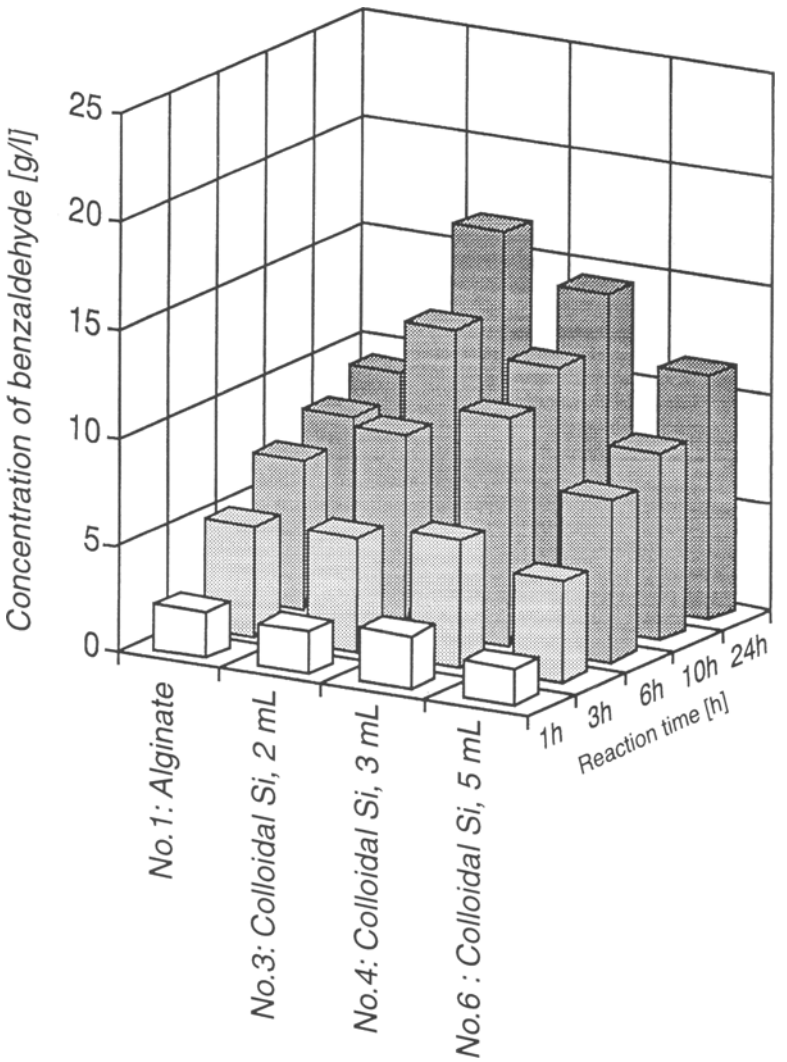

Fig. 2. Production pattern of benzaldehyde by whole cells of *Pichia pastoris* entrapped in hybrid gels composed of Ca-alginate and colloidal silica; effect of amount of colloidal silica added. Compositions of starting solutions; Nos. 1, 3, 4, and 6 in Table 1.

solutes were partitioned to a lesser extent into the hydrophilic-alginate gel, incorporation of silicate and methyl-modified silicate directed the partitioning of benzaldehyde toward the gel side, giving almost two and four times larger partition coefficients, respectively, as compared with the alginate single gel. Benzyl alcohol tended to be more partitioned into the gels than benzaldehyde, and an increase in the partition coefficients by hybridization was only 1.3 and 1.5 times for the incorporation of silicate and methyl-modified silicate, respectively.

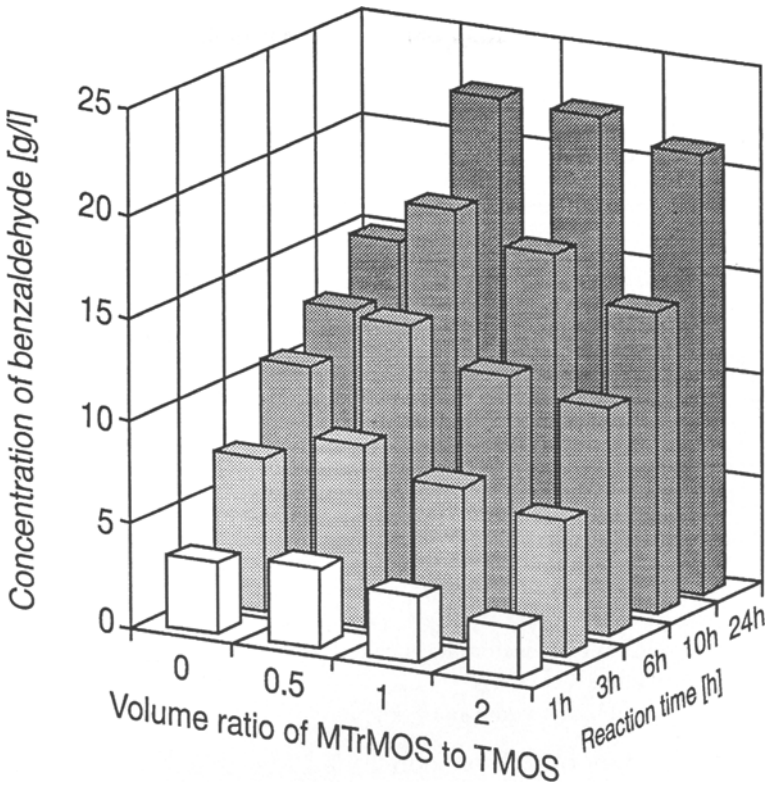

Fig. 3. Production pattern of benzaldehyde by whole cells of *Pichia pastoris* entrapped in hybrid gels composed of Ca-alginate and methyl-subsituted silicate; effect of volume ratio of MTrMOS to TMOS. Composition of starting solution; No. 3 in Table 1.

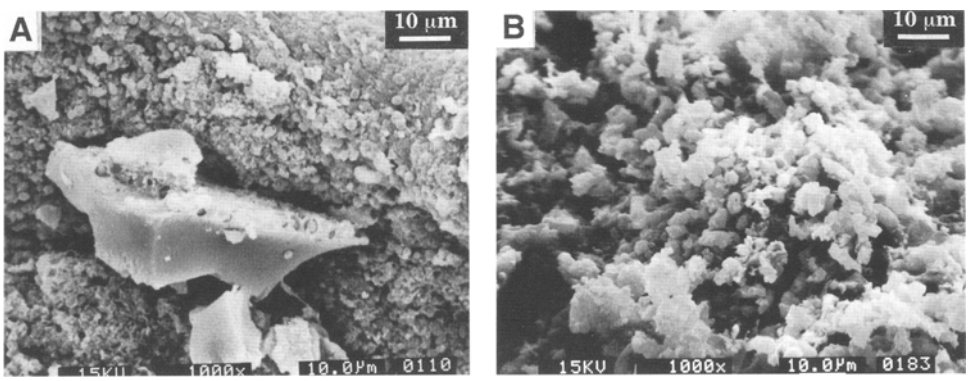

Fig. 4. Scanning electron micrographs of immobilized cells entrapped in a hybrid gel of **(A)** alginate-silicate derived from TMOS, and **(B)** alginate-organic silicate derived from TMOS + MTrMOS (2:1). Composition of starting solution; No. 3 in Table 1.

Table 2
Partitioning Equilibrium of Solutes Between Gel and Organic Medium (Xylene)

Partition coefficients[a]	$K_{benzylalcohol}$	$K_{benzaldehyde}$
Alginate	0.42	0.068
Alginate-Silicate (derived from TMOS[b])	0.53	0.12
Alginate-Organic Silicate (derived from TMOS + MTrMOS (2:1) mixture[b])	0.64	0.26

[a] Partition coefficients defined as the ratio of solute concentration in the gel phase to that in the xylene medium, $K = C_{gel}/C_{org}$. The value of C_{gel} was determined from a mass balance in the partition experiments. *See* experimental section.

[b] Composition of starting solution; No. 3 in Table 1.

In a previous report *(2)*, we concluded that this reaction was strongly influenced by both the substrate and product inhibitions, the production of benzaldehyde reached a maximum at the alcohol concentration of approx 29 g/L-aq, and diminished greatly with the accumulation of aldehyde at the concentration of ony 4–5 g/L-aq. As mentioned earlier, the present kinetic data indicated that by the addition of the silicates to alginate gel, the concentration of aldehyde produced after a longer reaction period was markedly increased, in spite that the equilibrium partitioning of aldehyde was shifted to the gel phase. The following explanation may be conceivable. Benzaldehyde produced would tend to be accumulated inside the cells owing to its low solubility in water. When a hydrophobic environment is given around the cells, benzaldehyde could rapidly diffuse out of the cells, and would be released to the organic phase through networks of the silicate.

The applicability of the present method to entrapment of other microorganisms is under study.

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