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Application of living microbial cells entrapped with synthetic resin prepolymers

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Summary. Living and growing microbial cells were immobilized by entrapping in synthetic resin gels prepared from their prepolymers, and used in the production of various useful substances. The production of the desired metabolites and also both the activity and the stability of the catalytic systems were seriously affected by the physico-chemical properties of the prepolymers, and those of the resin gels subsequently formed, such as gel network, hydrophilicity-hydrophobicity balance and ionic nature, as well as by the type of bioreactors. Hydroxylation of steroids and production of antibiotics, polypeptides and other biologically active substances, and the effects of gel properties on them are discussed as examples.
Key words. Immobilization; entrapment; immobilized cells; microbial cells; synthetic resin prepolymers; bioprocesses; bioconversion.

Introduction

With the development of enzyme engineering, immobilization of multienzyme systems has been attracting worldwide attention, because these systems can mediate complicated reactions for synthesis and conversion of various compounds, with regeneration of ATP and/or pyridine nucleotide coenzymes which participate in oxidation-reduction reactions. Microbial cells, especially living ones, contain metabolic systems that catalyze such complicated reactions. Therefore, immobilization of living cells means in effect the immobilization of multi-step and cooperative enzyme systems with coenzyme regeneration.

Immobilized living cells have several disadvantages, such as undesirable metabolic activities which reduce product yields and form by-products. The existence of a permeability barrier for various substrates and metabolites is another disadvantageous feature of the system. Nevertheless, immobilized living cells, especially immobilized growing cells, serve as renewable and self-proliferating biocatalysts, maintaining specific enzyme activities for a very long period. The application of immobilized living or growing cells was first demonstrated by Slowinski and Charm[22] in 1973 for the production of glutamic acid and, thereafter, developed by Larsson et al.[10] for the hydroxylation of hydrocortisone to yield prednisolone. Some aspects of immobilized cells, dead or alive, were reviewed by Chibata and Tosa[4] and by the present authors[6].

This article deals with the application of living microbial cells entrapped with synthetic resin prepolymers, mainly carried out in the authors' laboratory.

Entrapment of microbial cells with synthetic resin prepolymers

Microbial cells, dead or alive, can be immobilized by means of physical adsorption, ionic binding, entrapment etc. on or in inorganic materials, natural or modified polysaccharides, proteins and synthetic resins. We have developed novel immobilization methods, which are applicable to the entrapment of enzymes, cellular organelles, microbial cells, plant cells and animal cells, with synthetic prepolymers of photo-crosslinkable resins and urethane resins[5,7]. Specific features of these prepolymer

Application of microbial cells entrapped with synthetic resin prepolymers

Microorganism (condition)	Application	Ref.
Corynebacterium sp. (living)	9α-Hydroxylation of steroid	26
Rhizopus stolonifer (living)	11α-Hydroxylation of steroid	25
Curvularia lunata (living)	11β-Hydroxylation of steroid	23, 24
Sepedonium ampullosporum (living)	16α-Hydroxylation of steroid	9
Candida albicans etc. (living)	Asymmetric reduction of β-keto esters	1
Arthrobacter sp. (growing)	Production of muconic acid	19, 21
Corynebacterium glycinophilum (growing)	Production of L-serine	31
Streptomyces carbophilus (growing)	Production of pravastatin	3
Streptomyces rimosus (growing)	Production of oxytetracycline	15
Streptomyces peucetius (growing)	Production of daunorubicin	28
Agaricus campestris (growing)	Production of pyruvic acid	27
Methanogenic bacterium (growing)	Production of methane	2
Saccharomyces sp. (growing)	Production of ethanol	11, 13
Saccharomyces cerevisiae (growing)	Production of α-mating factor and a specific peptidase	16, 17

methods are simple immobilization procedures under very mild conditions and easy selection of gel properties, such as gel network size, hydrophilicity-hydrophobicity balance and ionic nature, by using different types of the prepolymers. Typical examples of living microbial cells entrapped with prepolymers are summarized in the table.

Applications of living microbial cells entrapped with prepolymers

Hydroxylation of steroids

Stereo- and regio-specific hydroxylation is one of the important processes in biotransformation of steroids for the synthesis of useful drugs. Since the enzymes involved in the hydroxylation systems are in general unstable, and a continuous supply of NAD(P)H is essential in these reactions, it is desirable to use living cells as catalysts for the processes. Various kinds of fungi can catalyze the hydroxylation reactions but it is difficult to entrap mycelial cells in a homogeneous state while retaining the enzyme activities.

Ohlson et al.[18] reported the entrapment of spores of *Curvularia lunata* in calcium alginate gels, in which the spores germinated and developed to form mycelia. The entrapped mycelia so prepared were demonstrated to have 11 β-hydroxylation activity toward cortexolone (Reichstein's compound S) (fig. 1). When the *C. lunata* spores were entrapped with hydrophilic photo-crosslinkable resin prepolymers of different chain-length, followed by incubation in a nutrient medium containing cortexolone as an enzyme inducer, development of the mycelia and, subsequently, the hydroxylation activity

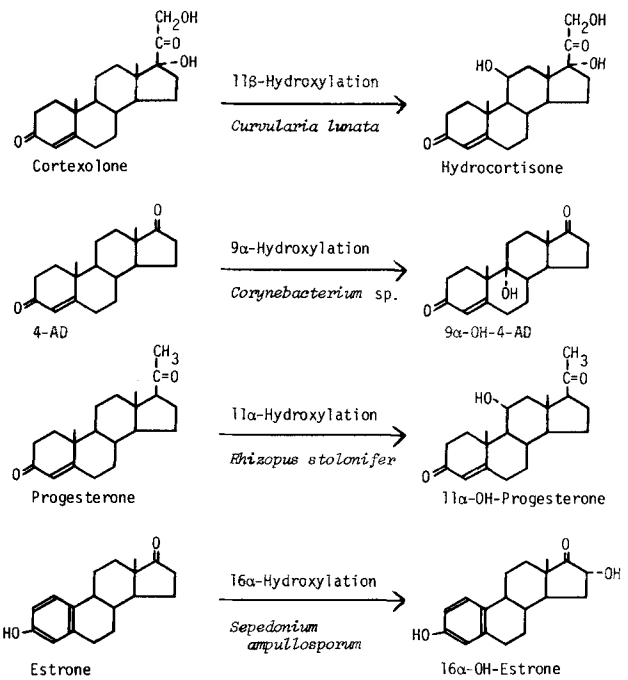

Figure 1. Hydroxylation of steroids catalyzed by living microbial cells entrapped with synthetic resin prepolymers.

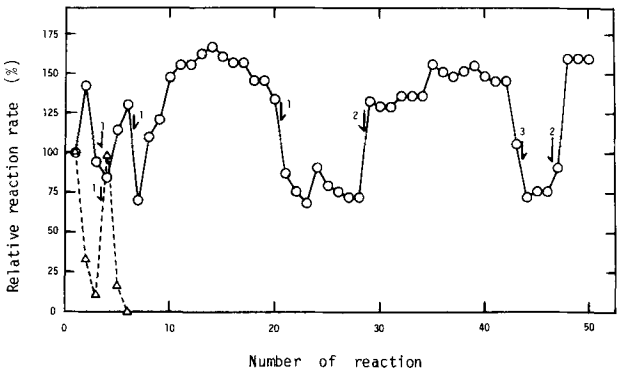

Figure 2. Repeated use of *C. lunata* mycelia entrapped with a photo-crosslinkable resin prepolymer of appropriate chain-length on 11 β-hydroxylation of cortexolone[24]. Each reaction was carried out for 2 days. Arrows indicate reactivation of the entrapped mycelia under various conditions. (○), Gel-entrapped mycelia; (△), free mycelia.

varied depending on the prepolymers used. That is, the mycelia did not develop well in the gels which had a tight network formed from shorter chain prepolymers, while abundant growth was observed with the gels with a much looser network prepared from longer chain prepolymers; however, there was leakage of mycelia from the gels. A high and stable 11 β-hydroxylation activity was obtained with mycelia entrapped with medium chain prepolymers. The enzyme system could be reactivated by incubating the cell-entrapping gels in a nutrient medium and the entrapped mycelia, once prepared, could be used repeatedly for 50 separate reactions over 100 days in a reaction medium containing 2.5% dimethylsulfoxide to dissolve the product (fig. 2)[23, 24]. The system was far more stable than that with mycelia entrapped in calcium alginate gels. On the other hand, free mycelia lost the activity very rapidly. This process could be combined with the Δ^1-dehydrogenation system using hydrophobic gel-entrapped acetone-dried *Arthrobacter simplex* cells to synthesize prednisolone from cortexolone[12].

11 α-Hydroxylation of progesterone by *Rhizopus stolonifer*[25] and 16 α-hydroxylation of estrone by *Sepedonium ampullosporum*[9] were also achieved successfully using mycelia entrapped with hydrophilic and hydrophobic prepolymers, respectively, of suitable chain-length (fig. 1).

Corynebacterium sp. ATCC 14887 cells entrapped by photo-crosslinkable resin prepolymers hydroxylated 4-androstene-3,17-dione to the corresponding 9 α-hydroxy derivative (fig. 1). The yield of the product was low when the reaction was carried out in an aqueous medium, particularly when the cells were entrapped with hydrophobic prepolymers. This arose from a further metabolism of the 9 α-hydroxy derivative accumulated inside the gels. Addition of dimethylsulfoxide at 15% (v/v) to the medium was found to be effective in extracting the desired product, improving the yield of 9 α-hydroxy-4-androstene-3,17-dione[26]. These results indicate that finding a means to remove the desired metabolite

from the vicinity of the cells is essential when living cells are used as biocatalysts.

Synthesis of pravastatin

Pravastatin is a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme involved in the biosynthesis of cholesterol, converting HMG-CoA to mevalonate. Pravastatin sodium could be synthesized via hydroxylation of sodium ML-236B-carboxylate, which was derived from ML-236B, a metabolite of *Penicillium citrinum*, by *Streptomyces carbophilus* (fig. 3). *Str. carbophilus* cells entrapped with hydrophobic photo-crosslinkable resin prepolymers have also been successfully applied to the hydroxylation reaction by the research group of Sankyo Co. Ltd, Japan[3].

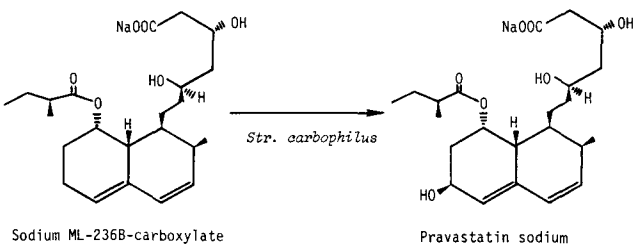

Figure 3. Hydroxylation reaction mediated by *Str. carbophilus*.

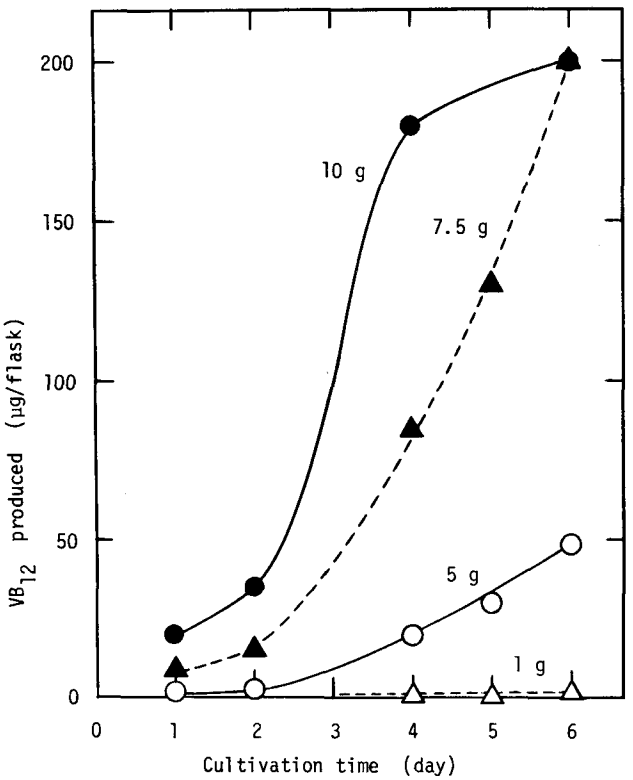

Figure 4. Effect of cell concentration on vitamin B_{12} production by gel-entrapped *Propionibacterium* sp. cells[32]. 1 (△), 5 (○), 7,5 (▲) or 10 g (●) of wet cells were entrapped with 1 g of a urethane resin prepolymer and cultivated statically in a flask holding 50 ml medium.

Vitamin B_{12}

Vitamin B_{12} is the most complicated non-polymer organic compound of known structure in nature, and is important as a medicine and as a human and animal nutrient. At present, production of vitamin B_{12} by chemical means is not practically feasible and only fermentation processes are industrially available. *Propionibacterium* sp. AKU 1251, which has a considerable activity in producing the vitamin extracellularly, was selected as a test organism. A hydrophilic urethane prepolymer was chosen as the most suitable gel material for the bacterial cells, on the basis of criteria such as convenience of preparation, stability of the cell-entrapping gels, cell leakage and vitamin B_{12} productivity. Satisfactory production was obtained when a high concentration of the cells were entrapped with the prepolymer (fig. 4). The entrapped cells could be used repeatedly for six separate fermentations over 18 days, with proliferation of the cells inside the gel matrix[32].

Production of antibiotics

As antibiotics are generally secondary metabolites not associated with growth, it is very difficult to produce them by continuous cultivation of microorganisms. However, application of immobilized growing cells may overcome this problem because control of the cultivation conditions to favor antibiotic production seems to be easier when the washout of the cells from the reactor can be avoided.

Streptomyces rimosus cells were entrapped with a hydrophilic urethane resin prepolymer of short chain-length, which was selected on the basis of several criteria, including oxytetracycline productivity and cell leakage from gels. Use of a glucose-free medium increased the production rate of the antibiotic and minimized the cell leakage by regulating the cell growth inside the gels. Continuous fermentation was successfully achieved in an air-bubbled reactor for at least 35 days, with reactivation by washing the cell-entrapping gels with saline to remove inactive cells from the gel surface[15]. Of different types of reactors tested, air-bubbled reactors (fig. 5), especially a draft-tube reactor (fig. 5A), were found to be suitable for the stable production of oxytetracycline (fig. 6). The system could be scaled up to 2 l of working volume in a stirred air-bubbled reactor equipped with gel films entrapping the cells (unpublished results).

Daunorubicin (daunomycin), which is known to be an antitumor anthracycline, was produced by the gel-entrapped cells of *Streptomyces peucetius*[28]. As gel materials, alginate and a photosensitive synthetic prepolymer[8] were found to be suitable. By the use of cultivation media free from insoluble components, these entrapped cells could be used at least five times for repeated daunorubicin production over 45 days. Entrapment significantly enhanced the excretion of the antitumor reagent, this

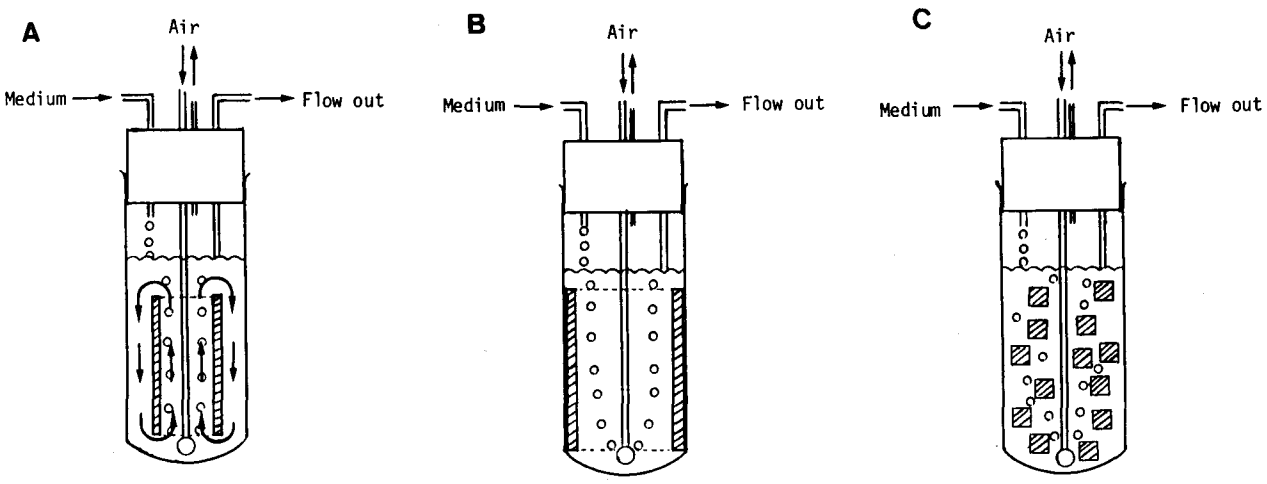

Figure 5. Air-bubbled reactors applied to continuous production of oxytetracycline. *A* Draft-tube type; *B* fixed-bed type; *C* moving-bed type. Hatched portions exhibit polyurethane resins entrapping *Str. rimosus* cells.

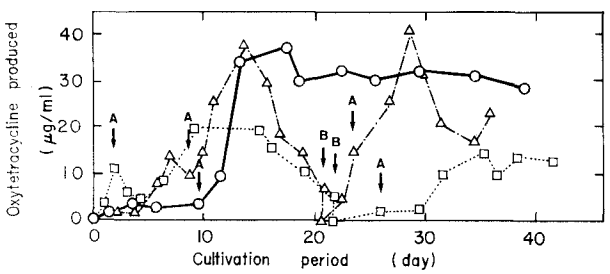

Figure 6. Continuous production of oxytetracycline by *Str. rimosus* cells entrapped with a urethane resin prepolymer in different types of reactors (see, fig. 5). (○), Draft-tube type (working volume, 190 ml); (△), fixed-bed type (working volume, 180 ml); (□), moving-bed type (working volume, 150 ml). *A* Start of continuous cultivation; *B* washing with saline for 2 days. Dilution rate, 0.030–0.037 h^{-1}; aeration, 3.7–6.7 VVM.

phenomenon being advantageous for the immobilized cell system.

Production of L-serine

L-Serine is a valuable amino acid as an additive in cosmetics and as a component of an intravenous nutrient solution. At present, practical production of L-serine, which is hard to obtain by direct fermentation from carbohydrates, is mainly carried out by bioconversion of glycine.

L-Leucine auxotrophic mutant cells of *Corynebacterium glycinophilum* with a high serine productivity were entrapped with various gel materials, such as synthetic resin prepolymers and natural polysaccharides. The entrapped cells were used for the production of L-serine in a medium supplemented with glycine as a precursor. Cells entrapped with a hydrophilic photo-crosslinkable resin prepolymer of an appropriate chain-length showed a high productivity and a low cell leakage, but the best results were obtained with cells entrapped in calcium alginate gels. Although an abundant supply of oxygen was required for the fermentation, L-serine was produced stably for at least 13 days on continuous cultivation in an air-bubbled moving-bed reactor (fig. 5C) equipped with a foam trap[31]. Such a continuous bioreactor system might be applicable to the production of other useful substances requiring highly aerobic conditions.

Production of cis, cis-muconic acid

In addition to production of fine chemicals, production of commodity chemicals by bioprocesses is one of the important and interesting targets of recent enzyme engineering. A research group of Mitsubishi Chemicals Co. Ltd., Japan, isolated a strain of *Arthrobacter* sp., which can degrade benzoic acid. From this parent strain they isolated a mutant converting benzoic acid to *cis, cis*-muconic acid (fig. 7) in the presence of sodium acetate as carbon source. Although the mutant cells entrapped with a photo-crosslinkable resin prepolymer produced *cis, cis*-muconic acid at a significant rate, the productivity was not so high as desired, probably owing to the insufficient diffusion of oxygen and the substrate into the gels. At present, *cis, cis*-muconic acid can be produced continuously at a rate of 80 $g \cdot l^{-1} \cdot day^{-1}$ in a membrane-type reactor under the optimized conditions[19,21]. Although the application of *cis, cis*-muconic acid has not been developed yet, this acid might be useful as a monomer component of specifically functional resins.

Asymmetric reduction of α-methyl β-keto esters

Bioconversion is becoming a useful synthetic means to prepare chiral synthons from prochiral compounds.

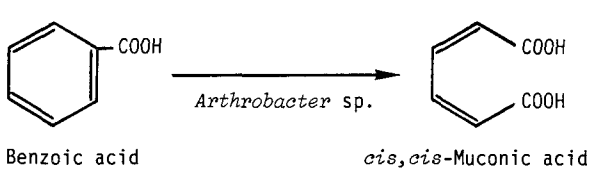

Figure 7. Conversion of benzoic acid to *cis, cis*-muconic acid catalyzed by a mutant of *Arthrobacter* sp.

Living yeast cells, such as *Candida albicans, Kloeckera saturnus, Saccharomyces delbrueckii* and *S. fermentati*, were entrapped with hydrophilic or hydrophobic photo-crosslinkable resin prepolymers and urethane resin prepolymers, and applied to the asymmetric reduction of α-methyl β-keto propionate derivatives[1]. Yields of the products and the enantioselectivity could be varied, depending on the yeast strains, the prepolymers used and the kind of substrates. In some cases, both chemical yield and optical purity could be improved by selecting more effective prepolymers for entrapment.

Production of biofuels

Yeast cells entrapped with photo-crosslinkable resin prepolymers were applied to the production of ethanol from molasses. The cell-entrapping gels were prepared in films and set up in a reactor with an alcohol production capacity of 250 l · day^{-1}. This reactor was successfully operated for 3000 h at an alcohol concentration of about 10% (v/v) with intermittent sterilization by introducing a hypochlorite or sulfite solution[13, 14]. However, this type of reactor was not suitable for further scaling up. Therefore, a suspended-bed reactor with a capacity of 5 kl · day^{-1} was constructed by using gel beads entrapping yeast cells[11]. The gel beads were prepared from a mixture of sodium alginate and a photo-crosslinkable resin prepolymer; the first gels were formed in a calcium chloride solution and the gelation of the prepolymer was carried out under photo-illumination[20]. A pilot-scale apparatus with the capacity to prepare 60 l of gel beads in an hour has been constructed. These systems have been developed by the research groups of Kansai Paint Co., Ltd and JGC Corporation, Japan.

Another type of biofuel, methane, was produced continuously from formate by a methanogenic bacterium entrapped with a urethane resin prepolymer. Entrapment proved to be effective in preventing the cells from being inactivated by oxygen[2].

Production of α-mating factor and a peptidase

α-Mating factor is a tridecapeptide of Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr excreted into the culture medium by α-mating type cells of *Saccharomyces cerevisiae*. Efficient production of α-mating factor with immobilized growing *S. cerevisiae* cells was investigated in order to establish an extracellular production system for useful foreign peptides using the α-mating factor secretion system of the yeast. Free yeast cells accumulated α-mating factor in the culture medium, but it was gradually degraded in situ. Entrapment of the cells with anionic photo-crosslinkable resin prepolymers was effective in protecting the peptide from degradation (fig. 8), while neutral prepolymers exhibited little effect and a cationic prepolymer no effect. Anionic resin gels were found to entrap selectively α-mating factor-degrading enzymes which were simultaneously produced by the cells. The entrapped yeast cells could be used successfully

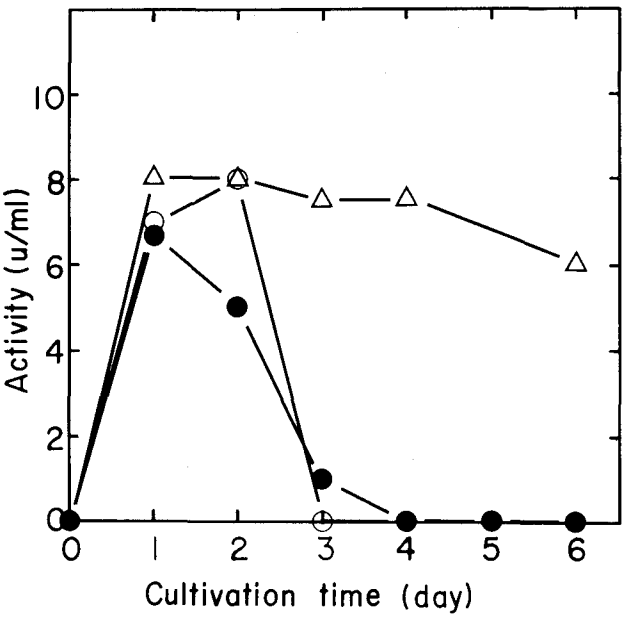

Figure 8. Effect of ionic property of gels on the production of α-mating factor by *S. cerevisiae* cells[16]. (○), Free cells; (△), cells entrapped *with* a mixture of neutral and anionic photo-crosslinkable resin prepolymers (8:2 w/w); (●), cells entrapped with a mixture of neutral and cationic prepolymers (8:2 w/w).

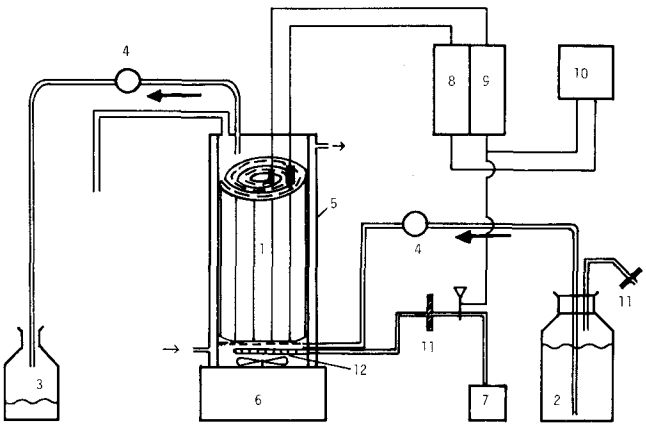

Figure 9. Continuous production system for α-mating factor[16]. 1, Air-bubbled reactor equipped with a spiral of photo-crosslinked resin gel films entrapping *S. cerevisiae* cells; 2, medium reservoir; 3, product reservoir; 4, pump; 5, water jacket; 6, stirrer; 7, air pump; 8, pH meter; 9, DO meter; 10, recorder; 11, air filter; 12, sparger.

for the continuous production of α-mating factor in a column reactor equipped with a spiral of the cell-entrapping gel films (fig. 9) for at least 30 days (fig. 10)[16].

When the yeast cells were entrapped with a neutral prepolymer at a high cell concentration, α-mating factor which had accumulated in the culture medium disappeared very rapidly. This phenomenon suggested the production of potent α-mating factor-degrading enzymes. In fact, the culture medium showed a high activity in cleaving the peptide. A main component of the enzymes was partially purified and characterized. The isolated enzyme was specific for internal Leu-Lys bonds of

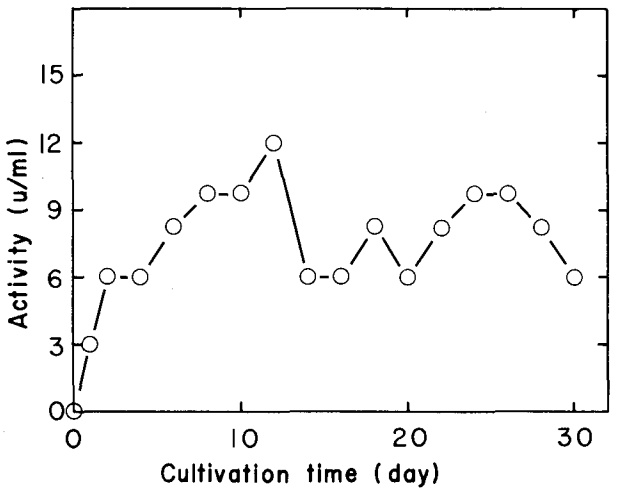

Figure 10. Continuous production of α-mating factor by gel-entrapped *S. cerevisiae* cells[16]. Cells were entrapped with a mixture of neutral and anionic photo-crosslinkable resin prepolymers.

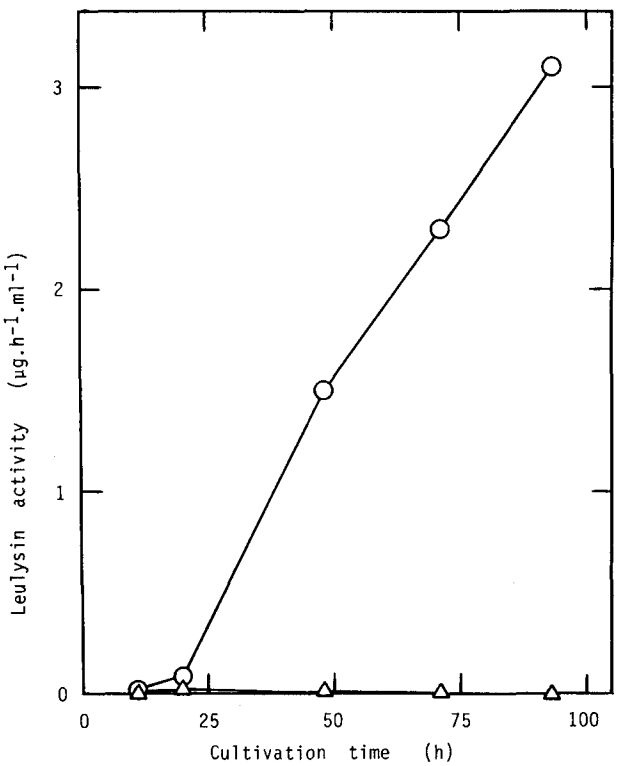

Figure 11. Production of Leulysin by *S. cerevisiae* cells. (○), Cells entrapped with a neutral photo-crosslinkable resin prepolymer; (△), free cells. Enzyme activity was expressed by the amount of 1–6 peptide formed from α-mating factor.

several peptides including α-mating factor, and was therefore named 'Leulysin'[17]. The fact that Leulysin was produced only when the cells were immobilized (fig. 11) suggested that conditions of stress, such as immobilization at a high cell concentration, might facilitate the synthesis and excretion of this peptidase. α-Mating type cells of *S. cerevisiae*, the recipient of α-mating factor, excreted much larger amounts of a Leulysin-like peptidase, which hydrolyzed the Leu-Lys bond of α-mating factor, than the α-mating type cells did (unpublished results). This specific peptidase will be useful as a restriction enzyme in protein research.

Conclusion

As summarized here, living or growing microbial cells entrapped with suitable gel materials can be applied to the production of different substances via biotransformation and biosynthesis. Entrapped cells have several advantages, such as stability of – or even increase in – the catalytic activities during operation; catalysis of complicated and multi-step reactions including coenzyme regeneration; and applicability to repeated batch or continuous system. Furthermore, entrapment seems to affect the physiology of microbial cells, facilitating the excretion of metabolites and other substances.

Extensive application of genetically improved cells is becoming an important objective in bioprocesses. Effective production systems should be constructed by selecting appropriate gel materials which have suitable properties, depending on the kinds of microorganisms and reactants involved, and by designing different types of reactors, suitable for various purposes.

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Solid state fermentation for cephalosporin production by *Streptomyces clavuligerus* and *Cephalosporium acremonium*

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Summary. Solid state fermentation systems were developed for the production of cephalosporins with *Streptomyces clavuligerus* and *Cephalosporium acremonium*. *S. clavuligerus* NRRL 3585 was grown on moistened barley under optimum solid state fermentation conditions for 7 days; approximately 300 µg cephalosporins per g substrate were extracted from the kernels. *C. acremonium* C-10 produced approximately 950 µg cephalosporin C per g substrate after 10 days of solid state fermentation.
Key words. Solid state fermentation; β-lactam antibiotics; cephalosporins; *Streptomyces clavuligerus*; *Cephalosporium acremonium*.

Introduction

As defined by Cannel and Moo-Young[4], solid state fermentation (SSF) refers to the growth of microorganisms on solid materials without the presence of free liquid. In SSF the moisture necessary for microbial growth exists in an absorbed state or complexed within the solid matrix. An exact definition of SSF with respect to free liquid is not easily established; while the minimum moisture level at which SSF can occur is about 12% (this level being that below which all microbial activities stop), the upper limit for SSF is more a function of absorbancy than moisture content. For example, free water becomes apparent in maple bark at about the 40% level, and in straw at about the 75% level[4]. Because of the low water content and a_w-value of the substrate, solid state fermentation systems provide a selective environment for a large number of filamentous fungi and a few bacteria, such as actinomycetes, which grow in mycelial form.

Solid state fermentations have been used for centuries in the Far East for the preparation of a variety of fermented foods such as miso, soy sauce, tempeh and others as described by Hesseltine[13]. Recently, this technology has been studied for the production of mycotox-