



Immobilization of Escherichia coli cells using porous support particles and application for bioconversion

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(Degree)

博士 (工学)

(Date of Degree)

2008-03-25

(Date of Publication)

2012-03-19

(Resource Type)

doctoral thesis

(Report Number)

甲4196

(URL)

<https://hdl.handle.net/20.500.14094/D1004196>

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神戸大学博士論文

**Immobilization of *Escherichia coli* cells using porous support
particles and application for bioconversion**

**大腸菌の新規固定化培養技術の開発と
有用物質生産に関する研究**

平成 20 年 1 月

神戸大学大学院自然科学研究科

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PREFACE

This is a thesis submitted by the author to Kobe University for the degree of Doctor of Engineering. The studies collected here were carried out between 2003 and 2007 under the direction of Professor Hideki Fukuda at the Laboratory of Biochemical Engineering, Division of Molecular Science, Graduate School of Science and Technology, Kobe University.

First of all, the author expresses the sincerest gratitude to a research advisor, Professor Hideki Fukuda, for his continuous guidance and invaluable suggestions during the course of his studies. The author also expresses hearty gratitude to Professor Akihiko Kondo for his enormously beneficial discussions and kind encouragement. The author wishes to acknowledge Professor Yasukiyo Ueda for his kind support. The author is also deeply grateful to Associate Professor Hideki Yamaji, all of the experiments were under his leading and guidance, and all of the experiment results can not be obtained successfully without his continuous guidance, invaluable suggestions and warm encouragement. The author further wishes to acknowledge Assistant Professor Tsutomu Tanaka and Ms. Yasuko Koshiba for their informative technical advice and kind support. The author also thanks Aion Co., Ltd for providing with polyvinyl formal (PVF) resin sponge sheets and biomass support particles (BSPs).

The author further pays sincerely acknowledges to Dr. Junya Narita (TOTO Ltd.), Dr. Shinji Hama (Bioenery Corp.), Dr. Takanori Tanino, Dr. Sriappareddy Tamalampdi, Mr. Jun Ishii, Ms. Tomoko Kawabuchi (Suzuki Co., Ltd.), Mr. Toshitaka Manabe (Pfizer Japan Inc.), Mr. Haruhito Michida (Kao Corp.), and all the members of Professor Fukuda's Laboratory for their technical assistance, informative advice and

encouragement.

And also the author expresses her hearty gratitude to Yanmar Co., Ltd. for the Yamaoka Scholarship in 2003 and Ministry of Education, Culture, Sports, Science and Technology, Japan for the support of Government-Financed Scholarship from 2004 to 2007.

Last but not least, the author expresses deep appreciation to her parents for their constant assistance and financial support, and also appreciates to her cousin's family for their kind encouragement and great support.

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INTRODUCTION

Enzymes are a kind of proteins that catalyze many kinds of chemical reactions (1). Enzymes, as biocatalysts, like all catalysts, work in normal temperatures, neutral and mild conditions, can dramatically accelerate the rate of the reaction. Most enzyme reaction rates are millions of times faster than those of comparable non-catalyzed reactions, and they are also extremely selective for their substrates and speed up only a few reactions from among many possibilities. Enzymes are known to catalyze about 4,000 biochemical reactions (2).

As enzymes can catalyze various kinds of reactions at the moderate conditions such as normal temperature and pressure and at around neutral pH, they are used as potential biocatalysts to design less energy consuming, environmentally friendly bioprocesses. Enzymes are used as catalysts in many fields, such as organic synthesis, clinical analysis, pharmaceuticals, detergents, food production, and fermentation, where extremely specific catalysts are required. In many industrial processes, for the synthesis of fine and commodity chemicals, pharmaceutical and agrochemical intermediates, and drug substances, the utilization of enzyme as biological catalysts continues to grow (3, 4). Over the past decade years, the industrial use of enzymes has developed rapidly because of their unique functions. The use is gathering increasing attentions, and particularly expected to lowering environmental problems (4). About industrial biocatalysts, in recent years there are many books and reviews have been published (5–7). All of these current developments can be attributed to recent progress in molecular biology, high-throughput screening techniques, advanced instrumentation, and engineering (3). Directed evolution (8) and metabolic engineering technologies (9)

have led to the production of stable biocatalysts with customized activity and selectivity (3, 10).

Biocatalysis may be carried out using whole cells or isolated enzymes in free or immobilized form. The advantage of using isolated purified enzymes is that undesirable byproduct by contaminating enzymes is avoided (11). However, extraction and purification of enzymes is also costly, and enzymes are frequently less stable in purified form than in a crude preparation or when in whole cells (12, 13). In recent years, in many industrial bioprocesses, the biocatalyst is used in the form of whole cells for greater cost effectiveness. Consequently the direct use of immobilized cells as whole cell biocatalyst has gained considerable attention (11).

Microorganisms, especially bacteria are holding various kinds of useful enzymes. As described above, it is considered that for the usage of bacterial enzymes as whole cell biocatalyst, simpler, economical, and environment-friendly immobilization technology for bacteria would increasingly develop. This thesis is about the development of an immobilization technology, that immobilize *Escherichia coli* cells, one of typical bacteria, using porous support particles coated with cationic polymers. Moreover, as the application of this immobilization technology, the production of aspartic acid using *E. coli* whole cell biocatalyst is also studied.

Industrial biocatalyst development

The use of biocatalyst for industrial is on the verge of significant growth in these years. Biocatalyst is looked as a tool in new and difficult syntheses, and this will lead to more industrial applications of biocatalysts (14, 15). Both isolated enzymes and whole cells are used in industry today. Isolated enzymes are typically used for

hydrolytic or isomerization reactions. Whole cells are often used for synthetic reactions that require cofactors which must be regenerated, because although cofactor regeneration in vitro is possible, it is generally easier and less expensive to regenerate cofactors in metabolically active cells (16). Enzymes are remarkable catalysts, and have so high selectivity. As a result, they afford efficient reactions with few by-products, are thought as environmentally friendly alternative to conventional chemical catalysts.

These attributes have resulted in many applications, especially in the food and pharmaceutical industries where high reaction selectivity on complex substrates is critical. The field of industrial biocatalysis is now experiencing a healthy growth, as judged by the steady flow of bioprocesses being transferred from the laboratory setting to manufacturing. There are many typical successful industrial examples: the production of high-fructose corn syrup, by the action of xylose isomerase which catalyses the isomerization of D-glucose to D-fructose (17), the preparation of semisynthetic penicillins catalysed by penicillin amidase (18). Recently years, enzymatic catalysis has also been developed in the extended of synthesis of specialty chemicals and polymers (19, 20), and of some bulk chemicals. For example, peroxidases are used to catalyze the synthesis of phenolic resins industrially for use as replacements of conventional phenol-formaldehydes (21), and nitrile hydratase is used to catalyze the hydration of acrylonitrile into acrylamide (22). Furthermore, in pharmaceutical industry, the use of biological catalysts in the synthesis of pharmaceutical intermediates, drug metabolites and drug products is now common. The growing list of compounds synthesized with the assistance of enzymes now includes anti-cancer, anti-viral, anti-infective, anti-psychotic, anti-arrhythmic, and

cholesterol-lowering agents, calcium channel blockers, ACE inhibitors and many others (23–26).

In these cases, nearly quantitative conversion from the reactants into products can be obtained, and the biocatalyst reaction conditions are far milder than the chemical reaction conditions. Most of the commercial enzymatic processes today contribute to several advancements including high product concentrations, productivities, no undesirable by-products, and enzymes that do not require expensive cofactors (16).

In consequence, a growing number of companies today consider biocatalyst as an interesting option. In the future, it is anticipated that in more large range of industrials, the utilization of industrial biocatalyst will grow rapidly.

Microbial enzymes

The application of the enzyme at first is not enzyme itself, it was beginning with the use of the microbe which had various kinds of enzymes maintained in the body. Until now, the use of enzyme usually means the use of microbe which maintain enzyme inside. Microbe is an organism that is microscopic (too small to be seen by the human eye). Microbes include bacteria, fungi, archaea or protists. Microbes live almost everywhere on earth where there is liquid water, including hot springs, on the ocean floor, and deep inside rocks within earth's crust. Once the favorable results of employing enzyme were established, a search for better, less expensive, and more readily available sources of enzymes had begun. It was found that certain microbes produce enzymes similar in action to the amylases of malt and pancreas, or to the proteases of the pancreas and papaya fruit. This led to the development of processes

for producing such microbial enzymes on a commercial scale (27).

The use of microbes in reaction has many advantages. Compare with other creature, the growth rate of microbes is much quickly than other higher organisms, therefore the productivity is very high. The microbial reactions are almost under normal temperatures, so the reaction security level is high. Of course, the characteristic diversity and versatility of microbial enzyme is great acquainted (28).

Since a long time ago, the enzymes from yeasts and fungus are used to produce fermented soybean paste, soybean sauce, alcoholic beverage and so on. Until now a great deal of enzymes derive from microbial are used in many fields. Microbial enzymes are useful catalysts for the synthesis of many biologically and chemically useful compounds (4, 28, 29). Uses of microbial enzymes in food, pharmaceutical, textile, paper, leather, and other industries are numerous and are increasing rapidly. The more important current uses are in the production of amino acids, antibiotics and fibers (30, 31). The development of current high biotechnologies, such as genetic engineering, protein engineering, and metabolic engineering and so on, will offer further exciting possibilities for the industrial use of microbial enzymes.

Whole cell biocatalyst

Ordinarily, in biocatalysis processes, the microbes are cultivated for a length of time and then the objective enzyme should be extracted from the culture broth or microbe self, after purification and condensation, then used for reactions. This kind of process is complicated and expensive. Furthermore, after these operations enzymes are frequently less stable than in a crude preparation (12). Correspondingly, in the last few years, the method of using microbes themselves directly as enzyme, that called whole

cell biocatalyst, has been investigated (11). There have been many studies that make on the concept of whole cell biocatalyst, utilization of whole microbial cells as biocatalysts during incubation (32–34). This kind of whole cell biocatalyst technology offer the abbreviation of the operations of extraction, purification and condensation of enzymes, and make the reaction process simple, energy saving and more economical.

Whole cell biocatalyst technology is used in wide areas to produce many popular materials economically, such as amino acid, biodiesel fuel and so on (36, 37). To build an energy-saving society, the utilization of whole cell biocatalyst is attracting the attention of industry.

Immobilization technology

Microbial and animal cells are used to produce various kinds of useful materials like alcohol, amino acids, fermented foods, antibiotics, enzymes, and physiologically active substances. Furthermore, the genetic engineering has made significant progress in recent years to produce various therapeutic proteins using recombinant microorganisms and other efficient gene expression systems.

Holding these kinds of cells in a limited space namely immobilization was practiced in bio-reaction process. Immobilization of cells can avoid difficult and expensive process for isolation and recovery of cells. It can also make the continuous reaction possible and improves the reaction rate per unit volume. In the case of immobilizing viable cells, because viable cells can multiply in the vehicle under some proper conditions, as a catalyst, their activity can be improved.

Especially in the utilization of whole cell biocatalyst, the immobilized cells holding enzymes can be used as whole cell biocatalyst directly. The immobilized

whole cell biocatalyst offer many advantages, including that the operations of extraction, purification and condensation of enzymes are unnecessary, the products can be separated from reaction system easily, holding a more stable enzyme than no immobilized enzyme in reaction process, and application to multiple enzyme reaction is possible (38–40). According these attractive advantages, immobilization of cells has been used in various processes.

Several methods have been reported for the immobilization of cells, all of them can be categorized to two kinds of methods: the active method and passive method (41).

Active methods that using reagent or gelatinizing agent to immobilize cells include cross-linkage method, covalent binding method and entrapping method. The cross-linkage method is using a chemical reagent that holding two or more than two functional groups to make the cell membrane or cell wall strong and build a bridge between cells to achieve immobilization (42) . The covalent binding method is base on the formation of covalent bonds between the enzyme and the support matrix. The functional groups that may take part in the binding are always amino group or carboxyl group (43). The entrapping method is a method that enwraps cells using polymer materials or coats cells by semi permeable polymer membrane (44, 45).

Passive methods are the kind of method like adsorption method and colony method. Adsorption method is using physical function between cells and supports to achieve immobilization (46). Colony method is by the utilization of adherence or associability of cells. The details of colony method will be described in following section.

Currently immobilized whole cell biocatalyst has already been used in

industrial scale successfully (47).

Immobilization using BSPs

As described above, all of immobilization methods can be categorized to two kinds of methods: active method and passive method. Active methods have been introduced in brief in above paragraph. In the immobilization process of colony method, cells are immobilized on porous biomass support particles naturally by their aggregation and adhesion characteristics during cells growth. This kind of particles are called BSPs elliptically and usually made by various types of particles such as polyester, polyurethane, and polyvinyl formal resin (48).

This technique was developed by Atkinson *et al.* in 1979 (49). This immobilization technique has many advantages such as easy in the immobilize process and aseptic operation, immobilized vehicle is physically firmly and it's also a suitable method for industry to scale-up. This technique has been applied to a wide variety of microorganisms like filamentous fungi, coherent yeast, insect cells, mammalian cells and plant cells (50–52). See Table 1 (48).

However, bacteria holding various kinds of useful enzymes and used as whole cell biocatalyst in industrial processes usually were unsuccessful to be immobilized with a high density in the BSPs because of the small size of about 0.5–2 μm . Therefore, the purpose in this thesis is to explore an efficient method to immobilize *E. coli* cells, which is widely used as a gene recombination host.

TABLE 1. Typical reported applications of biomass support particles (BSPs)

Organism	BSPs	Product and application	Reference
Microbial cell			
Mixed culture (aerobic)	PUF	Waste treatment	(53)
Mixed culture (anaerobic)	PUF	Methane	(54)
<i>Escherichia coli</i>	SF	Amylase	(55)
<i>Saccharomyces cerevisiae</i>	SS&	Ethanol	(50)
& <i>Saccharomyces uvarum</i>	PEF		
<i>Clostridium acetobutylicum</i>	NS	Acetone, butanol, ethanol	(56)
<i>Trichoderma viride</i>	SS	Cellulase	(57)
<i>Mucor ambiguus</i>	PUF	γ -Linolenic acid	(58)
<i>Rhizopus chinensis</i>	PUF	Lipase	(59)
<i>Rhizopus arrhizus</i>	PUF	Fumaric acid	(60)
<i>Phanerochaete chrysosporium</i>	PUF	Lignin peroxidase	(61)
<i>Penicillium chrysogenum</i>	PUF	Penicillin	(62)
<i>Botryococcus braunii</i>	PUF	Hydrocarbon	(63)
<i>Thiobacillus ferrooxidans</i>	PUF	Ferrous iron oxidation	(64)
Plant cell			
<i>Capsicum frutescens</i>	PUF	Capsaicin	(51)
<i>Humulus lupulus</i>	PUF	Hop flavous	(65)
<i>Coffea arabica</i> L	PUF	Caffeine	(66)

PUF, polyurethane foam; SF, silicone foam; SS, Stainless steel; PEF, polyester foam;

NS, Natural sponge

Escherichia coli

Escherichia coli is a kind of bacterium that is commonly found in the lower intestine of warm-blooded animals and it can live on a wide variety of substrates. *E. coli* is gram-negative, facultative anaerobic and non-sporulating. It can grow easily and its genetics are comparatively simple and easily-manipulated, these features make it one of the best-studied prokaryotic model organisms, and an important species in biotechnology. It has been used in the production of many substances. Especially in current, it is widely used in the field of genetic recombination. In this thesis, two strains of *E. coli* (K 12 and ATCC 11303) were used. *E. coli* K 12 was used in the study of immobilization, because it's widely use as host in genetic recombination. And *E. coli* ATCC 11303 cells that hold aspartase was used to study the production of L-aspartic acid.

L-aspartic acid production

Aspartic acid is a α -amino acid. The L-isomer is a one of the 20 proteinogenic amino acids. L-aspartic acid is widely used in food and pharmaceutical industry as an important amino acid. L-aspartic acid can be added into cool drinks as nutrition supplement. It is used in medicine as ammoniac detoxicating agent, hepar function accelerator or fatigue refresher. And it can also be used in biochemical reagent, medium and intermediate in organic synthesis. L-aspartic acid can synthesis polymer of aspartic acid which is widely used in detergent industry. From 1960s, the L-aspartic acid has been produced from fumaric acid and ammonia in industrial scale conventionally by the action of microbial enzyme: aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1). In this conventional method, there were many problems

in industrial scale. In past years, many efforts have been on more effectively reaction by immobilized aspartase and various kinds of immobilization method have been investigated (67– 69). In 1973, a method that include *E. coli* cells using polyacrylamide gel for continuous production of L-aspartic acid was realized industrially by Tanabe Seiyaku Co., Ltd. (67). Because the aspartase was an intracellular enzyme, the immobilization of microbes can promote reactions superior. The operation of extract enzymes from cells is unnecessary. And in industrial scale, immobilized enzymes are more suitable to continuous utilization. There is a successful industrial process of the production of L-aspartic acid using immobilized aspartase from *E. coli* as whole cell biocatalyst in Japan (70).

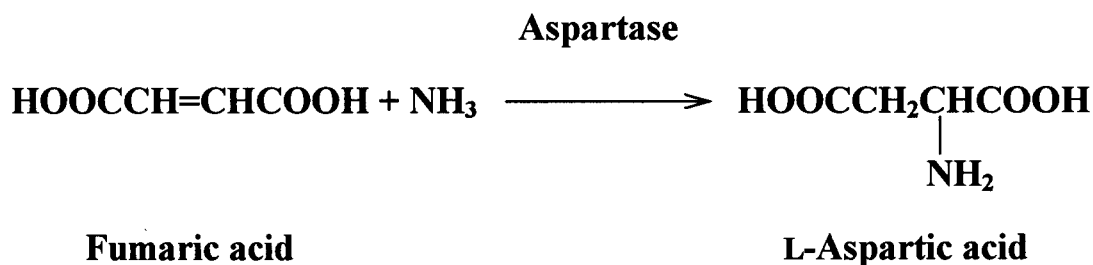


FIG. 1. Formation of L-aspartic acid using aspartase

In this thesis, to utilize bacterial cells as whole cell biocatalyst in a convenient and economical form for the production of useful chemicals, the immobilization of *E. coli* ATCC 11303 cells containing aspartase activity within BSPs was investigated. The use of BSP-immobilized *E. coli* cells as whole cell biocatalyst for the production of L-aspartic acid was also examined.

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SYNOPSIS

Part I

Immobilization of *Escherichia coli* cells using porous support particles coated with cationic polymers

The technique of cell immobilization using porous biomass support particles (BSPs), which is attractive from the point of view of simplicity and convenience, relies on the inherent ability of adhesive cells, as a consequence of their growth, to form films around the support material or the ability of flocculent cells to create flocculation within the porous structure. In the present study, the immobilization of *Escherichia coli* cells using BSPs was investigated. The density of immobilized cells within BSPs was evaluated by measuring intracellular lactate dehydrogenase (LDH) activity. Since *E. coli* K12 cells were not successfully retained within reticulated polyvinyl formal (PVF) resin BSPs with matrices of relatively small pores, coating the surface of BSPs with various polymers was examined as a way of promoting cell attachment. When positively charged polyamino acids such as poly-L-lysine, poly-L-arginine, poly-L-histidine, poly-L-ornithine and polyethyleneimine were adsorbed onto the particle surface, they were found to increase the immobilized cell density, while neutral and negatively charged polyamino acids including poly-L-asparagine and poly-L-glutamic acid were not effective. These results indicate that *E. coli* cells can be efficiently immobilized in PVF resin BSPs by electrostatic interaction between the negatively charged ions of the cell surface and the positively charged polymers adsorbed onto the BSP surface.

Part II

Preparation of porous support particles coated with cationic polymers for immobilization of *Escherichia coli* cells

Escherichia coli cells can be efficiently immobilized in reticulated polyvinyl formal resin (PVF) biomass support particles (BSPs) onto which cationic polymers including poly-L-lysine and polyethyleneimine are adsorbed. Particularly, a significantly high immobilized cell density is achieved in the BSPs coated with polyethyleneimine, a synthetic polymer with a high density of amino groups. To determine the optimal conditions for the surface coating of PVF resin BSPs, treatment time, polyethyleneimine concentration, and pore diameter of BSPs were investigated in shake-flask culture. Treatment time with polyethyleneimine did not affect the cell density within BSPs. Polyethyleneimine concentrations of 0.1 g/l and 0.2 g/l produced high levels of immobilized cell density. The highest cell density was obtained with BSPs with matrices of 60 μm pore diameter. Furthermore, it was found that a significantly high density of *E. coli* cells can be achieved in the BSPs simply prepared by autoclaving with the polymer solutions.

Part III

Production of L-aspartic acid using immobilized *Escherichia coli* cells as whole cell biocatalyst

L-Aspartic acid has been extensively used as a food additive and a raw material in the manufacture of medicines and a dipeptide sweetener known as aspartame. L-Aspartic acid can be produced by the activity of an enzyme aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) that catalyzes the conversion of fumaric acid and NH_4^+ to L-aspartic acid. The use of immobilized *Escherichia coli* cells containing aspartase activity has been practiced on an industrial scale. Polyacrylamide gel encapsulation and, more recently, entrapment of *E. coli* cells within κ -carrageenan gel have been used as the immobilization methods. In the present study, immobilization of *E. coli* ATCC 11303 cells within porous biomass support particles (BSPs) and the use of BSP-immobilized *E. coli* cells as whole cell biocatalyst for the production of L-Aspartic acid were investigated. A significantly high density of *E. coli* ATCC 11303 cells was achieved in polyvinyl formal resin BSPs coated with polyethyleneimine. The immobilized cells also showed a high level of aspartase activity.

Part I

Immobilization of *Escherichia coli* cells using porous support particles coated with cationic polymers

INTRODUCTION

In recent years, since they can catalyze various chemical reactions under mild conditions with great specificity, enzymes have come to be used increasingly for the industrial-scale production of commodity chemicals as well as fine chemicals (1–3). Biocatalytic conversion for the production of useful chemicals can be carried out using the extracellular or intracellular enzymes of microbial cells. Extracellular enzymes, however, require complicated and costly purification procedures that may limit widespread application. Furthermore, enzymes recovered through such operations are generally unstable and expensive. Accordingly, there has been considerable interest in the direct use of the intracellular enzymes of intact microbial cells as biocatalyst, known as whole cell biocatalyst (3).

To utilize microbial cells as whole cell biocatalyst in a convenient and economical form, the cells should be immobilized in such a way that they resemble the ordinary solid-phase catalysts used conventionally in synthetic chemical reactions. Of the various methods reported for cell immobilization, the passive immobilization technique using porous biomass support particles (BSPs), originally developed by Atkinson *et al.* (4), is attractive from the point of view of industrial application. The passive immobilization occurs when cells, as a consequence of their growth in culture, naturally form films around support material provided for that purpose or flocs within the porous structure of the material. This immobilization technique offers many advantages over other methods of immobilization, including ease of immobilization (natural entrapment), no need for chemical addition, large mass transfer rate in the particles, and ease of scale-up of the immobilization technique. This technique has

been successfully applied to a wide variety of microbial, plant, and animal cell systems (5–9).

Unfortunately, however, this technique does not allow dense immobilization of microbial cells that have no flocculent or adhesive characteristics, which include the *Escherichia coli* cells that extensively used as host cells in the expression of recombinant proteins such as various enzymes (6, 10). In mammalian cell culture, coating the culture surface of plastic dishes or flasks with a positively charged polymer such as poly-lysine has proved to be useful in promoting the attachment and spreading of many types of anchorage-dependent cells (11). Electrostatic interactions occur between the negatively charged surface ions of the cell membrane and the positively charged surface ions of attachment factors on the culture surface. When adsorbed onto the culture surface, poly-lysine increases the number of positively charged sites available for cell binding. Polyethyleneimine is a synthetic polymer with a high density of amino groups. It has been demonstrated to be a good poly-lysine replacement as a coating material to support the attachment and growth of neurons (12) and mammalian cell lines (13). A macroporous cellulose carrier whose surface was modified with polyethyleneimine has been successfully used for the high-density culture of hybridoma cells (14) and recombinant L-929 cells (15). A macroporous cellulose carrier treated with polyethyleneimine and stabilized by crosslinking has also been used for the immobilization of nitrifying bacteria and denitrification with immobilized cells (16, 17).

In the present study, the immobilization of *Escherichia coli* K12 cells within BSPs simply coated with various polymers was investigated in shake-flask culture. The density of the cells immobilized within BSPs was evaluated by measuring their

intracellular lactate dehydrogenase (LDH) activity. Our results indicate that *E. coli* cells can be efficiently immobilized in polyvinyl formal (PVF) resin BSPs by coating the surface with positively charged polyamino acids such as poly-L-lysine or the synthetic polymeric amine polyethyleneimine.

MATERIALS AND METHODS

Bacterial strain and culture medium

All experiments were carried out using an *E. coli* K12 strain, NovaBlue (Merck, Tokyo). Luria-Bertani (LB) medium was prepared with purified water which contained 10 g/l polypeptone, 5 g/l yeast extract, and 5 g/l NaCl. The *E. coli* cells were routinely maintained at 37°C on LB agar plates with 1.5% agar and LB medium.

BSPs and coating procedure

Reticulated PVF resin sponge sheets consisting of filter material (Aion, Osaka; pore diameter 60 μm ; porosity 0.88, apparent density 0.15 g/cm³) were cut into 2 × 2 × 2 mm cubes for use as BSPs and 250 units of each were autoclaved with purified water in 100-ml screw-capped Erlenmeyer flasks. After removal of the water, the BSPs were coated variously with the following polymers: poly-L-arginine hydrochloride (molecular weight (MW) 70,000–150,000; Sigma, St. Louis, MO, USA), poly-L-asparagine (MW 5000–15,000; Sigma), poly-L-glutamic acid sodium salt (MW 50,000–100,000; Sigma), poly-L-histidine hydrochloride (MW $\geq$ 5000; Sigma), poly-L-lysine hydrobromide (MW $\geq$ 300,000; Nacalai Tesque, Kyoto), poly-L-ornithine hydrobromide (MW > 100,000;

Sigma), and polyethyleneimine (MW 70,000; Wako Pure Chemical Industries, Osaka). All of the polymers were first dissolved at a concentration of 0.1 g/l in purified water and the solutions sterilized by filtration through a 0.22- μ m membrane filter. A 15 ml portion of each solution was then decanted into the Erlenmeyer flask and incubated with the BSPs for 4 h at 37°C on a reciprocal shaker (150 oscillations/min, amplitude 25 mm).

Shake-flask culture with BSPs

A colony of *E. coli* cells on an agar plate was transferred to a test tube containing 4 ml of LB medium and the cells were preincubated at 37°C on a reciprocal shaker (150 oscillations/min, amplitude 25 mm) for 12 h, after which the culture broth was diluted seven times with fresh LB medium. Fifteen milliliters of the resulting cell suspension was added to each of the Erlenmeyer flasks containing the BSPs after removal of the polymer solution. The cells and the BSPs were then incubated at 37°C on a reciprocal shaker (150 oscillations/min, amplitude 25 mm). At appropriate intervals, 1–10 BSPs were sampled from each flask for assessment of the density of cells entrapped within the BSPs. Reproducibility was checked to be satisfactory between separate culture experiments.

Cell density measurement

The density of free (nonimmobilized) cells was monitored in shake-flask culture by measuring the optical density of the cell suspension at a wavelength of 660 nm (OD_{660}) with a spectrophotometer (U-2001; Hitachi, Tokyo). The cell suspension was diluted with Dulbecco's phosphate buffered saline (PBS) to maintain OD_{660}

readings in the linear range below 1.0.

Assay of intracellular LDH activity

The cell density was also evaluated by measuring intracellular LDH activity (18–20) using an LDH Cytotoxicity Detection Kit (Takara Bio, Otsu). For measurement of the intracellular LDH activity of non-immobilized cells, the sampled cell suspension was diluted with PBS to maintain OD₆₆₀ below 1.0. Cells in 0.5 ml of the diluted cell suspension were washed twice with PBS in a microtube and sonicated for 20 s with a probe sonicator (Tomy Seiko, Tokyo). The cell lysate was incubated in the dark at room temperature for 30 min with 0.5 ml of a reaction mixture containing lactate, NAD⁺, diaphorase, and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which was supplied with the kit. LDH catalyzes the conversion of lactate and NAD⁺ to pyruvate and NADH. This reaction couples to the diaphorase-catalyzed re-oxidation of NADH by INT to regenerate NAD⁺ and form INT formazan. After the addition of 0.25 ml of 1 mol/l HCl to stop these reactions, the microtube was centrifuged. The absorbance of INT formazan in the supernatant was measured with a spectrophotometer using a test wavelength of 492 nm and a reference wavelength of 620 nm and the difference between the two absorbances ($A_{492} - A_{620}$) was calculated.

For measurement of the intracellular LDH activity of BSP-immobilized cells, appropriate numbers of BSPs were washed twice with PBS in a microtube to which PBS was then added to give a total volume (BSPs + PBS) of 0.5 ml. After sonication for 20 s with a probe sonicator, the preparation was incubated with 0.5 ml of the same reaction mixture as above for 30 min in the dark at room temperature. Following the

addition of 0.25 ml of 1 mol/l HCl and sonication for 30 s with a probe sonicator, the microtube was then centrifuged. The absorbance of the supernatant was measured as described above. The number of BSPs used for the assay was adjusted to maintain the value of $A_{492} - A_{620}$ in the linear range below 0.6.

Scanning electron microscopy

E. coli cells immobilized in BSPs were fixed for scanning electron microscopy (7). BSP-immobilized cells, washed well with PBS, were first fixed for 2 h in a mixture of 2% formaldehyde/4% glutaraldehyde in PBS. After washing thoroughly with PBS, the immobilized cells were fixed for 1 h more in 1% osmium tetroxide and again washed with PBS. The cells within the BSPs were then dehydrated through a graded series of ethanol soaks (50%, 60%, 70%, 80%, 90%, 95%, and 100% ethanol, using 100% ethanol twice) and the BSPs were dried at room temperature, soaked in liquid nitrogen then cut open immediately and coated with a film of platinum/palladium. Observations were made using a scanning electron microscope (SEM) (JSM-5610; JOEL, Tokyo).

RESULTS AND DISCUSSION

Intracellular LDH activity measurement to evaluate cell density

It is clearly important to monitor the growth of immobilized cells in immobilized cell culture. For foam BSPs (say 6 mm cubes) and stainless steel BSPs (6 mm spheres), dry cell weight measurements have been employed to determine the

biomass content of the material (6). In the case of the $2 \times 2 \times 2$ mm cubic BSPs used in the present study, however, it is virtually impossible to accurately measure dry cell weight given the small numbers of particles and an alternative procedure is therefore needed. The cytoplasmic enzyme LDH has been used to monitor the growth and death of mammalian cells immobilized within BSPs (18, 19). When the intracellular LDH content of viable cells is constant, the density of viable cells immobilized within the BSPs can be estimated by measuring LDH activity after lysing the cells with surfactants or by sonication. In the present study, the application of intracellular LDH activity measurement to *E. coli* cells immobilized within BSPs was examined.

Shake-flask culture of nonimmobilized *E. coli* cells was carried out to measure the intracellular LDH activity. Figure 1 shows the relationship between OD_{660} and $A_{492} - A_{620}$ measured in the culture broth. The absorbance of INT formazan ($A_{492} - A_{620}$) and thereby intracellular LDH activity increased linearly with the optical density, indicating that the intracellular LDH content of *E. coli* cells was almost constant in shake-flask culture. In the present study, the density of *E. coli* cells immobilized within BSPs was therefore evaluated in terms of OD_{660} from the absorbance of INT formazan formed by immobilized cells, based on the linear correlation determined with nonimmobilized cells (Fig. 1).

Effect of BSP surface coating on cell immobilization

Even when reticulated PVF resin BSPs with matrices of relatively small pores (pore diameter 60 μ m) were used, *E. coli* K12 cells were not successfully retained within the particles in shake-flask culture (indicated with crosses in Fig. 2). In mammalian cell culture, coating the plastic culture surface with a positively charged

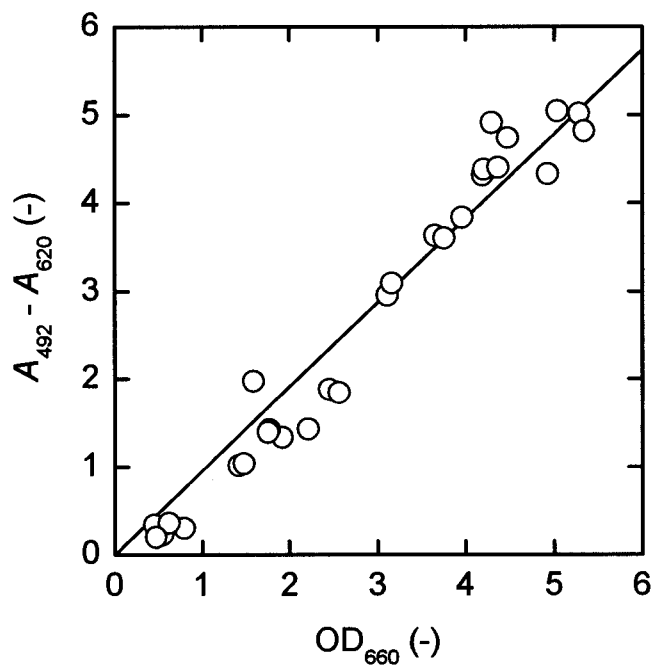


FIG. 1. Relationship between optical density at wavelength of 660 nm (OD_{660}) in culture broth taken from shake-flask culture of nonimmobilized *Escherichia coli* K12 cells and difference in absorbance at 492 nm and at 620 nm ($A_{492} - A_{620}$) in intracellular lactate dehydrogenase (LDH) activity measurement.

polyamino acid such as poly-lysine has been used to facilitate the attachment and spreading of many types of anchorage-dependent cells (11). Coating the surface of the PVF resin BSPs with various polyamino acids was therefore examined as a way of promoting the attachment of *E. coli* cells.

When the BSPs were pretreated with poly-L-lysine, a considerably higher cell density within the BSPs was obtained than in nontreated BSPs (Fig. 2). Treatment with poly-L-arginine, poly-L-histidine, or poly-L-ornithine was also effective in increasing the cell density in BSPs, while poly-L-histidine was less effective than the others. In contrast, poly-L-asparagine and poly-L-glutamic acid did not affect the cell density within the BSPs. All the polyamino acids effective in increasing the cell density within the BSPs, poly-L-arginine, poly-L-histidine, poly-L-lysine, and poly-L-ornithine, consist of basic amino acids with a positively charged side chain under neutral pH conditions, while poly-L-glutamic acid is negatively charged and poly-L-asparagine is uncharged. Hence, the following mechanism is suggested to account for the increase in the cell density in BSPs: when adsorbed onto the surface of uncharged PVF resin BSPs, positively charged polyamino acids produce positively charged sites on the BSP surface to which *E. coli* cells can be efficiently attached due to electrostatic interactions with their negatively charged surface ions.

Coating the BSP surface with polyethyleneimine, a synthetic polymer with a high density of amino groups, was also examined. Figure 2 shows the effect of surface coating with polyethyleneimine on the *E. coli* cell density within PVF resin BSPs in shake-flask culture. As can be seen from the figure, polyethyleneimine was highly effective in increasing the cell density within the BSPs. Since the amino groups of polyethyleneimine can be protonated, the high *E. coli* cell density within the BSPs

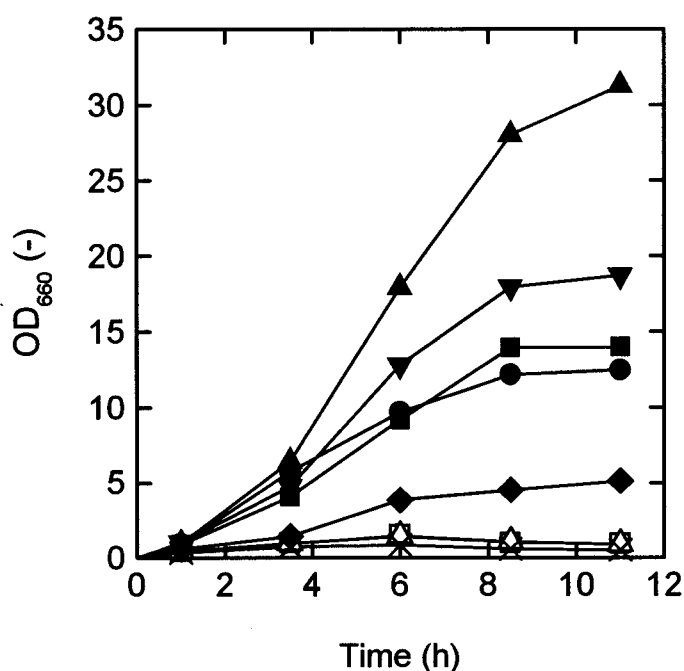


FIG. 2. Effect of coating particle surface with various polymers on entrapment of *E. coli* cells in reticulated polyvinyl formal (PVF) resin biomass support particles (BSPs) in shake-flask culture. Before shake-flask culture, 250 BSPs with matrices of 60 μm pore diameter were incubated for 4 h at 37°C on a reciprocal shaker in Erlenmeyer flasks with 15 ml of 0.1 g/l solution of poly-L-arginine (circles), poly-L-asparagine (open squares), poly-L-glutamic acid (open triangles), poly-L-histidine (diamonds), poly-L-lysine (inverted triangles), poly-L-ornithine (closed squares), or polyethyleneimine (closed triangles). Untreated BSPs are indicated with crosses.

probably results from electrostatic interactions between the negative charge of the *E. coli* cell surface and the positively charged sites of the polyethyleneimine adsorbed onto the BSP surface. Polyethyleneimine is attractive because its cost is significantly lower than that of polyamino acids like poly-L-lysine.

Scanning electron microscopy of immobilized cells

The interiors of BSPs retaining *E. coli* cells were observed using an SEM. Figure 3 shows a scanning electron micrograph of *E. coli* cells entrapped in reticulated PVF resin BSPs coated with polyethyleneimine after 11 h in shake-flask culture. As can be seen from this photograph, cells were attached to the surface of the reticulated PVF resin. This observation may support the mechanism proposed above to account for the increase in cell density within the BSPs, particularly for polyethyleneimine.

Accumulation of cells within BSPs

To evaluate the extent of accumulation of cells within BSPs, shake-flask culture of *E. coli* cells using BSPs with or without polyethyleneimine pretreatment was carried out together with shake-flask culture of nonimmobilized cells. Figures 4a and 4b show the time course of the density of cells entrapped in the BSPs and the density of free (nonimmobilized) cells in the culture broth in shake-flask cultures with polyethyleneimine-treated or untreated BSPs. For comparison, the time course of the culture broth cell density in shake-flask culture without BSPs is shown in Fig. 4c. Cell density in Fig. 4 is expressed as OD₆₆₀ based on intracellular LDH activity measurement. The total biomass content (immobilized and nonimmobilized cells) of each flask at 11 h was calculated and found to be almost the same regardless of whether BSPs

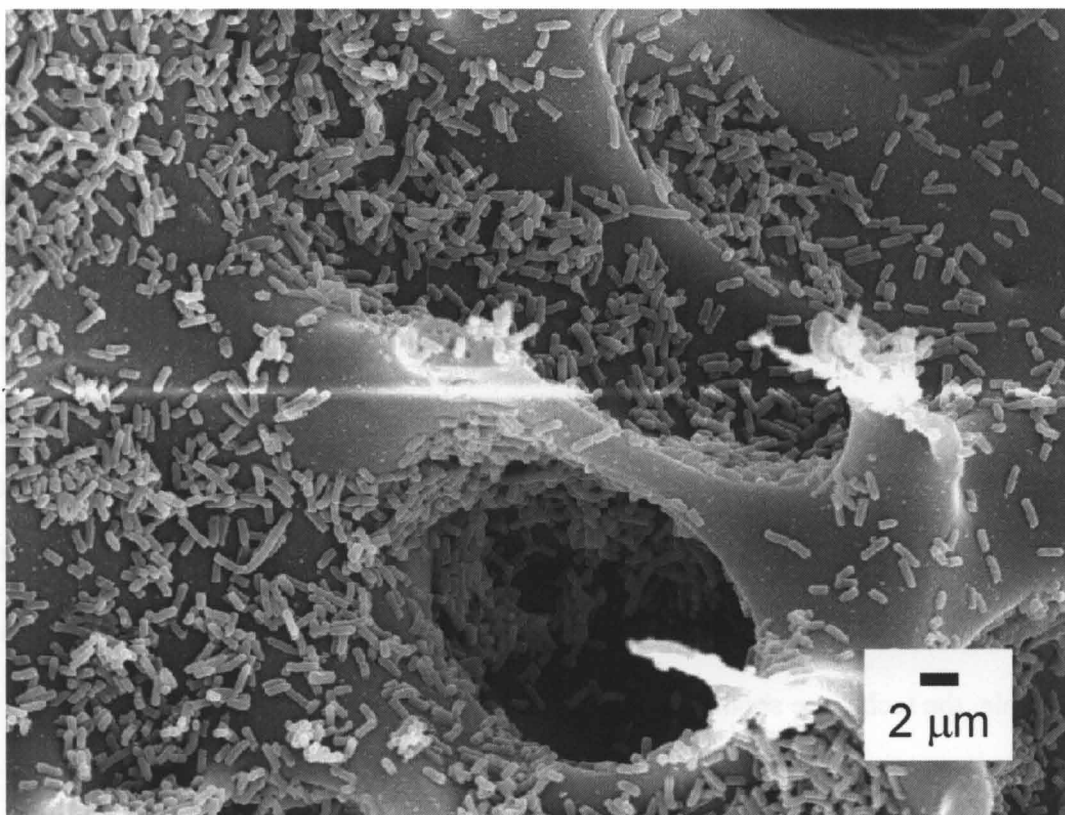


FIG. 3. Scanning electron micrograph of *E. coli* cells immobilized in reticulated PVF resin BSPs coated with polyethyleneimine after 11 h in shake-flask culture.

were present or were treated with polyethyleneimine. This finding indicates that the growth of *E. coli* cells was not influenced by BSPs or by polyethyleneimine-coated BSPs. The density of cells entrapped in polyethyleneimine-treated BSPs was nearly five times higher than that in shake-flask culture without BSPs. Furthermore, almost 70% of the cells were evaluated to be present in the BSPs coated with polyethyleneimine (Fig. 4a).

From the results obtained in the present study, it is concluded that *E. coli* K1 cells can be efficiently immobilized within PVF resin BSPs by simply coating the particle surface with polyethyleneimine or polyamino acids such as poly-L-arginine, poly-L-histidine, poly-L-lysine, and poly-L-ornithine. In particular, polyethyleneimine is an attractive coating polymer because of its relatively low cost. Preparation of BSPs coated with these polymers is simple and can be easily carried out. Cell immobilization can be achieved naturally and aseptically through adsorption of cells onto the particle surface. Since reticulated PVF resin BSPs are mechanically strong and stable, the technique should be simple to scale up.

FIG. 4. Growth of *E. coli* cells in shake-flask culture using reticulated PVF resin BSPs with (a) and without (b) polyethyleneimine treatment. Symbols: circles: cell density in BSPs; squares: cell density in culture broth. BSPs with matrices of 60 μm pore diameter were used after treatment with 0.1 g/l polyethyleneimine solution for 4 h; (c) cell density in shake-flask culture without BSPs.

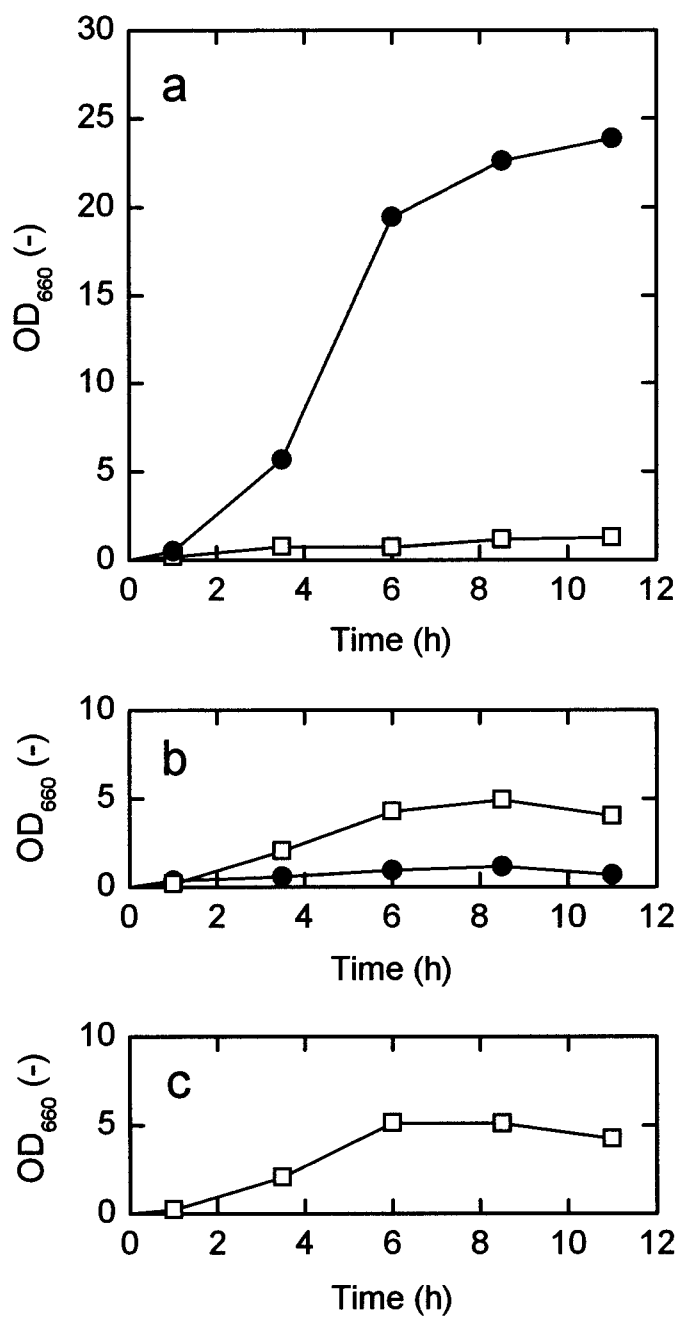


FIG. 4. (legend on page 38)

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Part II

**Preparation of porous support particles coated with cationic polymers
for immobilization of *Escherichia coli* cells**

INTRODUCTION

The technique of cell immobilization using porous support particles (biomass support particles, BSPs) has been successfully applied to a variety of microbial, plant, and animal cell systems (1–4). This immobilization technique is attractive from the point of view of simplicity and convenience since passive immobilization occurs when films or flocculation of cells form naturally around or within support material provided for that purpose (5–9). This technique, however, does not allow dense immobilization of bacterial cells that have no flocculent or adhesive characteristics, such as *Escherichia coli* cells (6, 10).

In a previous study (11), the immobilization of *E. coli* K12 cells using BSPs was investigated in shake-flask culture. Since the *E. coli* cells were not successfully retained within reticulated polyvinyl formal (PVF) resin BSPs with matrices of relatively small pores (pore diameter 60 μm), coating the surface of the BSPs with various polymers was examined as a way of promoting cell attachment (12–16). When positively charged polyamino acids including poly-L-lysine, poly-L-arginine, poly-L-histidine, and poly-L-ornithine and polyethyleneimine were adsorbed onto the particle surface, they were found to increase the immobilized cell density, while neutral and negatively charged polyamino acids were not effective. It was concluded that *E. coli* cells can be efficiently immobilized in PVF resin BSPs by electrostatic interaction between the negatively charged ions of the cell surface and the positively charged polymers adsorbed onto the BSP surface. A significantly high immobilized cell density was achieved in the BSPs coated with polyethyleneimine, a synthetic polymer with a

high density of amino groups.

In the present study, to determine the optimal conditions for the surface coating of PVF resin BSPs, treatment time and polyethyleneimine concentration were investigated in shake-flask culture. While reticulated PVF resin BSPs with matrices of 60 μm pore diameter were used in a previous study (11), the effect of BSP pore size was also examined in the present study.

Furthermore, in the present study, procedures for preparing BSPs coated with cationic polymers were investigated. Thus far a macroporous cellulose carrier treated with polyethyleneimine and stabilized by crosslinking has been developed for the immobilization of nitrifying bacteria (17) and mammalian cells (18–20). In a previous study (11), we prepared reticulated PVF resin BSPs onto which cationic polymers including poly-L-lysine and polyethyleneimine were adsorbed in the following way: the BSPs were first sterilized by autoclaving with water and then incubated with filter-sterilized polymer solutions for 4 h at 37°C on a reciprocal shaker. From the point of view of industrial application, however, a simpler and easier method of preparing BSPs coated with cationic polymers is required. In the present study, we examined another approach to coat the BSP surface with cationic polymers. After autoclaved with polyethyleneimine or poly-L-lysine solution, reticulated PVF resin BSPs were found to successfully retain a significantly high density of *E. coli* cells. This approach eliminates the processes of filter sterilization of polymer solutions and incubation with cationic polymers in preparing the BSPs.

MATERIALS AND METHODS

Bacterial strain and culture medium

All experiments were carried out using an *E. coli* K12 strain, NovaBlue (Merck, Tokyo). Luria-Bertani (LB) medium was prepared with purified water which contained 10 g/l polypeptone, 5 g/l yeast extract, and 5 g/l NaCl. The *E. coli* cells were routinely maintained at 37°C on LB agar plates with 1.5% agar and LB medium.

Preparation of BSPs

Six types of reticulated PVF resin sponge sheet consisting of filter material (Aion, Osaka; pore diameter 60, 80, 130, 200, 300, or 700 μm ; porosity 0.88–0.91; apparent density 0.11–0.15 g/cm³) were cut into 2 × 2 × 2 mm cubes for use as BSPs. The BSPs were coated with polyethyleneimine (MW 70,000; Wako Pure Chemical Industries, Osaka) or poly-L-lysine hydrobromide (MW $\geq$ 300,000; Nacalai Tesque, Kyoto) in two different ways. In the first way, as previously described (11), the polymers were first dissolved at a concentration of 0.1 g/l in purified water and the solutions sterilized by filtration through a 0.22- μm membrane filter. Meanwhile, 250 BSPs were autoclaved with purified water in 100-ml screw-capped Erlenmeyer flasks. After removal of the water, a 15 ml portion of each polymer solution was then decanted into the Erlenmeyer flask and incubated with the BSPs for 4 h at 37°C on a reciprocal shaker (150 oscillations/min, amplitude 25 mm). In this way polyethyleneimine concentrations of 0.1 g/l and 0.2 g/l produced the highest levels of immobilized cell density in the BSPs (11). In the other way of coating, 250 BSPs were directly autoclaved for 20 min at 121°C with 15 ml of each polymer solution at different

concentrations in the Erlenmeyer flask.

Shake-flask culture with BSPs

A colony of *E. coli* cells on an agar plate was transferred to a test tube containing 4 ml of LB medium and the cells were preincubated at 37°C on a reciprocal shaker (150 oscillations/min, amplitude 25 mm) for 12 h, after which the culture broth was diluted seven times with fresh LB medium. Fifteen milliliters of the resulting cell suspension was added to each of the Erlenmeyer flasks containing the BSPs after removal of the polymer solution. The cells and the BSPs were then incubated at 37°C on a reciprocal shaker (150 oscillations/min, amplitude 25 mm). At appropriate intervals, 1–10 BSPs were sampled from each flask for assessment of the density of cells entrapped within the BSPs.

Cell density measurement

The density of free (nonimmobilized) cells was monitored in shake-flask culture by measuring the optical density of the cell suspension at a wavelength of 660 nm (OD₆₆₀) with a spectrophotometer (U-2001; Hitachi, Tokyo). The cell suspension was diluted with Dulbecco's phosphate buffered saline (PBS) to maintain OD₆₆₀ readings in the linear range below 1.0.

The cell density was also evaluated by measuring the intracellular LDH activity using an LDH Cytotoxicity Detection Kit (Takara Bio, Otsu) for the measurement of intracellular LDH activity of both non-immobilized cells and BSP-immobilized cells (11, 21–23).

RESULTS AND DISCUSSION

Effect of polyethyleneimine treatment conditions

To determine the optimal conditions for the surface coating of PVF resin BSPs, treatment time and polyethyleneimine concentration were investigated in shake-flask culture using BSPs with matrices of 60 μm pore diameter (Fig. 1). Treatment time with polyethyleneimine did not affect the time course of cell density within BSPs (Fig. 1a), indicating that the rate of polyethyleneimine adsorption onto the surface of the reticulated PVF resin was rapid. Polyethyleneimine concentrations of

FIG. 1. (a) Effect of treatment time on immobilization of *E. coli* cells within reticulated PVF resin BSPs in shake-flask culture. Before shake-flask culture, 250 BSPs with matrices of 60 μm pore diameter were incubated with 15 ml of 0.1 g/l polyethyleneimine solution in Erlenmeyer flasks at 37°C on a reciprocal shaker for 1 h (circles), 2 h (squares), 4 h (triangles), 8 h (diamonds), or 24 h (inverted triangles). Untreated BSPs are indicated with crosses. (b) Effect of polyethyleneimine concentration on cell immobilization within BSPs in shake-flask culture. Before shake-flask culture, 250 BSPs with matrices of 60 μm pore diameter were incubated for 4 h at 37°C on a reciprocal shaker in Erlenmeyer flasks with 15 ml of polyethyleneimine solution at the following concentrations: 0.05 g/l (circles), 0.1 g/l (squares), 0.2 g/l (triangles), 0.5 g/l (diamonds), or 1 g/l (inverted triangles). Untreated BSPs are indicated with crosses.

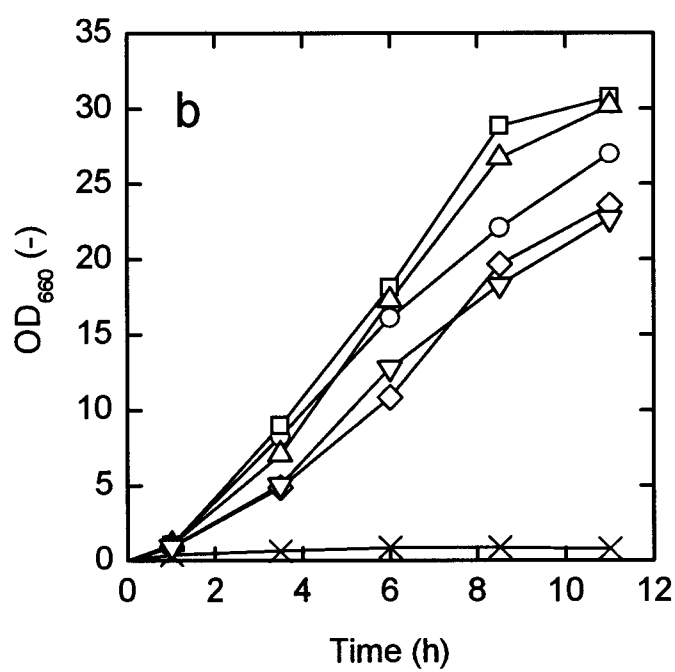
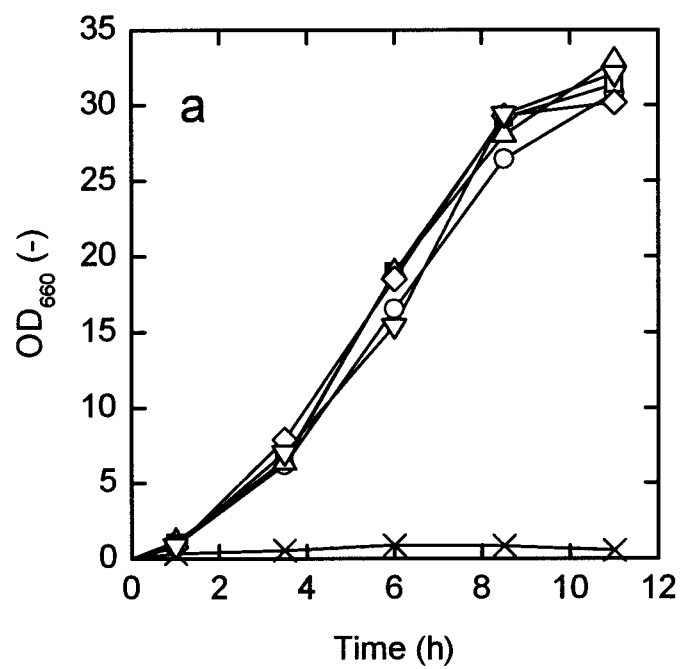


FIG. 1. (legend on page 48)

0.1 g/l and 0.2 g/l produced slightly higher levels of immobilized cell density in BSPs than the others (Fig. 1b).

Effect of BSP pore size and specific surface area

Next, shake-flask culture using reticulated PVF resin BSPs with matrices of 60–700 μm pore diameter was performed following treatment of the BSPs with 0.1 g/l polyethyleneimine solution for 4 h (Fig. 2). The highest cell density was obtained with BSPs with matrices of the smallest pore diameter, 60 μm . Although the BSP pore size appears to influence cell density, the size of the *E. coli* cells is much smaller than that of the BSP pores.

Generally decreasing the pore diameter of porous material leads to an increase in the specific surface area. This is not necessarily in the case for reticulated PVF resin BSPs since they differ in porosity as well as pore diameter. It is however considered that the immobilized cell density within BSPs may be affected by the specific surface area of the BSPs rather than the pore size. Table 1 shows the specific surface area of reticulated PVF resin BSPs. Since the structure of the BSPs with matrices of 700 μm pore size is different from the other BSPs, the specific surface area of this type of BSPs is larger than that of BSPs with matrices of 200 μm pore diameter. As expected, the immobilized cell density increased with increasing the specific area of BSPs (Fig. 3).

Taken together, the BSPs with matrices of 60 μm pore diameter were selected for use in subsequent investigations after treatment with 0.1 g/l polyethyleneimine solution for 4 h.

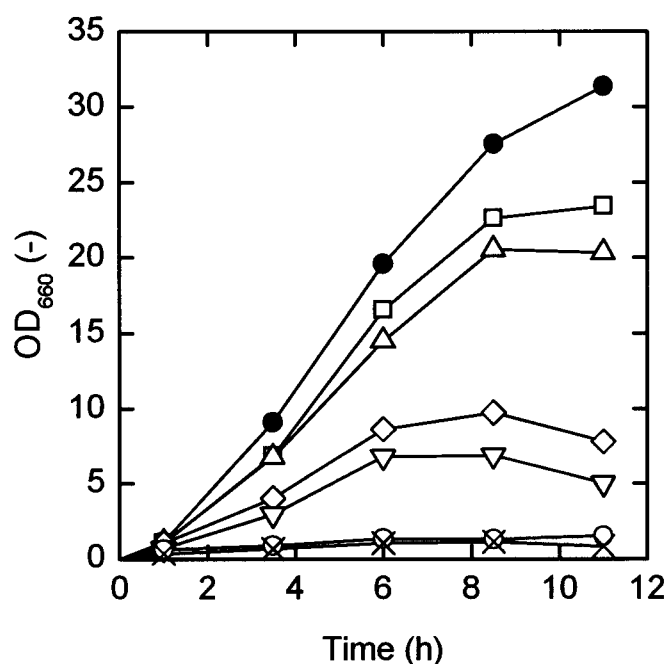


FIG. 2. Effect of pore size on immobilization of *E. coli* cells within reticulated PVF resin BSPs in shake-flask culture. Before shake-flask culture, 250 BSPs with matrices of various pore diameters were incubated for 4 h at 37°C on a reciprocal shaker in Erlenmeyer flasks with 15 ml of 0.1 g/l polyethyleneimine solution. Pore diameter: 60 μm (closed circles), 80 μm (squares), 130 μm (triangles), 200 μm (diamonds), 300 μm (inverted triangles), or 700 μm (crosses). Untreated BSPs with matrices of 60 μm pore diameter are indicated with open circles.

TABLE 1. Pore diameter and specific surface area of reticulated PVF resin BSPs

Pore diameter	Specific surface area
(μm)	(m^2/g)
60	0.834
80	0.528
130	0.389
200	0.234
700	0.282

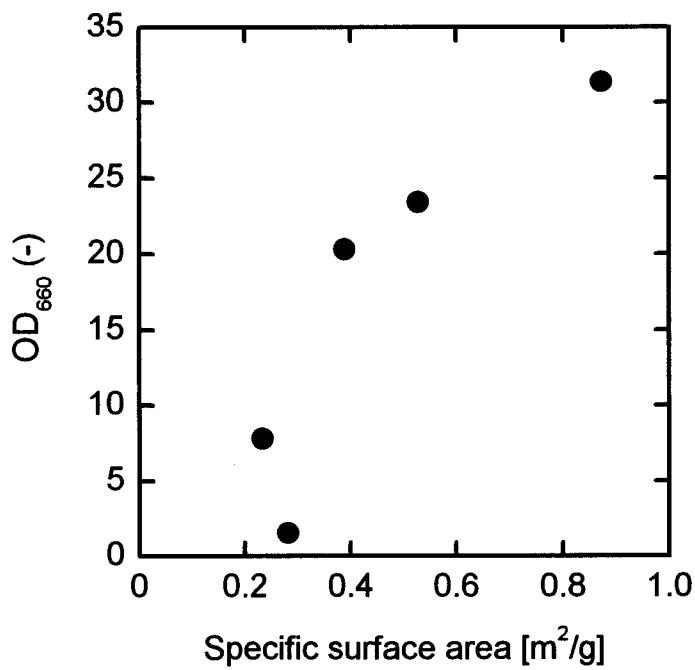


FIG. 3. Effect of specific surface area on immobilization of *E. coli* cells within reticulated PVF resin BSPs in shake-flask culture. Before shake-flask culture, 250 BSPs with matrices of various pore diameters were incubated for 4 h at 37°C on a reciprocal shaker in Erlenmeyer flasks with 15 ml of 0.1 g/l polyethyleneimine solution.

Comparison of polyethyleneimine treatment procedures

Next, we examined different approaches to coat the BSPs surface with cationic polymers. First, reticulated PVF resin BSPs with matrices of 60 μm pore diameter were treated with synthetic polymeric amine polyethyleneimine in different ways and the densities of *E. coli* K12 cells immobilized within the BSPs were evaluated in shake-flask culture (Fig. 4). When the BSPs were prepared by directly autoclaving with 0.1 g/l polyethyleneimine solution, a considerably higher cell density within the BSPs was obtained than in nontreated BSPs, indicating that this simple preparation procedure is effective in increasing the immobilized cell density. However, the immobilized cell density remained half of that within the BSPs prepared by the method previously described, that is, by autoclaving with water and then incubating in 0.1 g/l filter-sterilized polyethyleneimine solution. Before autoclaved with water, reticulated PVF resin BSPs with matrices of 60 μm pore diameter float on the water since they retain air within the pores. Once autoclaved with water, the BSPs sink in the water. While the BSPs float on the polyethyleneimine solution during autoclaving, polyethyleneimine might be adsorbed inefficiently onto the BSP surface. Hence, the BSPs were prepared by autoclaving first with water and then in 0.1 g/l polyethyleneimine solution.

As can be seen from Fig. 4, the density of cells immobilized within the resultant BSPs increased but was still lower than within the BSPs prepared by incubation in the polyethyleneimine solution after autoclaving.

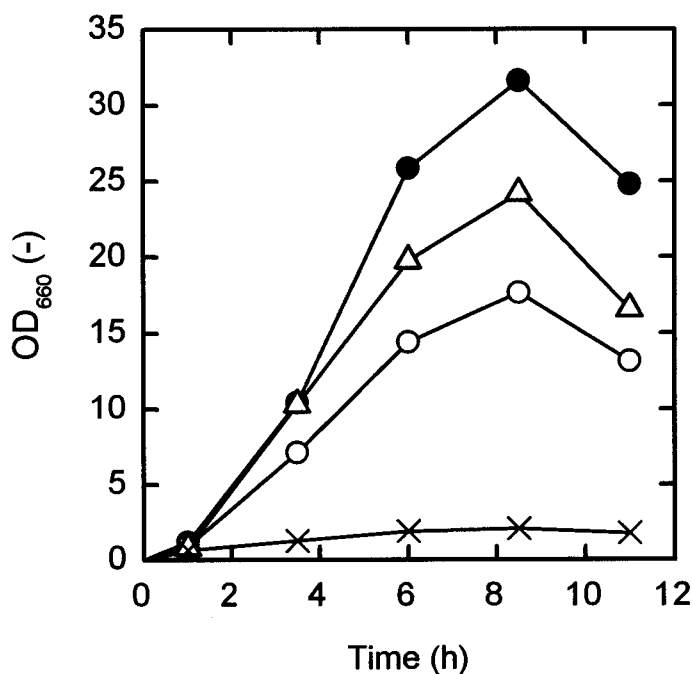


FIG. 4. Effect of polyethyleneimine treatment procedure on immobilization of *E. coli* cells within reticulated polyvinyl formal (PVF) resin biomass support particles (BSPs) in shake-flask culture. Symbols: closed circles, BSPs were autoclaved with purified water and then incubated for 4 h at 37°C with 15 ml of 0.1 g/l filter-sterilized polyethyleneimine solution before shake-flask culture; open circles, BSPs were directly autoclaved with 15 ml of 0.1 g/l polyethyleneimine solution before shake-flask culture; triangles, BSPs autoclaved with purified water were autoclaved again with 15 ml of 0.1 g/l polyethyleneimine solution; crosses, untreated BSPs.

Next, the effect of polyethyleneimine concentration during autoclaving on immobilization of *E. coli* cells was examined (Fig. 5). As reported above, when reticulated PVF resin BSPs were autoclaved with water and then incubated in filter-sterilized polyethyleneimine solutions, polyethyleneimine concentrations of 0.1 g/l and 0.2 g/l produced the highest levels of immobilized cell density in the BSPs. By contrast, when the BSPs were prepared by directly autoclaving with polyethyleneimine solutions, the cell density in the BSPs increased with the polyethyleneimine concentration up to 0.5 g/l (Fig. 5). Interestingly, the immobilized cell densities in the BSPs directly autoclaved with 0.5 g/l and 1 g/l polyethyleneimine solutions were 1.3-fold higher than the density within the BSPs incubated in 0.1 g/l polyethyleneimine solution after autoclaving. The reason why the higher cell densities were obtained by directly autoclaving the BSPs with polyethyleneimine solutions is not known. The amount of polyethyleneimine adsorbed onto the surface of the BSPs is presumably increased by directly autoclaving the BSPs with polyethyleneimine solutions, though further investigations are required to elucidate the mechanism.

Autoclaving the BSPs with poly-L-lysine solutions was also examined (Fig. 6). When the BSPs were prepared by directly autoclaving with 0.1 g/l poly-L-lysine solution, the immobilized cell density remained as high as in the BSPs prepared by autoclaving with water and then incubating in 0.1 g/l filter-sterilized poly-L-lysine solution. This result indicates that after autoclaved, poly-L-lysine is still effective in promoting cell attachment. When the BSPs were directly autoclaved with 0.5 g/l poly-L-lysine solution, the immobilized cell density achieved was approximately six times higher than that obtained in the BSPs incubated in the poly-L-lysine solution after autoclaving. As a result, the maximum immobilized cell density achieved in the

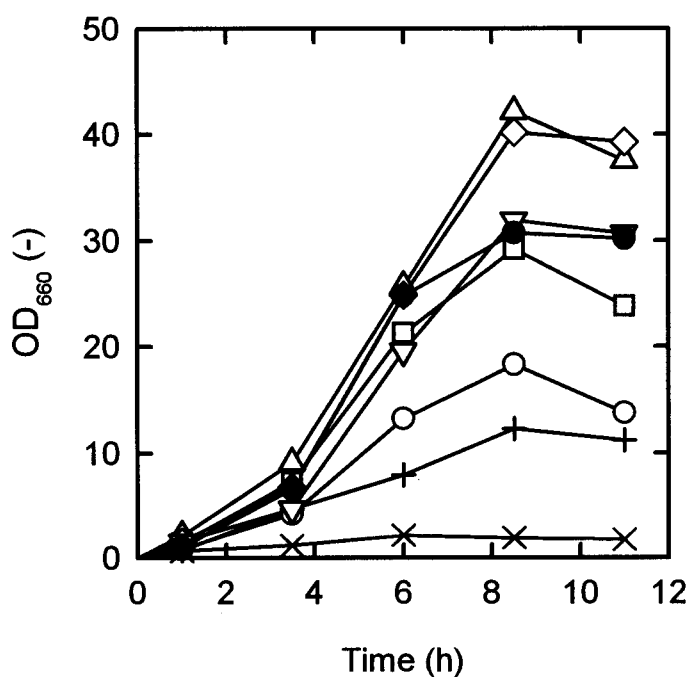


FIG. 5. Effect of polyethyleneimine concentration during autoclaving on immobilization of *E. coli* cells within reticulated PVF resin BSPs in shake-flask culture. Before shake-flask culture, BSPs were autoclaved with 15 ml of polyethyleneimine solution at the following concentrations: 0.05 g/l (plus symbols), 0.1 g/l (open circles), 0.2 g/l (squares), 0.5 g/l (triangles), 1 g/l (diamonds) or 1.5 g/l (inverted triangles). BSPs were autoclaved with purified water and then incubated for 4 h at 37°C with 15 ml of 0.1 g/l filter-sterilized polyethyleneimine solution before shake-flask culture (closed circles). Untreated BSPs are indicated with crosses.

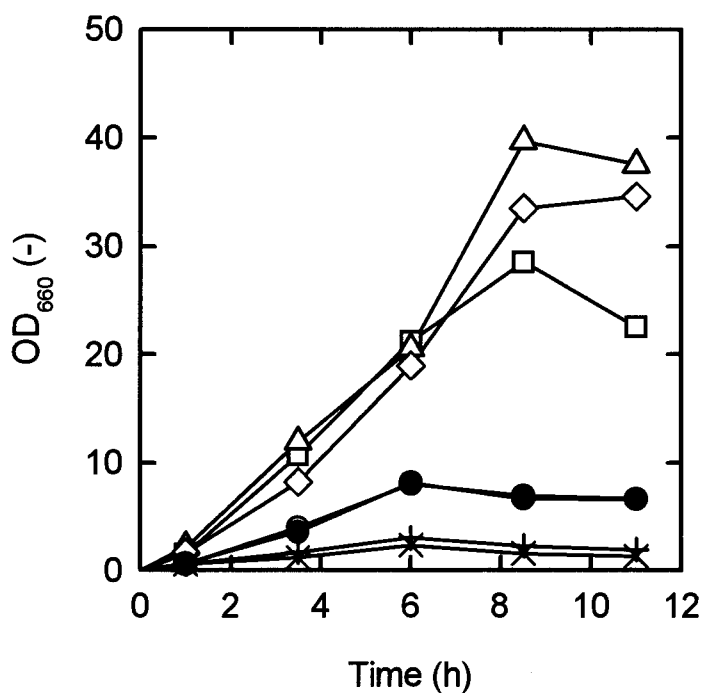


FIG. 6. Effect of poly-L-lysine concentration during autoclaving on cell immobilization within BSPs in shake-flask culture. Before shake-flask culture, BSPs were autoclaved with 15 ml of poly-L-lysine solution at the following concentrations: 0.05 g/l (plus symbols), 0.1 g/l (open circles), 0.2 g/l (squares), 0.5 g/l (triangles), or 1 g/l (diamonds). BSPs were autoclaved with purified water and then incubated for 4 h at 37°C with 15 ml of 0.1 g/l filter-sterilized poly-L-lysine solution before shake-flask culture (closed circles). Untreated BSPs are indicated with crosses.

poly-L-lysine-treated BSPs was comparable to that obtained in the polyethyleneimine-treated BSPs (Figs. 4, 5).

In conclusion, *E. coli* K12 cells can be densely immobilized within reticulated PVF resin BSPs simply prepared by autoclaving with polyethyleneimine or poly-L-lysine solution. While comparable immobilized cell densities were obtained within the BSPs autoclaved with poly-L-lysine and polyethyleneimine solutions, polyethyleneimine is attractive because its cost is significantly lower than poly-L-lysine. This preparation procedure of the BSPs is simpler and can be more easily carried out than that described in a previous study (11). Since the technique should be simple to scale up, the cell immobilization using polyethyleneimine-coated BSPs is promising for industrial application.

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Part III

Production of L-aspartic acid using immobilized *Escherichia coli* cells as whole cell biocatalyst

INTRODUCTION

L-Aspartic acid has been extensively used as a food additive and a raw material in the manufacture of medicines and a dipeptide sweetener known as aspartame. L-Aspartic acid can be produced by the activity of an enzyme aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) that catalyzes the conversion of fumaric acid and NH_4^+ to L-aspartic acid. (Fig. 1). Since 1960s, this method has been used in industrial-scale production of L-aspartic acid. However, the use of aspartase derived from microbial cells such as *Escherichia coli* cells has several drawbacks. Especially microbial enzymes require complicated and costly purification procedures. Accordingly, there has been considerable interest in the direct use of the intracellular enzymes of intact *E. coli* cells containing aspartase activity as whole cell biocatalyst. The use of immobilized *E. coli* cells has also been examined and practiced on an industrial scale. Polyacrylamide gel encapsulation and, more recently, entrapment of *E. coli* cells within κ -carrageenan gel have been used as the immobilization methods (1–4).

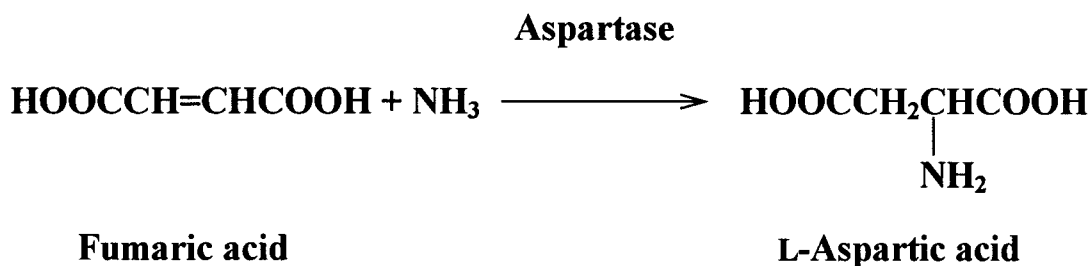


FIG. 1. Formation of L-aspartic acid using aspartase

To utilize microbial cells as whole cell biocatalyst in a convenient and economical form, the cells should be immobilized in such a way that they resemble the ordinary solid-phase catalysts used conventionally in synthetic chemical reactions. Of the various methods reported for cell immobilization, the passive immobilization technique using porous biomass support particles (BSPs), originally developed by Atkinson *et al.* (5), is attractive from the point of view of industrial application. The passive immobilization occurs when cells, as a consequence of their growth in culture, naturally form films around support material provided for that purpose or flocs within the porous structure of the material. This immobilization technique offers many advantages over other methods of immobilization, including ease of immobilization (natural entrapment), no need for chemical addition, large mass transfer rate in the particles, and ease of scale-up of the immobilization technique. This technique has been successfully applied to a wide variety of microbial, plant, and animal cell systems (6–10). In a previous study, this immobilization technique has been successfully used to immobilize *E. coli* cells within polyvinyl formal (PVF) resin BSPs coated with positively charged polyamino acids such as poly-L-lysine or the synthetic polymeric amine polyethyleneimine (11). In the present study, the immobilization of *E. coli* ATCC 11303 cells containing aspartase activity within BSPs was investigated. The use of BSP-immobilized *E. coli* cells as whole cell biocatalyst for the production of L-aspartic acid was also examined.

MATERIALS AND METHODS

BSPs and reagents

Reticulated PVF resin BSPs ($2 \times 2 \times 2$ mm cubes; pore diameter 60 μ m) were obtained from Aion, Osaka. The culture media reagent, Difco nutrient broth (BD 234000) was obtained from Becton Dickinson, Tokyo. Polyethyleneimine (MW 70,000) was obtained from Wako Pure Chemical Industries, Osaka. Fumaric acid monosodium salt and fumaric acid were obtained from Nacalai Tesque, Kyoto.

Bacterial strain, culture medium, and shake-flask culture with BSPs

E. coli ATCC 11303 was grown under aerobic conditions at 37°C with a medium containing ammonium (1.5%), fumaric acid (1.5%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05%), KH_2PO_4 (0.2%), and Difco nutrient broth (0.8%) (pH adjusted to 7.0) (12–14). After preincubation in a test tube containing 4 ml of the medium at 37°C on a reciprocal shaker (150 oscillations/min, amplitude 25 mm) for 16 h, 30 μ l of the resulting cell suspension was added to 100-ml screw-capped Erlenmeyer flasks containing 15 ml of fresh culture medium with or without BSPs. The cells and the BSPs were then incubated at 37°C on a reciprocal shaker (150 oscillations/min, amplitude 30 mm).

BSPs coating procedure

In the present study, polyethyleneimine was used to coat the surface of BSPs. Two-hundred and fifty BSPs were directly autoclaved for 20 min at 121°C in 15 ml of 0.5 g/l polyethyleneimine solution in the Erlenmeyer flask. After removal of the polymer solution, the polyethyleneimine-treated BSPs were used for immobilized cell

culture. In the case of nontreated BSPs, 250 BSPs were autoclaved for 20 min at 121°C in 15 ml of purified water in the Erlenmeyer flask.

Cell density measurement

The density of free (nonimmobilized) cells in shake-flask culture was monitored by measuring the optical density of the cell suspension at a wavelength of 600 nm (OD_{600}) with a spectrophotometer (U-2001; Hitachi, Tokyo). The cell suspension was diluted with Dulbecco's phosphate buffered saline (PBS) to maintain OD_{600} readings in the linear range below 0.8. The density of cells immobilized within BSPs was calculated from the difference between OD_{600} in the culture broth without BSPs and OD_{600} with BSPs and the volume of the culture broth and BSPs.

Assay of aspartase activity

Aspartase activity was determined spectrophotometrically by measuring the formation of fumarate at a wavelength of 240 nm. Due to the strong absorbance of fumarate at 240 nm, the amination reaction was followed by measuring the disappearance of fumarate (15–17).

L-Aspartic acid production

After 24 h of shake flask culture of nonimmobilized *E. coli* cells, the suspension was concentrated 10-fold in phosphate-buffered saline by centrifugation. Two milliliter of the resultant cell suspension or 250 BSPs (total volume 2 cm³) were incubated at 37°C for 1 h with 17 ml of 1.0 M ammonium fumarate solution (pH adjusted to 9.0) containing 1 mM Mg²⁺ and 0.2% (w/v) Triton X-100. The reaction

was stopped by immersing the reaction mixture in boiling water for 5 min, and the cell debris was removed by centrifugation (12, 18). The resulting supernatant was diluted with phosphate-buffered saline to measure the absorbance at a wavelength of 240 nm. The molar extinction coefficient of $2530 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 240 nm for fumarate (19, 20) was used. The decreased concentration of fumaric acid represents the L-aspartic acid concentration produced. One unit (U) of aspartase activity is defined as the amount of enzyme required to consume 1 μmol of fumarate per minute at 37°C and pH 9.0.

RESULTS AND DISCUSSION

Immobilization of *E. coli* ATCC 11303 cells

Reticulated PVF resin BSPs with matrices of 60 μm pore diameter were prepared by directly autoclaving with 0.5 g/l polyethyleneimine solution and *E. coli* ATCC 11303 cells were cultured with the BSPs in shake-flask culture. Shake-flask culture of the *E. coli* cells using BSPs without polyethyleneimine pretreatment was also carried out together with shake-flask culture of nonimmobilized cells. After 24 h of cell culture, OD_{600} of the culture broth was measured by a spectrophotometer. As can be seen from Fig. 2, the optical density of the culture broth obtained with untreated BSPs was almost equivalent to that obtained in the absence of BSPs. On the contrary, the optical density of the broth obtained with polyethyleneimine-treated BSPs was significantly lower than the others. Since the total biomass content (immobilized and nonimmobilized cells) in each flask is considered to be the same regardless of whether BSPs were present or were treated with polyethyleneimine, the decrease in the optical

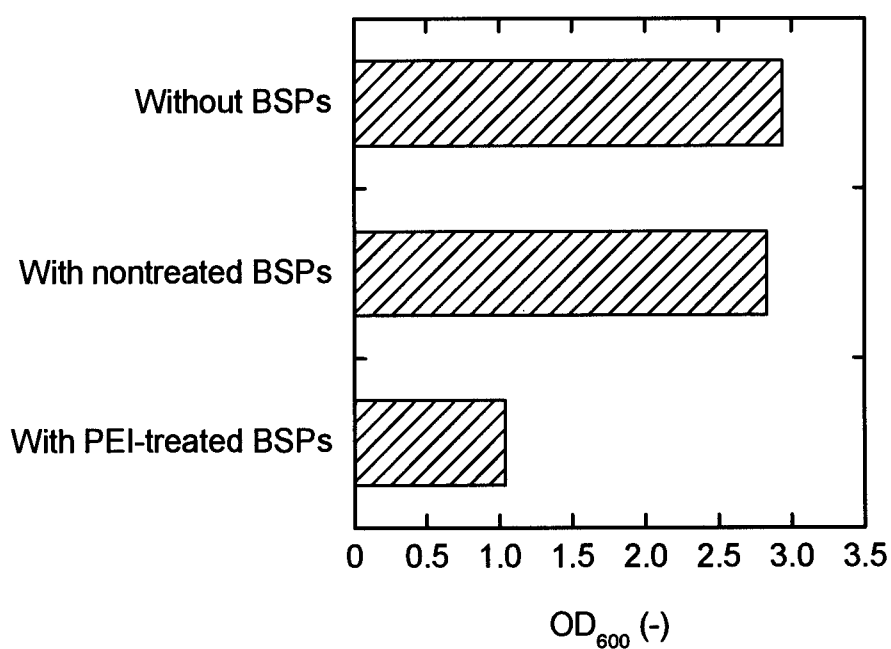


FIG. 2. Optical density at 600 nm (OD₆₀₀) of culture broth in shake-flask culture. *Escherichia coli* ATCC 11303 cells were cultivated for 24 h with polyethyleneimine (PEI)-treated biomass support particles (BSPs) and nontreated BSPs or without BSPs.

TABLE 1. Density of cells immobilized within BSPs in shake-flask culture

BSPs	OD ₆₀₀ (–)
Untreated BSPs	0.79
Polyethyleneimine-treated BSPs	14.24

density of the culture broth with polyethyleneimine-treated BSPs should result from entrapment of cells within the BSPs.

The densities of cells entrapped within BSPs were calculated on the mass balance of cells in shake-flasks with and without BSPs and expressed as OD₆₆₀ (Table 1). An optical density more than 10 was found to be obtained in the polyethyleneimine-treated BSPs. Although this result was obtained under suboptimal conditions, it indicates that the *E. coli* ATCC 11303 cells as well as *E. coli* K12 cells were successfully immobilized within the BSPs treated with polyethyleneimine.

Production of L-aspartic acid

L-Aspartic acid production was carried out using nonimmobilized *E. coli* ATCC 11303 cells and the cells immobilized within polyethyleneimine-treated BSPs as whole cell biocatalyst. When a 10-fold concentrated suspension of nonimmobilized *E. coli* cells was added to the reaction solution, decrease in the concentration of fumaric acid was spectrophotometrically detected in the reaction solution. When the cells

TABLE 2. Specific aspartase activity of nonimmobilized and immobilized cells

<i>E. coli</i> cells	Specific activity* (U)
Nonimmobilized cells	168.3
Cells immobilized within polyethyleneimine-treated BSPs	340.1

* Aspartase activity per OD₆₀₀ (–)

immobilized within polyethyleneimine-treated BSPs were used, consumption of fumaric acid was also observed. However, when cells were incubated with untreated BSPs, fumaric acid concentration did not decrease.

Specific aspartase activities of nonimmobilized cells and cells immobilized within polyethyleneimine-treated BSPs were calculated (Table 2). The specific aspartase activity of immobilized cells was found to be approximately twice that of nonimmobilized cells. Whereas these results were obtained under suboptimal conditions and further investigations are required, it is suggested that cells immobilized within polyethyleneimine-treated BSPs show a high specific aspartase activity.

Taken together, *E. coli* ATCC 11303 cells can be densely immobilized within reticulated PVF resin BSPs prepared by autoclaving with polyethyleneimine solution. Compared with entrapment in polyacrylamide gel and κ -carrageenan gel, the

immobilization method using BSPs is simple and natural and can be easily carried out aseptically. The cells immobilized within polyethyleneimine-treated BSPs were also found to show a high specific aspartase activity. Since the technique should be simple to scale up in a bioreactor system, the immobilization of *E. coli* cells using BSPs would be useful for industrial scale production of L-aspartic acid.

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GENERAL CONCLUSION

In this thesis, to utilize bacterial cells as whole cell biocatalyst in a convenient and economical form for the production of useful chemicals, the immobilization of *Escherichia coli* cells using porous support particles (biomass support particles, BSPs) was investigated. *E. coli* cells have been extensively used as host cells in the expression of recombinant proteins such as various enzymes. The use of *E. coli* cells immobilized within BSPs as whole cell biocatalyst for the production of L-Aspartic acid was also examined.

The technique of cell immobilization using BSPs is attractive from the point of view of simplicity and convenience since passive immobilization occurs when films or flocs of cells form naturally around or within support material provided for that purpose. This technique, however, does not allow dense immobilization of bacterial cells that have no flocculent or adhesive characteristics, such as *E. coli* cells. Actually, *E. coli* K12 cells were not successfully retained within reticulated polyvinyl formal (PVF) resin BSPs with matrices of relatively small pores (pore diameter 60 μm). Therefore, coating the surface of the BSPs with various polymers was first examined as a way of promoting cell attachment. When positively charged polyamino acids such as poly-L-lysine, poly-L-arginine, poly-L-histidine, and poly-L-ornithine were adsorbed onto the particle surface, they were found to increase the immobilized cell density, while neutral and negatively charged polyamino acids including poly-L-asparagine and poly-L-glutamic acid were not effective. These results indicate that *E. coli* cells can be efficiently immobilized in PVF resin BSPs by electrostatic interaction between the negatively charged ions of the cell surface and the positively

charged polymers adsorbed onto the BSP surface. A significantly high immobilized cell density was also achieved by coating the surface of the BSPs with the synthetic polymeric amine polyethyleneimine.

Next, procedures for preparing BSPs coated with cationic polymers were investigated with *E. coli* K12 cells. To determine the optimal conditions for the surface coating of PVF resin BSPs, treatment time, polyethyleneimine concentration, and pore diameter of BSPs were examined in shake-flask culture. Treatment time with polyethyleneimine did not affect the cell density within BSPs. Polyethyleneimine concentrations of 0.1 g/l and 0.2 g/l produced a high density of immobilized cells. The highest cell density was obtained with the BSPs with matrices of 60 μm pore diameter. Furthermore, it was found that a significantly high density of *E. coli* cells can be achieved in the BSPs simply prepared by autoclaving with polyethyleneimine or poly-L-lysine solution. The procedure eliminates the processes of filter sterilization of polymer solutions and incubation with cationic polymers in preparing the BSPs. Since the technique should be simple to scale up in a bioreactor system, the cell immobilization using BSPs coated with cationic polymers would be useful for industrial application.

E. coli cells containing aspartase activity have been used for the conversion of fumaric acid and NH_4^+ to L-aspartic acid on an industrial scale. Finally, the immobilization of *E. coli* ATCC 11303 cells within BSPs and the use of *E. coli* cells immobilized within BSPs as whole cell biocatalyst for the production of L-aspartic acid were investigated. *E. coli* ATCC 11303 cells were found to be densely immobilized within reticulated PVF resin BSPs prepared by autoclaving with polyethyleneimine solution. It was also indicated that the cells immobilized within

polyethyleneimine-treated BSPs show a high specific aspartase activity, comparable to that of nonimmobilized cells. Since the technique should be simple to scale up in a bioreactor system, immobilization of *E. coli* cells using BSPs would be useful for industrial scale production of useful chemicals.

PUBLICATION LIST

Part I

Juan Huang, Hideki Yamaji, and Hideki Fukuda

Immobilization of *Escherichia coli* cells using porous support particles coated with cationic polymers.

Journal of Bioscience and Bioengineering. Vol. 104 (2007), No.2, p.98-103.

Part II

Juan Huang, Hideki Yamaji, and Hideki Fukuda

Simple preparation of porous support particles coated with cationic polymers for immobilization of *Escherichia coli* cells.

Submitted.

Part III

Juan Huang, Hideki Yamaji, and Hideki Fukuda

Production of L-aspartic acid using immobilized *Escherichia coli* cells as whole cell biocatalyst.

In preparation.

