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Development of a biofilm bioreactor for enhanced propionic and acetic acid production

by

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For the Graduate College

In memory of my father, Nejat Yilmaz Özadali

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APPENDIX C. MEDIA COMPARISONS

ABSTRACT

A biofilm is a natural form of cell immobilization that results from microbial attachment to solid supports. Ten support materials including plastic-composite supports and six strains of propionibacteria were tested for their possible use in biofilm systems for enhanced production of propionic and acetic acid by fermentation. From screening experiments *Propionibacterium thoenii* strain P20 and fire bricks were chosen for further investigations.

Propionibacterium thoenii P20 resists low-pH conditions, produces acid rapidly, forms luxuriant biofilms, and resists solvent inhibition better than other strains. Fire bricks are inexpensive, reusable, and compare favorably to commercial supports in ease of use and structural stability. A modified "lifesaver" shape for the individual fire brick particles was found to provide increased available surface area for biofilm formation and better flow patterns of the medium through and around supports. The attachment mechanism of bacterial cells to the supports was sought by measuring zeta potentials of both organisms and support materials by hydrophobicity (MATH test) analysis, and by scanning electron microscopy (SEM) examination.

To hold support materials and to provide better flow of medium through and around the supports, a novel stainless steel basket was designed to fit into the fermenter. The basket, termed the BioCage, holds support materials in four separate compartments, with provision for introduction of acid or base for pH

control through a central channel, and with agitation at the base and at the center of the basket.

When repetitive fed-batch fermentations were performed with the empty basket in the fermenter, and with fire brick supports in the basket, the bacterial biofilm formed preferentially on the fire bricks. However, a "hairy" biofilm covered the outside of the empty basket.

In repetitive fed-batch fermentations, average yields of propionic and acetic acid from substrate lactate were about 52 and 20% for pH 6.9 and 53 and 21% for pH 5.5. Average productivities for propionic acid were about 0.18 (pH 6.9) and 0.14 g/l/h (pH 5.5) and for acetic acid were 0.04 (pH 6.9) and 0.03 g/l/h (pH 5.5), respectively.

For the repetitive fed-batch biofilm fermentations over four consecutive batches of acid production with the basket and fire brick supports, the average productivity and yield coefficient values were about 0.1 g/l/h and 27% for acetic acid and 0.26 g/l/h and 71.5% for propionic acid, respectively.

Two immobilization methods, biofilm formation and calcium alginate entrapment, were compared in a mini reactor to determine the rates of substrate consumption and acid production per unit of the immobilized systems. Average substrate consumption and propionic acid production rates were 0.09 and 0.06 g/l/h for reactors with biofilm and 0.14 and 0.09 g/l/h for reactors with calcium alginate beads, respectively. Acid production rate increased in sequential batches in the biofilm system. Even though acid production with beads was

higher than in the biofilm system, beads dissolved at the end of the first batch and started clogging outlet lines.

Overall results indicate that *P. thoenii* P20 is an excellent biofilm former, and that biofilm fermentations can maintain high acid productivities even at low pH values.

CHAPTER 1. GENERAL INTRODUCTION

Introduction: General explanation of the problem and rationale for the research

Today, propionic acid is a valuable chemical which is currently produced from petroleum sources. Because of its potency as a mold inhibitor, propionic acid is a very effective preservative of high-moisture grains and various food products.

Propionic acid can also be produced via fermentation processes. The main problem with this type of production is that propionic acid-producing organisms are slow growers and batch fermentations may take up to two weeks to reach 2-3% propionic acid levels. The main goal of current fermentation research is to develop a cost-effective fermentation process by reducing the cost of the raw material, increasing the productivity of the organisms, and/or improving acid recovery methods. Genetically altering the characteristics of the organism can also be an alternative for improvement of the process parameters. Natural production of the propionic acid has some advantages over chemical production such as finding alternatives for depleting petroleum resources and avoiding more stringent labeling requirements for artificial preservatives.

There have been several attempts to increase propionic acid production via fermentation. Although they obtained promising results, the fermentation process does not yet compete with chemical production.

Our overall approach to this problem is to improve the process productivities by using immobilized cells and improved product recovery systems. Reaching higher cell densities through immobilization may overcome the long-process-time problem by increasing the utilization rate of the substrate. As an end product, propionic acid is inhibitory to its producer strain. To overcome this problem, *in situ* product recovery has been suggested by several investigators (54, 63, 75).

Whole-cell immobilization is a well-proven method to increase fermentation productivity and yield. The main idea is to achieve high cell concentrations in the reactor without significant loss of cells from the reactor due to system upsets. Such upsets would include pH and temperature changes, or high flow rates. Cell immobilization can be described as the attachment of cells to or entrapment in a distinct solid phase that allows exchange of substrates, products, or inhibitors, but at the same time separates the cells from the bulk phase in which substrates are dispersed (83).

There are problems associated with conventional immobilized-cell bioreactors. Productivity of the nongrowing immobilized cells declines during the process due to loss of cell viability. If the system contained the growing immobilized cells, the whole system could become clogged or the bed could

expand from biofilm growth. High pressure drop, gas entrapment, and accumulation of dead cells in the packed-bed reactors may also lead to a loss of production capability (55).

By comparing various fermentations performed in our laboratory, it was determined that fed-batch processes that involved periodic addition of substrate produced the highest final concentration of organic acids (about 4% propionic acid in defined medium), but the productivity was not much faster than in simple batch fermentation. Continuous culture systems gave higher acid productivities, but the concentration of acid in the broth was low (66). The most promising method tried was cell immobilization, in which a high concentration of active cells could be maintained in the fermenter by trapping them in calcium alginate gel. Higher propionic acid concentrations (about 5%) were obtained in immobilized cultures in a fed-batch or a repeated-batch mode (64, 69).

Results of these studies led us to seek additional cell immobilization techniques. After our preliminary tests with *Propionibacterium* strains for biofilm formation in continuous fermentation systems, it was decided to further investigate the biofilm system for propionic acid fermentation.

After screening for the best support-strain combination, fire bricks and *Propionibacterium thoenii* were selected and tested in larger scale repetitive fed-batch fermentations. Those experiments were carried out in novel basket reactors with and without fire brick support materials. These materials have

several advantages over regular packed-bed immobilized cell systems such as better agitation, pH control, and compartmentalization.

Surface characteristics such as hydrophobicity and charges of the support materials and the organisms were also investigated to support our results from biofilm experiments.

Since the effects of differential changes might be difficult to observe in a large (2-liter) reactor, a mini-scale immobilized cell reactor was constructed to follow the small increments in acid production and substrate consumption per mass unit of support or biofilm.

Dissertation organization

This dissertation is composed of a literature review and three chapters. The first chapter describes the preparation of different biosupports and evaluation of these biosupports for enhanced acetic and propionic acid production in biofilm reactors by using six strains of propionibacteria. The second chapter deals with fed-batch fermentations by *Propionibacterium thoenii* strain P20 for acetic and propionic acid production in novel basket biofilm reactors with fire brick support materials. The last chapter concerns the evaluation of biofilm and cell-loaded alginate bead systems in a cell-free circulated mini-reactor to determine propionic acid production and substrate consumption rates per unit immobilization. Following the third chapter is a general summary and conclusions. The American Society for Microbiology

format was used in general sections of the dissertation, and the instructions of the appropriate journals were followed for the possible future submissions. The cited references from Chapter 1 are listed at the end of this dissertation.

Literature Review

Propionic acid

Propionic acid is a three-carbon volatile fatty acid (CH_3CH_2COOH) which is well known as a potent natural mold inhibitor and industrial chemical. Cellulose propionate is an important thermoplastic, and esters of propionic acid are used in the perfume industry. Calcium and sodium propionates are mainly used as antifungal agents in breads and other foods. In addition to these major uses, propionic acid and its derivatives have been used to manufacture antiarthritic drugs, flavors, plasticizers, and solvents (11, 68).

As of July 1994 annual propionic acid demand was predicted as 172 million pounds in 1995 with 3 to 4 percent annual growth rate (11). The late-1996 price of propionic acid was $0.44 per pound delivered in tanks (12).

Use of propionic acid in chicken and other animal feeds to prevent infection from moldy food has high growth potential in farm markets. Development of new phenoxypropionate herbicides has increased both domestic and export demand for propionic acid and its 2-chloropropionic acid derivative (11).

The most common commercial process for the production of propionic acid is by the oxidation of liquid-phase propane. Also, propionic acid is produced by the oxidation of propionaldehyde. The propionaldehyde is obtained by the reaction of ethylene and carbon monoxide at high pressure. Direct oxidation of propanol with nitric acid is another process for the production of propionic acid (70). Producers such as Eastman (Kingsport, Tennessee), Union Carbide (Texas City, Texas), and Hoechst Celanese (Pampa, Texas) produce propionic acid as a co-product of acetic acid via n-butane oxidation. Physico-chemical properties of the propionic acid are summarized in Table 1.

Propionic acid can also be produced biologically by the fermentation of sugars using species of *Propionibacterium* (52). Fermentation processes have not been used commercially, primarily because separation of the product acids from the fermentation medium and concentration of the acids have proved too expensive. The generalized pathways from glucose to the major fermentation products such as propionate and acetate are shown in Figure 1. Formation of propionate is usually accompanied by formation of acetate. Dicarboxylic acid pathway is the most common pathway for the formation of propionic acid. Lactate is used preferentially to glucose as a substrate by most propionic acid-producing bacteria. Propionate may be formed from lactate by either the dicarboxylic acid pathway or the acrylic pathway (68).

Table 1. Physico-chemical properties of propionic acid (17, 88)

Formula	CH_3CH_2COOH
Form and color description	Colorless oily liquid. Slightly pungent, disagreeable, rancid odor
Synonym	Propanoic acid
Formula weight	74.08
Dissociation constant (@25°C)	pK_1 : 4.87
Specific gravity (@20°C)	0.993 (referred to water @ 4°C)
Melting point	-20.8°C
Boiling point	141.4°C
Solubility	∞ (in 100 parts of water, alcohol, or ether)
Refractive index (n_D)	1.3865 (@20°C)
Viscosity ($mN \cdot s \cdot m^{-2}$)	1.175 (@15°C)
Dielectric (ε)	3.44 (@40°C)
Dipole moment (D)	1.75 (@20°C)
Surface tension (dyn/cm)	a. 28.68 b. 0.0993 $\gamma = a - bt$ (@20°C)
Critical temperature and pressure	339.5°C and 53 atm

The simplified summary equation is:

3 glucose ⟶ 4 propionic acid + 2 acetic acid + 2 CO_2 + 2 H_2O + 12 ATP

The enzyme, S-methylmalonyl-SCoA: pyruvate transcarboxylase, is a key to the cyclic nature of the dicarboxylic acid pathway, since it enables a carboxylic group to be transferred from S-methymalonyl-SCoA to pyruvate to form oxaloacetate and propionyl-SCoA (68).

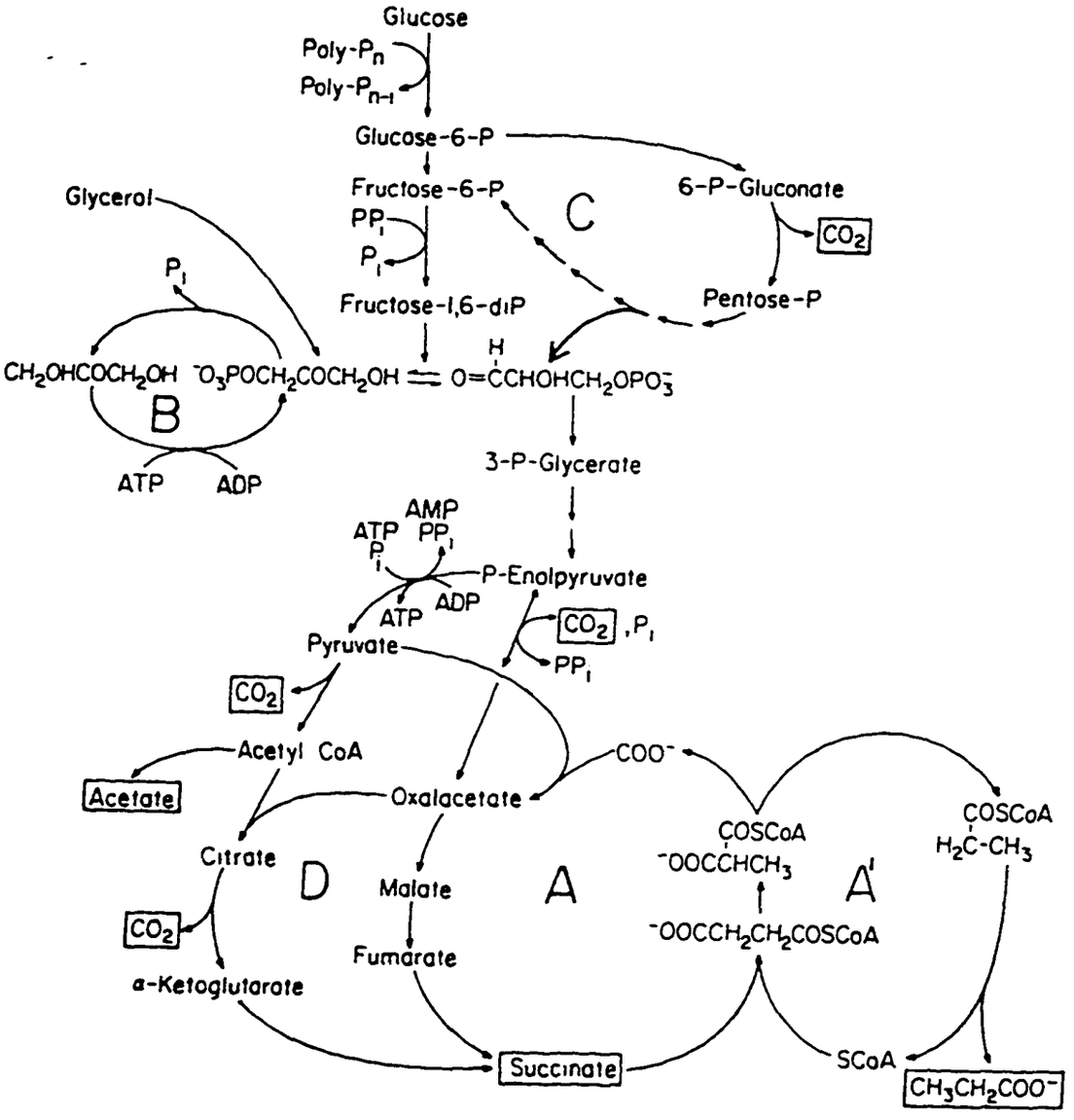

Figure 1. Pathway of the propionic acid fermentation showing the transcarboxylase cycles [A and A'], the futile dihydroxyacetone cycle [B], the pentose pathway [C], and the citrate pathway [D] (89, 90).

Acetic acid

Acetic acid (CH_3COOH) production is an incomplete oxidation rather than a true fermentation, because the reducing power which is produced is transferred to oxygen (15). During the oxidation, 1 mole of acetic acid is produced from 1 mole of ethanol. From 1 liter of 12% (v/v) alcohol, 1 liter of 12.4% (w/v) acetic acid is produced (15). Acetic acid is also produced by many fermentative bacteria including propionibacteria through pathways given in the previous section. Physico-chemical properties of acetic acid are summarized in Table 2.

Acetic acid's uses can be fractionated as follows: vinyl acetate monomer, 59%; acetic anhydride including production of cellulose acetate, 15%; esters, 10%; textiles, 2%; chloroacetic acid, 1%; other, 5%. Its projected demand in 1996 was 4.18 billion pounds with 3 percent annual growth rate (11). The 1996 price of acetic acid was $0.40 per pound (delivered in tanks) (12). Acetic acid is widely used in manufacturing of acetates, acetyl compounds, cellulose acetate, acetate rayon, plastics and rubber in tanning, printing calico and dyeing silk, preserving foods, solvent for gums, resins, volatile oils, and many other substances (88).

History of propionic acid fermentation

Propionic acid production from fermentation was first observed by Strecker (1854). Pasteur (1879) also found propionic acid as one of the products formed in the fermentation of calcium tartrate. In 1878 Fitz was the first to

Table 2. Physico-chemical properties of acetic acid (17, 88)

Formula	CH_3COOH
Form and color description	Colorless liquid. Pungent odor
Synonym	Ethanoic acid
Formula weight	60.05
Dissociation constant (@25°C)	pK_1 : 4.76
Specific gravity (@20°C)	1.049 (referred to water @ 4°C)
Melting point	16.6°C
Boiling point	118.1°C
Solubility	∞ (in 100 parts of water, alcohol, or ether)
Refractive index (n_D)	1.3719 (@20°C)
Viscosity ($mN \cdot s \cdot m^{-2}$)	1.314 (@15°C)
Dielectric (ε)	6.15 (@40°C)
Dipole moment (D)	1.74 (@20°C)
Surface tension (dyn/cm)	a. 29.58 b. 0.0994 $\gamma = a - bt$ (@20°C)
Critical temperature and pressure	321.3°C and 57.1 atm

determine the quantitative relationship of products formed by *Propionibacterium* and formulated the Fitz equation (30, 37, 68):

$$3 \text{ lactate} \longrightarrow 2 \text{ propionate} + 1 \text{ acetate} + 1\ CO_2 + H_2O$$

The first workers to advocate industrial production of propionic acid by fermentation were Sherman and Shaw (73). They used a slow-growing species of propionibacteria and suggested that fermentation could be accelerated by using a mixed inoculum of a lactic acid-producing organism with the chosen propionibacteria species.

In 1933 Stiles and Wilson (76) obtained one of the highest acid concentrations ever recorded by using a patented two-step fermentation, consisting of a lactic acid stage (*Lactobacillus*) and propionic acid stage (*Propionibacterium*). They used molasses and starch hydrolysate as substrate. Even though the production from molasses was low, they managed to obtain over 40 g/l propionic acid from starch hydrolysate.

In the 1970s, commensalistic interaction between *Lactobacillus* species and *Propionibacterium* species was demonstrated by various groups. Lee et al. (52) indicated that propionibacteria preferentially use lactic acid when presented with a medium containing both glucose and lactic acid. In a study on the dynamics of mixed cultures of *Lactobacillus plantarum* and *Propionibacterium shermanii*, Lee et al. (53) predicted that the average growth rate and maximum density of *P. shermanii* would be less when grown with *L. plantarum* than when grown in pure culture, because of limited substrate concentration. These results suggested that the interaction observed would depend on the rate of lactic acid production. Parker and Moon (67) reported that *L. acidophilus* and *P. shermanii* displayed a commensalistic response when grown in mixed culture.

El-Hagarawy et al. (25) studied the effect of strain, pH, source of carbohydrates, and intermediates of fermentations on propionic and acetic acid production in batch culture. They showed that sodium lactate stimulated acid production, which reached a maximum in two days. In contrast, acid production

was maximum only after eight days on lactose. These results suggested that lactate may be the precursor of propionic acid.

Wayman et al. (86) developed a continuous process based on waste sulfite liquor in which propionibacteria were immobilized on limestone pebbles. The liquor was recycled, which helped to reduce mold growth, assisted with buffering, and improved acid yield. Clausen (9) studied the fermentation of propionic and acetic acids using *P. acidipropionici* in batch and continuous systems and drew a kinetic model about the batch and continuous fermentations of mixed glucose and xylose for the production of propionic acid. Clausen and Gaddy (10) investigated techniques to increase the rate of production of propionic and acetic acids, while decreasing the fermentation time. They compared the performance of a continuously stirred tank reactor (CSTR) and of an immobilized cell reactor (ICR) for the production of propionic acid using *P. acidipropionici*. The fermentation in the CSTR was shown to be about four times faster than that in a batch culture. Fermentation time could be shortened in ICR at the same conversion rate of substrates.

To improve the rate of organic acid production, cell recycle systems have been used. High cell concentrations have been obtained by continuous filtration of fermentation medium in microfiltration or ultrafiltration systems with cell recycle. Production of propionic acid from whey permeate by sequential fermentation, ultrafiltration, and cell recycling was studied by Colomban et al. (13). They suggested that their sequential system would allow cell

multiplication in the first cycles at a neutral pH, and acidification in the next ones to enhance yield as previously reported by Hsu and Yang (42). Hsu and Yang (42) studied the effect of pH on fermentation of lactose and indicated that even if neutral pH is optimum for the growth of *Propionibacterium acidipropionici*, the propionic acid yield is low. On the other hand, in the acidic pH range, the growth rate is low, but the yield is doubled.

Propionic acid production from glycerol in a continuous fermentation with a membrane bioreactor was studied by Boyaval et al. (4). They suggested that fermentation of glycerol by propionic acid bacteria leads only to propionic acid with no acetic acid.

Immobilized cell systems for organic acid and ethanol production

Most free-cell reactor systems have the difficulties of maintaining stability and preventing washout from the reactor system. To minimize these problems, cell immobilization techniques have been proposed since the beginning of the 19th century. Cell immobilization improves reactor productivity by allowing reactor operation at high dilution rates without cell washout. The cell population is also separated from products in solution (55, 72).

Some advantages of immobilized-cell over free-cell fermentations include maintenance of stable and active biocatalysts, reuse of biocatalysts, accelerated

reaction rates, high volumetric productivities, improved process control, and improved production efficiency (48).

One of the most common immobilization techniques is viable cell entrapment in various polymers such as alginate, polyacrylamide, gelatin, κ-carrageenan, and agarose. Generally, a cross-linking agent such as calcium is needed to form the polymeric network. Production of propionic acid in immobilized systems besides biofilm systems was extensively studied in our laboratory (64, 65, 69). In-depth literature reviews on this subject were covered by Paik (64) and Rickert (69).

Entrapment of cells represents a type of immobilization that does not depend on cellular properties (i.e., flocculation, aggregation, appendages). In this case, cells are held either within the interstices of porous materials or by the physical restraints of membranes or encapsulating gel matrices (72). Simply, in immobilization techniques, entrapment includes both enclosure of a catalyst behind a membrane and within a gel structure.

Potential mass transfer limitations are always present with an entrapment system, either across the gel matrix or gel occlusion, or across the system membrane in membrane reactors. In a gel entrapment system the most active cells are at the gel surface (50a); agitation of the beads can lead to loss of activity due to leakage of the outer layer (50a).

Calcium alginate entrapment is one of the most common immobilization techniques. Alginate is a glycuronan consisting of residues of D-mannuronic

acid and L-glucuronic acid arranged in a blockwise fashion along a polymer chain (44). In the presence of multivalent cations gel formation occurs. Stenrous et al. (77) investigated lactic acid production with entrapped *Lactobacillus delbrueckii* in calcium alginate beads and reported that immobilized cells produced a maximum of 12 g/l lactic acid with the productivity of 0.2 g/l/h.

Ethanol production from glucose by calcium alginate-entrapped yeast cells was investigated by McGhee et al. (57). They observed that the older yeast cells were much more efficient ethanol producers than were younger cells.

A major disadvantage of calcium alginate as an immobilized support is that moderate concentrations of calcium chelating agents and certain cations such as phosphates, EDTA, Mg^{+2}, and K^{+} disrupt the gel by solubilizing the calcium (8). Some workers have reported shrinkage and decreased strength of calcium alginate beads during lactic acid production (24, 71).

Cell entrapment in polyacrylamide gels involves the polymerization of an aqueous solution of acrylamide monomers in which microorganisms are suspended. The porosity of the gel is a function of the degree of cross-linking, which in turn depends on the relative amounts of the acrylamide monomer and the bi-functional cross-linking agent used (45).

Polyacrylamide has some disadvantages to use in immobilized cell systems. It may cause denaturation of enzymes. Irregular shapes and sizes of the gel pellets are hard to pack uniformly in a column, which leads to uneven

flow and the development of high, flow-induced pressure drops. Presence of high biomass loading may hinder the polymerization of the acrylamides (72).

Development of fermentation system under nonsterile conditions has been investigated by several groups (62, 78, 87). Sterilization is one of the major line-items for the total cost of the fermentation processes. Some methods such as addition of inhibitory substances (32, 33) and control of pH optima (60) were studied. Ohta et al. (62) suggested a process involving co-immobilization of the fermentation microorganism with castor oil and suppression of contaminant growth by addition of an anti-microbial substance (0.1% *n*-butyl, *p*-hydroxybenzoate, POBB and Preventor GD) to the fermentation medium. The effectiveness of a vegetable oil in protecting the immobilized cells against an inhibitory substance depends on the partition coefficient of the inhibitory substance between the oil and the aqueous phase (78).

Fast colonization of the macroporous glass beads with *Zymomonas mobilis* in fluidized-bed reactors and the conversion of nonsterile hydrolyzed B-starch to ethanol was studied by Weuster-Botz et al. (87). Their system managed to convert 99% of the glucose in nonsterile hydrolyzed B-starch, to a final ethanol concentration of 50 g/l.

Ethanol production by whole-cell immobilization using lignocellulosic materials was studied by Das et al. (16). They found that rice straw was the most suitable among four carriers in terms of ethanol production. They reported that the maximum productivity of 17.84 g/l/h corresponded to a dilution rate of

0.39 h^{-1}, with the ethanol concentration at 45.8 g/l. They also obtained 12.55% productivity increase with a rhomboidal bioreactor compared to a cylindrical column reactor.

Continuous propionic acid fermentations of lactate by *Propionibacterium acidipropionici* were studied in spiral-wound fibrous bed bioreactors by Lewis and Yang (55). They claimed that the immobilized-cell bioreactor was scalable and suitable for industrial production of propionate. They reported a high cell density of 37 g/l and four-fold greater reactor productivity than that from a conventional batch process. It was also suggested that the reactor could accept low-nutrient and low-pH feed without sacrificing much in reactor productivity.

Biofilm concept

Many cells have the ability to adhere to solid surfaces. This type of attachment, which may be either natural or induced, can frequently form the basis for an inexpensive but effective immobilization technique. Biofilms as a natural form of cell immobilization are dynamic microenvironments, encompassing processes such as metabolism, growth, and product formation, and finally detachment, erosion, or "sloughing" of the biofilm from the surface (6, 7). The rate of biofilm formation depends on the physicochemical properties of the interface, the physical roughness of the surface, and physiological factors of the attached microorganisms (27). Shear forces generated by fluid velocity

may be important in the release of biofilms from the surfaces (6). Some of the industrial fermentations using biological films are listed in Table 3.

Biofilms have been a big problem for several industries, including nuclear power plants, marine transportation, and water distribution systems. Dental plaque is also a biofilm. Unwanted biofilms have been called "biofouling" (84).

Biofilms have been studied because they can be good as well as bad and we can learn from both types. Biofilms have presented opportunities for bioprocessing applications, especially in the area of environmental control technology where naturally occurring microbial films are used in fixed-film bioreactors (5).

Some microorganisms can adhere directly to the surface via appendages that extend from the cell membrane; other bacteria form a capsular material of extracellular polysaccharides (EPS), sometimes called a *glycocalyx*, that anchors the bacteria to the surface (3, 14, 29). However, some types of organisms do not effectively attach to surfaces on their own but can rely on the symbiotic actions of other attachment organisms that might exist in a mixed culture (72). The following figure demonstrates a composite of all processes contributing to biofilm accumulation (Figure 2).

Production of valuable products via biofilm systems

Biofilm systems for other valuable acids such as lactic acid and ethanol

Table 3. Industrial applications of biofilm systems (1)

Process	Objective	General characters
Trickling filter	Biological oxidation of industrial and domestic effluent	Nonaseptic, microbial growth occurs in a packed bed. Wastewater distributed intermittently over the packing. Aerobic; packing supported on a grid structure, enhancing aeration by natural convection.
Rotating disc	Biological oxidation of industrial and domestic effluent	Microbial growth on discs rotating in a vertical plane, the disc dipping into a trough of wastewater. Microbial growth is alternately in contact with nutrients and air.
"Quick" vinegar process	Oxidation of alcohol by acetic acid bacteria	Similar in principle to the trickling filter, but with forced aeration. Wine or other feed liquor recirculated over beechwood chips or similar packing. Batch process (4-5 days).
Animal tissue culture	Growth of animal cells in a surface layer for the culture of viruses	Animal tissue minced and reduced to single cells by enzyme action. The cells adhere to surfaces provided and grow as a film in the presence of a suitable medium. Can be used subsequently for virus culture. Strictly aseptic.
Bacterial leaching of ores	Recovery of metals from sulfide ores using iron and sulfur oxidizing bacteria	Bacteria used, *in situ*, in dumps of low-grade or waste ores. Possibility of tank-leaching methods.

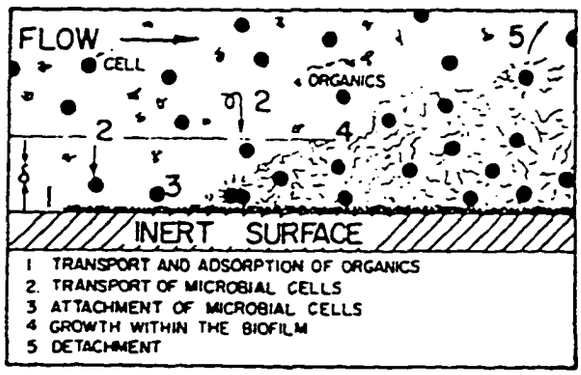

Figure 2. Processes affecting biofilm formation (6)

have been studied in the department Food Science and Human Nutrition, Iowa State University (20-22, 49, 50, 38-40). Demirci and Pometto (22) recommended that plastic-composite supports can be used for pure-culture lactic acid production in long-term repeated-batch fermentation. They also reported that a pure-culture bioreactor with *Lactobacillus casei* subsp. *rhamnosus* produced significantly more lactic acid than did a mixed culture with a biofilm-producing *Streptomyces*; an earlier study (20) showed better production in mixed-culture reactors.

Demirci and Pometto (22) evaluated plastic supports consisting of polypropylene blended with oat hulls/soybean flour or oat hulls/zein as supports for mixed- and pure-culture, repeated-batch, lactic acid fermentations in biofilm reactors. *Lactobacillus casei* subsp. *rhamnosus* was used for L-lactate production and *Streptomyces viridospores* was used to form a biofilm for mixed-culture fermentations. Demirci and Pometto reported higher concentrations of lactic acid in the mixed- and pure-culture biofilm reactors with plastic-composite

supports (55 g/l and 60 g/l, respectively) than with polypropylene supports (48 g/l for both mixed and pure cultures). However, they found that the percentage yields, maximum productivity, glucose consumption rates, and growth rates were not significantly different among reactors. They also suggested that agricultural material blended with the polypropylene stimulated biofilm formation on the support surface by serving as a carbon and/or nitrogen source, by presenting a favorable surface energy, and/or by increasing the absorption of microorganisms to the solid supports. Their earlier study (20) also showed that continuous lactic acid production rates in biofilm reactors were two to five times faster than those of the suspension culture for the pure- and mixed-culture bioreactors. Again they used *Streptomyces viridosporus* to form biofilm and *Lactobacillus casei* subsp. *rhamnosus* for lactic acid production.

Ho et al. (40) studied ingredient selection for plastic-composite supports for lactic acid biofilm fermentation by *Lactobacillus casei* subsp. *rhamnosus*. They evaluated the effects of different agricultural components on the properties of the plastic-composite supports. They suggested that incorporation of yeast extract into plastic-composite supports enhanced growth of free and immobilized cells. They also concluded that plastic-composite supports containing soybean hulls, yeast extract, soybean flour, bovine albumin, and mineral salts gave the highest biofilm population.

Ho et al. (39) also studied leachate bioavailability, leaching rate, and lactic acid accumulation properties of plastic-composite supports in large-scale

long-term lactic acid fermentations. They found no correlation between lactic acid production and lactic acid accumulation in plastic-composite supports. They also suggested that plastic-composite supports with only yeast extract as the minor agricultural ingredient had high leaching rates; 51 to 60% of the total nitrogen was leached from the supports during the first repeated-batch fermentation.

In another study, Ho et al. (38) optimized lactic acid production by using ring and disc shape plastic-composite supports in repeated-batch biofilm fermentations. They suggested that plastic-composite supports can stimulate biofilm formation, supply nutrients to attached and free cells, and reduce medium channeling in the reactor. They also claimed an excellent improvement of the fermentation rate with reduced complex-nutrient addition.

Kawabata et al. (46) studied continuous production of L-aspartic acid from ammonium fumarate using cells immobilized by capture on the surface of nonwoven cloth coated with pyridinium-type polymer. The basicity of the supporting material that captures the microbial cells was reduced by coating the nonwoven cloth with poly(*N*-benzyl-4-vinylpyridinium chloride-co-styrene.) Continuous operation of a fixed-bed column reactor containing 21.7 g/l of the immobilized cells on the nonwoven cloth produced L-aspartic acid in 95% yield from ammonium fumarate. Yield of L-aspartic acid increased with increase of coated polymer.

Goncalves et al. (31) used four inert adsorbent supports in continuously recycled packed reactors to immobilize *Lactobacillus rhamnosus*. They claimed that sintered glass beads were the best in terms of volumetric lactic acid productivity. They indicated that pHs above or below the optimum for suspended cell systems could be used in the immobilized reactor and still maintain lactic acid productivity. They also reported that zeta potentials of *L. rhamnosus* showed the cells to be negatively charged at all pHs studied, with the change becoming less negative with increasing ionic strength.

Environmental use of biofilm systems

Biofilm systems are widely used in environmental biotechnology, especially in wastewater treatment and degradation processes. The most significant variable in anaerobic digestion in an anaerobic fluidized-bed reactor (AFBR) is the selection of the support medium for microbial adhesion. The fluidized bed biofilm reactor (FBBR) represents an innovation in biofilm processes. Immobilization of microorganisms on the small, fluidized particles of the medium results in a high reactor biomass holdup which enables the process to be operated at significantly higher liquid throughputs with the practical absence of biomass washout (1). Reduction in process size while maintaining performance makes this technology attractive in biological wastewater treatment (47).

Kida et al. (47) used eight kinds of media (cristobalite, zeolite, vermiculite, granular active carbon, granular clay, pottery stone, volcanic ash, and slag) as immobilization matrix. They suggested that good performance as a support medium was associated with rougher surfaces rather than with larger surface areas. They found that microorganisms, which are generally negatively charged, could adhere more easily to cristobalite and zeolite because of the positive charge of the cristobalite. They also suggested that a suitable medium for adherence of microorganisms in the AFBR should have a rough and positively charged surface rather than a large surface area.

Balaguer et al. (2) studied an anaerobic fluidized bed reactor, with sepiolite as support, for the treatment of distillery wastewater. Six different steady states at hydraulic retention times between 0.5 and 2.48 days were studied; a COD removal efficiency of between 70.5 and 88.6% was achieved.

Removal of nitrogen compounds from air, water and soil is a problematic area. The nitrification process can only be accomplished by special species of autotrophic bacteria, *Nitrosomonas* and *Nitrobacter*. The problems are mainly due to the very slow growth of these bacteria, which means that they are easily washed out of a bioreactor. Immobilization was suggested as the obvious solution, and the best nitrifying technical installations were claimed as biofilm reactors in which the nitrifying biomass was attached (naturally) to a solid support (82).

Many sources of drinking water, especially in areas of intensive agriculture, contain intolerably high levels of nitrate ion (World Health Organization limits: 11.3 mg NO^{3-} -N/l or 50 mg NO^{3-}/l). One possible process for nitrate removal is biological denitrification. Denitrification refers to the biological process by which microorganisms use oxygen in nitrate to oxidize a carbon source to CO_2, reducing NO^{3-} to N_2 (51).

Precoating the growth support media with denitrifying biofilms has been found to be effective for startup of a full-scale anaerobic fluidized bed reactor treating soft drink bottling wastewater (41, 74). A major objection to the use of anaerobic processes for industrial wastewater treatment is the long time required for startup due to the low growth rates of the methanogens. Startup can be defined as the time required by a bioreactor to attain stable performance at a designated loading from initial reactor inoculation (43).

Denitrifying and methanogenic bacteria in the biofilm of a fixed-film reactor operated with methanol/nitrate were studied by Zellner et al. (91). A denitrifying bacterial biofilm population established on a polypropylene substratum of a fixed-film reactor was characterized by microscopy, scanning electron miscroscopy, and immunofluorescence. The reactor with synthetic wastewater containing methanol/nitrate achieved a denitrification rate of 0.24 mol NO^{3-}/l/day with a removal efficiency for nitrate of 95-99% at an organic loading rate of 0.325 mol methanol/l/day. The biofilm contained mainly cells of *Methanobrevibacter arboriphilus*.

System upsets and shocks are very important factors in waste treatment processes. Porous characteristics of the support materials provide protection for organisms against system upsets and shocks (23, 34-36). Durham et al. (23) claimed to develop inorganic matrices for fixed-film bioreactors affording protection to microorganisms and preventing loss of bioreactor productivity during system upsets. They tested these biocarriers, designated Type-Z, against plastic and diatomaceous earth biocarriers. They suggested that Type-Z biocarriers represent an immobilization medium that provides an amenable environment for microbial growth and has the potential for improving the reliability of fixed-film biotreatment processes.

Degradation of cyanuric acid, a herbicide derivative, by adsorbed *Pseudomonas sp.* was studied in a continuous system (26). Cyanuric acid in high concentrations (15 mM) was degraded completely by *Pseudomonas sp.* in a two-stage process with granular clay as a carrier material.

Meta-Alvarez and Llabres (56) suggested that the anaerobic digestion of animal wastes offers heat and energy production, reduction of the pollution load on the environment, the removal of odor problems, and a digested product which can be used as a fertilizer when sprayed on the land. They used a high-rate digestion system, the down-flow stationary fixed film (DSFF) digester, to treat piggery waste. They suggested 50 m^2/m^3 specific support surface as optimal, because yields did not differ when higher values were used, and because the possibility of clogging was reduced.

Evaluation methods for bacterial adhesion

To explain the attachment and detachment processes during biofilm formation, several methods can be used. Cell adhesiveness depends on several factors between biocarriers and cells. Hydrophobic interaction, hydrogen bonding, and ionic interaction are some of the mechanisms affecting the cell's adhesiveness. Surface charge and surface wettability of the biocarriers are also two important factors for attachment processes (61).

A zeta potentiometer measures the zeta potential and conductance of colloidal particles by determining the rate at which these particles move in a known electric field in electrophoresis. Since the particles are observed with a microscope, it is common to refer to this method as microelectrophoresis. The colloid (cell or support particle) is placed in a cell consisting of two electrode compartments and a connecting chamber. A voltage applied between the electrodes produces a uniform electric field in the connecting chamber; charged particles respond by moving to one or the other electrode. The speed of the particle is directly related to the magnitude of the particle charge or zeta potential (26).

Nishizawa et al. (61) studied the effect of the surface wettability and zeta potential of bioceramics on the adhesiveness of anchorage-dependent animal cells (mouse-derived cell line). They suggested that the affinity and adhesiveness of the cells to the ceramics were regulated by the surface potential. They also concluded that a negative potential on the ceramic surface was

effective in increasing the adhesiveness, even though living cells have negative charges. It was speculated by Nishizawa et al. (61) that cell adhesiveness decreases with increasing positivity in the zeta potential of calcium-phosphate ceramic carriers because of a difference in the selectivity of serum protein adsorption, or a difference in the adsorption of Mg^{2+} or Ca^{2+}.

Microbial adhesion to hydrocarbons (MATH) is the most commonly used method to determine microbial cell surface hydrophobicity (28). Geertsema-Doornbusch et al. (28) demonstrated the involvement of electrostatic interactions in MATH by measuring hydrophobicities and by comparing the zeta potentials of the microorganisms (hydrophilic and hydrophobic strains of *Streptococcus salivarius*) and of hexadecane droplets.

Although hydrophobicity is an important factor in microbial adhesion to surfaces, adhesion is thought to be determined by a complicated interplay among hydrophobicity, Van der Waals forces and electrostatic interactions (85). Geertsema-Doornbusch et al. (28) demonstrated a highly negative zeta potential for hexadecane droplets in aqueous suspensions, although the source of the negative charge was not exactly known. They explained the highly negative zeta potentials as oriented adsorption of water molecules to the hexadecane droplets by attractive Van der Waals forces and adsorption of miscellaneous anions. Maximal adhesion (hydrophobicity) of bacteria to hexadecane was found to be due to the undisturbed action of the attractive Van der Waals forces only in

the absence of significant electrostatic interactions (i.e., around the pH of the isoelectric points of hexadecane and/or of the bacteria).

Mozes et al. (59) suggested that the adhesion of hydrophilic microorganisms is controlled essentially by electrostatic interactions. They indicated that the only way to obtain adhesion is to reduce strongly the cell-support electrostatic repulsion and to create electrostatic attraction by making the surface of the support or the cells positively charged.

Adhesion of hydrophobic cells is favored on hydrophobic supports; this illustrates the importance of interfacial energy. The influence of cell-cell and cell-support electrostatic repulsion is illustrated by the influence of pH on the density of adhering cells (59). The adhesion of *Saccharomyces cerevisiae*, *Acetobacter aceti*, and *Moniliella pollinis* to different materials (glass, metals, plastics), some of which were treated by an Fe(III) solution, was compared (59). The only way to obtain cell adhesion was to reduce strongly the cell-support electrostatic repulsion and, eventually, to create electrostatic attraction by making the surface of the support or the cells positively charged. Cell flocculation (cell-cell association) competed with adhesion (cell-support association), depending on the cell concentration and on the procedure used to bring the cells in contact with the support.

Goncalves et al. (31) claimed that the surface charge of the cells did not control adhesion. They also commented on the influence of hydrophobicity on the effect of surface charge in explaining the adsorption of negatively charged

cells to negatively charged supports. The surface of the bacterium they used in this work, *Lactobacillus rhamnosus*, was negatively charged for all the pHs studied. Since the glass surface of the support was also negatively charged, the adsorption to the supports or to the glass walls of the fermentors was ascribed to: high ionic strength of the culture medium, resulting in less negatively charged cell surfaces; modification of the cell surface charge and/or support surface charge by some components of the fermentation medium; or increased cell hydrophobicity occurring during exponential growth or at high growth rates in a chemostat, without marked change in zeta potential. They also quoted the study of Thonart et al. (81), which stated that the adsorption of negatively charged cells to negatively charged supports can be significantly increased in the presence of starch in the medium. Goncalves et al. (31) concluded that one cannot predict microbial adsorption in complex media based on zeta potential alone.

Biofilm formation and adhesion can also be evaluated by determining the weight change of the support, observing the clumping characteristics of the supports after drying at 70°C overnight, obtaining a Gram stain reaction, and plating the disrupted cells of the biofilms for viable counts (21, 49, 50). Supports were dried to obtain weight change information, then were shaken vigorously for an evaluation of clumping strength. Supports with good biofilm formation resisted separation, whereas supports with no biofilm formation separated

easily. Biofilm-coated supports developed a much darker blue color in the Gram stain than did uninoculated supports.

The influence of calcium on specific growth rate, extracellular polymeric substance (EPS) formation rate, biofilm detachment rate, and biofilm calcium concentrations was determined in a RotoTorque reactor (a continuous-flow stirred tank reactor, CFSTR) with *Pseudomonas aeruginosa* (84). No increase in EPS formation rate by changing calcium concentration was seen. However, lower relative detachment rates were observed at increased calcium concentration, probably because of increased cohesiveness of the biofilm.

Processes governing primary biofilm formation were also discussed by Bryers et al. (5). They summarized the process in three stages: 1) transport and adhesion of soluble components and microbial cells to the surface; 2) metabolic conversion within the biofilm including growth, maintenance, and decay processes; 3) detachment of portions of the biofilm and reentrainment in the bulk fluid.

Bryers et al. (5) also suggested that biofilms develop in a sigmoidal fashion with transport and biological processes such as adsorption of dissolved organics at the wetted surface, transport of microbial particles to the surface, microorganism adhesion to the surface, biofilm production, and biofilm detachment.

Molin (58) observed that the viable count of the attached cells was of the same magnitude as those in suspension. The attached cells seemed to have a

significantly higher maximum growth rate than did the suspended cells. Molin studied *Pseudomonas putida* in a continuous culture at various dilution rates with asparagine as the carbon source, and reported that the attachment capacity of the culture increased with increasing dilution rates (up to about 1.0 h^{-1}). It was claimed that the amount of carbon source did not have a critical influence on the attachment. Molin also concluded that the cells in a batch culture had higher attachment capacity in the exponential growth phase than in the lag or declining phase.

CHAPTER 2. EVALUATION OF VARIOUS SUPPORTS FOR ENHANCED PROPIONIC AND ACETIC ACID PRODUCTION IN BIOFILM REACTORS

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Abstract

Six strains of propionibacteria were tested for the ability to form biofilms on different support materials: chips of pure polypropylene; chips composed of 75% polypropylene + 25% agricultural materials (various combinations of corn starch, corn hulls, oat hulls, zein, and soy protein); glass beads; ceramic saddles; stainless steel wool; fire bricks; extraction sockets (thimbles); and commercial biocarriers. With successful biofilm formation, flow rates of media in continuous fermentation systems were increased significantly to dilution rate (D) 8.64 h^{-1}. All reactors with supports showed better performance by all measurements (OD, pH, and acid content of exiting medium) than was seen in the control (free cell) reactor. Fire bricks and *Propionibacterium thoenii* strain P20 were selected for further investigation. Propionic acid concentrations in the medium ranged from 2 to 4 g/l in reactors containing biofilms. Acid productivities ranged from 2.22 to

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22.03 g/l/h for propionic acid and from 0.84 to 4.27 g/l/h for acetic acid. Not all of the substrate was consumed in a single-pass system. To better understand the mechanism of adherence, surface characteristics of the support materials and *P. thoenii* were also investigated. Cell surfaces were found to be negatively charged whereas the surfaces of the selected support materials were positively charged.

Introduction

The long-term objective of this study is to improve the economics of production of propionic acid by fermentation. The use of novel biofilm reactors to maintain high cell concentrations in the fermenter can lower fermentation costs and increase productivity. Propionic acid is a three-carbon fatty acid well known as a natural mold inhibitor; it also has several uses as an industrial chemical. Propionic acid is made commercially by the oxidation of liquid phase propane or propionaldehyde, but acetic and propionic acids may also be produced biologically by the fermentation of sugars by various bacteria, especially the propionibacteria (27).

Retention times and stability of the microorganisms in the reactors are factors that directly affect the feasibility of the fermentation process. Especially in continuous systems, microorganisms can easily be washed out from the reactor at high flow rates; this dramatically decreases the overall performance.

Many microorganisms, primarily bacteria, tend to adsorb to and colonize surfaces submerged in aquatic environments. A biofilm is a natural form of cell immobilization that results from microbial attachment to solid support. Biofilms as a natural form of cell immobilization are dynamic micro-environments, encompassing processes such as metabolism, growth, and product formation, and finally detachment, erosion, or "sloughing" of the biofilm from the surface (5).

Fixed-film or biofilm systems, which are generally packed-bed systems filled with various support materials such as stoneware or plastic packing, are seeded once during their startup period and are generally operated upflow to increase contact time and to permit concurrent flow of liquids and gases. In a biofilm system, the film affords the bound organisms some protection from toxic materials and sudden changes in the feed (30).

While a number of groups have investigated production of propionic acid by immobilized cells (16, 19, 32), there has been little work on the production of propionic and acetic acids by biofilms. In this study, several strains of propionibacteria known to produce high levels of propionic acid were tested for their ability to form biofilms. Novel solid supports, consisting of polypropylene blended with various agricultural materials, commercially available inert materials such as Type-Z and Type-CZ Grace biocarriers, and porous materials such as fire bricks and paper filters were compared as possible supports.

Hydrophobicity and surface charges of the support materials and *P. thoenii* were also investigated for better understanding of the attachment process.

Materials and Methods

Microorganisms and media

Six strains of propionibacteria, *P. acidipropionici* strains P9 and P200910 and *P. thoenii* strains P4, P20, P38, and P127 were obtained from the culture collection of the department of Food Science and Human Nutrition at Iowa State University. The basal medium used in some of the continuous fermentations contained (g/l): D-glucose (20), yeast extract (10) (Difco Laboratories, Detroit, MI), peptone (10) (Difco), KH_2PO_4 (0.25), $MgSO_4 \cdot 7H_2O$ (0.20), $MnCl_2 \cdot 4H_2O$ (0.05). Sodium lactate broth (NLB) used in repetitive-batch (RB) and in some continuous fermentations contained 1% (v/v) sodium lactate 60% syrup, 1% (w/v) yeast extract, and 1% (w/v) Trypticase soy broth (Baltimore Biological Laboratories, BBL, Cockeysville, MD). Salts, glucose, and sodium lactate syrup were reagent grade and were obtained from Fisher Scientific Co., Pittsburgh, PA. Propionibacteria strains were maintained at 4°C on sodium lactate agar (NLA) plates as previously described by Woskow and Glatz (33).

Support materials

Plastic-Composite Supports

All plastic-composite supports (PCS) contained 75% polypropylene (Quantum USI Division, Cincinnati, OH) and 25% agricultural materials, which contained major [ground (20 mesh) soy hull (Iowa State University Center for Crops Utilization Research, Ames, IA), ground (20 mesh) corn hull (Penford Products Co., Cedar Rapids, IA), ground (20 mesh) and dried (20 mesh) oat hull (National Oats Co., Cedar Rapids, IA), or corn starch (American Maize Products Co., Hammond, IN), at least 20% by wt] and minor [zein (Sigma Chemical Company, St. Louis, MO) or soy protein (Archer Daniels Midland Company, Decatur, IL), 5% by wt] components (7, 8).

Plastic-composite supports were prepared by high-temperature extrusion in a Brabender PL2000 twin-screw extruder (C. W. Brabender Instruments, Inc., South Hackensack, NJ). The barrel temperatures were 200, 210, and 220°C, the die temperature was 220°C and the screw speed was 20 rpm. Each agricultural material was vacuum-dried for 48 h at 110°C prior to use. The composite material was extruded as 3-mm-diameter rods, air-cooled, and cut into chips 2 to 3 mm in length (7, 8).

Other support materials

Other inert support materials used were: pure polypropylene (Quantum USI), glass beads, ceramic saddles (Fisher Scientific Co.), cotton cellulose

extraction thimbles (single thickness, 1 mm; ID x height, 22x80 mm) (Fisher), stainless steel wool (generic brand for household use, Iowa State University Central Stores, Ames, IA), fire bricks (Al_2O_3 - obtained as blocks from the Department of Material Science and Engineering at Iowa State University, Ames, IA), and the commercially available biosupports (W. R. Grace & Co., Columbia, MD).

Ceramic saddles (Fisher), glass beads (Ace Glass, Vineland, NJ), and stainless steel were used as obtained without any modifications or pretreatments. Fire bricks were cut into 6 x 1.5-cm cylindrical pieces and then into 1.5-1.8-cm pieces with 6-7-mm holes (BioLifeSaver - patent pending). Pure cotton cellulose soxhlet extraction thimbles (Fisher) were lengthened with a small cylindrical piece of the same material to fit the size of the 60-ml syringe bioreactor and five holes were made at the conical bottom of the thimbles to increase the internal medium flow.

Commercially available biocarriers, Type-Z and Type-CZ, were generously supplied by Grace Research (W.R. Grace & Co). These biocarriers were preconditioned by submerging them in 0.04% (w/v) NaOH for 30 min. After rinsing with two volume changes of distilled water, the biocarriers were added to appropriate growth medium. Target pH at this point was 7.0. An average of 144 biocarrier particles with a total average weight of 32 g were packed into 54 ml volume (including void volume) of the 60-ml syringe bioreactors.

Biofilm evaluations

Biofilm formation on the plastic-composite supports was evaluated by determining extent of clumping of the support after drying at 70°C (7), and Gram-staining. Supports with good biofilm formation resisted separation even after vigorous shaking, and developed a dark blue color after Gram-staining.

Biofilm visualization with scanning electron microscopy (SEM)

Biofilms formed on different support materials were analyzed using the JEOL JSM-35 scanning electron microscope (Japanese Electric and Optical Laboratory, Tokyo, Japan) in the Iowa State University Electron Microscopy Facility. Sample preparation was as follows. Biofilms were fixed on the surface of the support materials in the series of 4% glutaraldehyde (Sigma), 3% paraformaldehyde (Sigma), and then stored overnight in 0.1 M cacodylate buffer (Sigma) (pH 7.2) at 4°C. The fixed biofilms with supports were washed in the same buffer three times for approximately 10 min total. Samples were then fixed in 1% osmium tetroxide (OsO_4) (Sigma) in the same buffer for 1 h at 4°C. The washing step was repeated three times in the same buffer for approximately 10 min. After that the fixed and washed biofilms on the surfaces were dehydrated in a series of ethanol concentrations of 50, 70, 80, 95, 100, 100, and 100% for 10 min each. Samples in absolute ethanol were dried in a critical point drying apparatus (DENTON DCP-1 - Denton Vacuum Corporation, Cherry Hill, NJ) with CO_2. They were mounted on brass discs with double-stick tape and

silver paint and coated with gold-palladium (60:40) in a Polaron E5100 Sputter Coater. Biofilms and individual organisms were observed and photographed by using a JEOL JSM-35 SEM at maximum 20 kV. Polaroid type 665 film was used to record the images.

Surface characterizations

Zeta potentials of the support materials and the bacteria were measured at room temperature with a Lazer Zee Model 500 (Pen-Kem, Inc., Bedford Hills, NY), which uses scattering of incident laser light to detect particles (bacteria or support material) at relatively low magnifications. The absolute electrophoretic mobilities can be derived directly from the velocities of the particles in the applied electric field, the applied voltage, and the dimensions of the electrophoresis chamber (15, 31).

The culture, *P. thoenii* strain P20, was grown in NLB at 32°C and harvested during the late exponential phase (24 h). Cells were pelleted in a Beckman model J2-21 centrifuge (Beckman, Palo Alto, CA) at 15,000 x *g*, 25°C for 10 min, and washed twice with 10 mM NaCl aqueous solution. Cells were resuspended in 10 mM NaCl aqueous solution.

For the zeta potential measurements, 10 mM phosphate-buffered saline (PBS, containing 0.142 g of Na_2HPO_4 and 0.526 g of NaCl in 1 L distilled water) was used as the medium. The pH of the buffer was adjusted to vary from 5.0 to

7.0 with 0.5 pH unit increments by adding 20 mM HCl or NaOH to maintain a constant ionic strength. A small amount of bacterial suspension (25-50 mL) in 10 mM NaCl was added to 10 mM PBS (5-10 ml) to a concentration of 10^7 cells/ml.

Selected support materials (fire bricks, Type-Z, and Type-CZ) were prepared by grinding to a powder with a Fisher mortar grinder, model 155 (torque = 0.12 cm/g and rpm=47-57) (Fisher Scientific, Pittsburgh, PA) for 30 min. After test runs to find the optimum particle concentration, 0.1 g of powdered support material was suspended in 200 ml of sterile sodium lactate broth. Particles were mixed in the solution by sonication (Vibra Cell sonicator, Sonics & Materials, Inc., Danbury, CT) for 2 min. After a 5-min waiting period, the suspension was equally distributed into five 100-ml beakers, the pH of the suspension in each beaker was adjusted to a pre-set value between pH 5.0 and 7.0 with concentrated HCl (36.5-38%), and the suspension in each beaker was sonicated for 60 s. Zeta potentials for each suspension were determined at the upper stationary level of a flat rectangular quartz cell with rotary-prism system fitted to a microscope (Nikon, Tokyo, Japan). At least two readings per filling of the electrophoresis chamber were made by approaching from both negative and positive sides.

Hydrophobicity

The microbial adhesion to hydrocarbons (MATH) test was performed according to Lichtenberg et al. (20) on microbial cells and ground support

materials. Strain P20 was suspended to an absorbance at 550 nm (A_o) of between 0.4 and 0.6 in 10 mM potassium phosphate buffer, with the pH adjusted to 2.0, 3.0, 4.0, 5.7, 5.9, 6.1, 6.5, 6.95, 7.37, 7.75, and 7.9 by the addition of HCl or KOH. An aliquot (150 ml) of hexadecane was added to 3 ml of bacterial suspension, after which the two-phase system was vortexed for 10 s and allowed to settle for 10 min. The absorbance (A_t) of the water phase was then measured. This procedure was repeated until the total vortexing time amounted to 60 s. The log (A_t/A_o x 100) was plotted against the vortexing time, and a linear least-squares fit of the initial declining part of the plot subsequently yielded the initial removal rate (R_o) per minute as a measure of the adhesion of the cells to hexadecane.

Repetitive-batch propionic acid fermentations in culture tubes

The six strains of propionibacteria were tested on 10 different support materials for biofilm formation and organic acid production in test tubes (25 x 200 mm with screw cap) with an average 30 ml working volume. Initial inoculation was 2 ml of 24-h cultures. Cultures were incubated at 32°C for 36 h to allow growth and biofilm formation. After this initial growth period, liquid contents were aseptically drained every 48 h and replaced with fresh NLB. A control culture of free cells was maintained in 40 ml of NLB. Each time the immobilized cultures were drained, the free cells were centrifuged at 13,800 x *g* for 15 min and resuspended in fresh NLB.

Continuous propionic acid fermentation in biofilm reactors

Biofilm reactors were 60-ml plastic syringes filled with 50 ml of support materials and connected to a reservoir of fresh medium. A CO_2 line fitted with a filter was connected to the medium inlet line (Figure 1). The reactors were incubated in a water bath at 32°C and inoculated with 1.5 ml of a 24-h culture of strains P9, P127, or P20. Fermentation was started as a batch for 36 to 48 h and then switched to continuous feed. A reactor containing 25 ml of free-cell culture was used as a control. Medium was pumped at various flow rates (0.06, 0.12, 0.24, 0.48, 0.96, 2.00, 3.00, and 3.60 ml/min) to give dilution rates of 0.144, 0.288, 0.576, 1.152, 2.304, 4.8, 7.2, and 8.64 h^{-1}. The pH, cell density (absorbance at 550 nm), and concentrations of propionic acid, acetic acid, and substrate in the effluent were analyzed every 5-12 h. The pH of the medium was adjusted in the reservoirs prior to each experiment, but pH was not controlled in the reactors.

Analytical methods

The suspended free-cell density in the reactors was measured by absorbance at 550 nm by using a Spectronic 20 spectrophotometer (Milton Roy, Rochester, NY). Concentrations of glucose and lactic, acetic, and propionic acids were determined by using a high-performance liquid chromatography system (HPLC, Waters, Milford, MA) equipped with a Waters Model 401 refractive

index detector, column heater, autosampler, and computer controller. Separation was achieved on a Bio-Rad Aminex HPX-8711 column (300x7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) with 0.012 N H_2SO_4 as mobile phase at a flow rate of 0.8 ml/min. The injection volume was 20 ml and the column temperature was 65°C. At these settings, one should expect the following peak sequence (retention time): glucose (~7 min), lactate (~10 min), acetate (~12 min), and propionate (~14 min). Average percent deviations for glucose, lactate, acetate, and propionate over three sets of injection data were 3.8, 2.4, 3.2, and 1.5%, respectively.

Results

Repetitive-batch fermentations

Six strains of propionibacteria were tested on several support materials for acetic and propionic acid production in repetitive-batch biofilm fermentations (Table 1a-c). *P. thoenii* strain P38 produced little acid and was disqualified from further investigations. Acid production in successive batches was not significantly different among the other strains on the various supports. Biofilm formation was observed on both fire bricks and plastic-composite supports (PCS). Acid production in biofilm reactors matched that in reactors with free cells, in which all cells were retained by centrifugation between batches (Table 1a-c).

Strains P127 and P20 were selected for further study on the basis of their acid production and biofilm formation abilities. When the supports with these strains were Gram-stained (7), a dark color indicative of biofilm formation was observed (Table 2). Another advantage of strain P20 was its resistance to organic solvents (12). In addition to fire bricks and thimbles, the corn starch-zein PCS was also selected for further study as a representative of the plastic-composite supports.

During these studies it was observed that strain P4 produced extensive slimy by-products throughout the fermentation process. Even though P4 is also a *P. thoenii* strain, the consistency and characteristics of its slime were totally different from the biofilm formed by strains P20 and P127. It is hard to call the film formed by P4 a biofilm; the broth became opaque, slimy, and highly viscous. The yellowish turbidity of the cells was visible in the broth with strain P4, in contrast to the red film accumulated on all surfaces in fermentations with strains P20 and P127.

Tested strains could be categorized either as nonbiofilm-formers (P38, P4) or biofilm-formers (P9, P200910, P20, P127). Even among biofilm-formers there were noticeable differences; for instance, the reddish biofilms formed by strains P20 and P127 were more sticky and slimy than were the yellowish biofilms formed by strains P9 and P200910.

Continuous fermentations

Because it has been previously observed that movement of the medium might have an impact on biofilm formation, strains P127 and P9 were tested in one-pass continuous flow fermentation systems (Table 3 and 4). Strain P9 had been used previously in our lab for propionic acid fermentations (24, 25, 29, 33). Acid production was greater in reactors with supports than in the control (without support) reactor.

Continuing acid production, clumping, weight gain, and cell viability at increasing dilution rates were evidence of continued survival even at low pH (Table 5). The average pH of the medium exiting the reactors was about 4.5, which is at or below the pH value at which inhibition of free cells is generally seen (32). If there had not been any growth or acid production in the reactor, the pH of medium exiting the reactor would be expected to approach the pH of the fresh medium in the reservoir (pH 6.9).

Strains P9 and P127 were next compared for biofilm formation and acid production when supported by a corn starch-zein PCS, fire bricks, or glass beads (Figure 2). Cells grown on fire bricks and corn starch-zein PCS produced the most acid. The increase in acid concentration with increasing dilution rates seen with the cultures grown on corn starch-zein supports was unexpected, and is unexplained. In comparison with free-cell reactors, the biofilm reactor systems increased the overall retention time of the cells in the reactors. Even

when dilution rates were increased up to 8.64 h^{-1} in subsequent experiments, cells continued to grow, produce acetic and propionic acids, and form biofilm.

Because it was a good biofilm former and acid producer, and also because of its resistance to the solvent used for acid extraction from the broth (12), *P. thoenii* strain P20 was selected for further studies. Biofilm formation and acid production were followed when fire bricks were used as supports with this organism over six dilution rates up to 8.64 h^{-1} (Table 6). In continuous fermentation mode, about 4 g/l propionic and 1.5 g/l acetic acids were produced in this system; productivities ranged from 2.22 g/l/h to 22.03 g/l/h and from 0.84 g/l/h to 4.27 g/l/h for propionic and acetic acids, respectively.

Tables 3, 4, and 6 summarize the acid production results for different strain-support combinations over a range of dilution rates. According to previous studies in our lab, the maximum dilution rate for free-cell reactors was around 0.27 h^{-1} (25). The biofilm reactors in the current study allowed dilution rates at least three times higher to be used, and thus much higher acid productivities were achieved. Propionic and acetic acids were produced at 3:1 (weight:weight) ratio.

Commercial support materials, Grace Type-Z and Type-CZ, were also tested with *P. thoenii* strain P20. Even though these materials demonstrated competitive performance in terms of acid production (Figure 3), decomposition of supports during fermentation was a major problem. The Type-CZ support completely disintegrated into a sandy structure in the pH range used here.

Some of the sandy particles were washed out along with the organisms. Supports were not in a reusable condition after the first run.

Surface characteristics

Attachment of cells of *P. thoenii* strain P20 onto selected support materials (fire bricks, thimbles, Type-Z, and Type-CZ) was observed via scanning electron microscopy at several magnifications. At the end of a normal fermentation, extracellular polysaccharides (EPS) fully covered all surfaces so that individual cells and the cell-surface interface in a fully formed biofilm could not be seen. Special fermentation runs were performed for just 24 h for observation by SEM (Figures 4 and 5). Commercial biocarriers were also observed under SEM (Figure 6). The SEM pictures demonstrate that cells form a thick biofilm layer after proper colonization on the support materials.

Surface charges of cells and support materials as measured with the zeta potentiometer over the range of pH 4-8 are shown in Figure 7. Zeta potential (mV) values of support materials were higher (i.e., more positive) as pH decreased. Cells had negative zeta potential which would cause them to be attracted toward positively charged support surfaces. The zeta potential of the cells became less negative as pH decreased.

The MATH test determined the combined effect of hydrophobicity and surface charges over a wide pH range (pH 2-8). Strain P20 showed hydrophilic behavior at all tested pH values except pH 2 and 3 (Figure 8). Adhesion of cells

and support material particles to hexadecane (i.e., an indication of their hydrophobicity) can be evaluated by calculating their initial removal rates R_o (min^{-1}) from the aqueous phase in the MATH test. These rates are plotted in Figure 9. Values observed here are indicative of hydrophilic behavior, and are what would be expected given the zeta potentials of these particles. The R_o values for *P. thoenii* increased at extremely low pH levels, where zeta potentials were lower.

Discussion

Most of our previous work has been with *P. acidipropionici* strain P9, and its propionate-tolerant variant strain P200910. Strain P9 was identified as a strong acid producer in a survey of strains in our culture collection (2) and strain P200910 was isolated after prolonged exposure of strain P9 to high concentrations of propionate (33). However, a number of other strong acid producers had also been identified in previous surveys, and other traits such as ability to clump or form extracellular polysaccharides, or tolerance to solvents used for acid extraction, could prove to be equally beneficial to the overall goal of most efficient acid production.

We have accomplished one of our main goals: to find a good strain-support combination for biofilm formation. Most of the tested propionibacteria strains formed biofilm on selected surfaces; however, some strains (P127 and P20) have

shown much better adhesion characteristics than others. These also had the advantage of better visibility because of their red color. Our indicators for biofilm formation were color development by accumulation of pigmented cells, microscopic examination (light microscopy and SEM), color intensity after Gram-staining of supports (Table 2), acid production by biofilms (Tables 3, 4, and 6), and weight gain and clumping characteristics of supports after the biofilm fermentations (Table 5).

Initial screening of different combinations of strains and supports was performed in repetitive batch biofilm fermentations in large screw-cap culture tubes. Although no significant differences in acid production were seen among different combinations, consistency of acid production over many repeated batches suggested that sufficient biofilm had formed on the support materials to successfully maintain the fermentation through each cycle. Biofilm reactors produced as much acid as did reactors with cell-recycled free-cell reactor, even though all free cells were retained in the control reactor by centrifugation between batches.

Another observation made in this phase of the study was that the propionibacteria tended to form clusters and accumulate as a pellet at the bottom of the reactors. After this observation was made, reactors were kept in a horizontal position and rotated 180° daily until sufficient biofilm had formed on all surfaces.

Selected strains and supports were then tested in a continuous fermentation system. At this point, a representative of the plastic-composite supports (corn starch-zein), fire bricks, and *P. thoenii* strain P20 were chosen for further studies.

Even though the commercial Type-Z and Type-CZ biocarriers supported biofilm development and comparable acid production, some disadvantages to their use were noted. The requirement for pretreatment to adjust pH was a drawback; the process took much longer than the reported 30 min. Also, the biocarriers, especially Type-CZ, lost significant amounts of material from their structure during the course of fermentations. This might indicate acid sensitivity of the zeolite structure of the biocarriers. At the end of the process, the remaining biocarriers fused to form one large mass. This precluded reuse of these biocarriers.

Modified fire brick supports (called BioLifeSavers) performed well as a support material. By all measurements, apparent biofilm formation was observed. The structure of the support was stable through extended and repeated use. Even though extraction thimbles also supported biofilm formation, problems in reusability were a drawback. Complete washing of the biofilm from the cellulosic surfaces of the thimbles was impossible and after drying the thimbles became hard and brittle.

With thick extracellular polymeric materials covering the support surfaces, it was not possible to observe the cell-support interface. A slimy, red-

brown biofilm grew into the void volume of the reactors, with thickness varying according to flow characteristics at different locations in the reactor. To enhance the flow and substrate distribution, CO_2 gas was bubbled into the reactor in an upward direction parallel to the medium flow. This also helped keep air out of the reactor. Rate of gas flow was controlled at the lowest possible level because high gas flow rates or sudden fluctuations could cause detachment and washout of the biofilm.

The most important factors for the selection of good support materials are the ease of preparation, chemical reactivity, performance in the fermentation, reusability, and cost. "Life-saver" shaped fire bricks were selected as an alternative inert support for natural immobilization of *Propionibacterium* as biofilm. They were stable from one run to another, and could be used repeatedly without decrease in performance. Their life-saver shape helped the flow regime in the reactor by allowing flow through the center of each support piece and increasing the accessible surface area for the bacteria to form biofilm. Although PCSs also demonstrated good performance, shape and reusability were two factors that favored fire bricks over PCSs. Acid production by cultures grown with fire bricks was higher than that produced by cultures grown with PCSs at all except the highest dilution rate (Figure 3A). Since PCSs are considerably smaller than fire bricks, packing them in a reactor can decrease the available void volume and cause problems such as channeling in the flow regimes. Reactors with PCSs can eventually become clogged with excessive biofilm

accumulation. In contrast, ring-shaped fire bricks can reduce channeling and improve flow patterns.

Age of the biofilm on the support materials is another important factor in this study. Dilution rates were not tested randomly. Rather, flow rates started low and were gradually increased. Therefore, fermentations at higher dilution rates were performed with older, more established biofilms. With constant detachment and attachment processes going on in a typical biofilm system, cells of different ages and in different metabolic states might be present under all of the conditions tested. However, it is likely that the fermentations conducted last (i.e., those at higher dilution rates) were performed with more cells in the established biofilm.

Even though the mechanisms behind the attachment process are not very well known, it is generally accepted that hydrophobicity, surface charges, various forces (e.g., electrostatic, electrodynamic, hydrodynamic, aerodynamic, gravitational), and microbial physiology are the major factors contributing to biofilm formation (6, 10, 20, 22, 23, 28, 31).

Zeta potential and hydrophobicity, both related to the overall chemical composition and structure of microbial cell surfaces, are important properties with respect to microbial adhesion (4, 23). Generally, hydrophilic cells are expected to adhere preferentially to hydrophilic substrata, while hydrophobic cells should adhere preferentially to hydrophobic substrata (31). In addition,

when substratum and cell surface charges are the same, repulsive electrostatic interactions can discourage adhesion (31).

The magnitude and sign of the particle charge can be determined by observing the speed and direction of the particle movement under the influence of the applied field. However, there is one important complicating factor. When the voltage is applied, not only do the particles move with respect to the fluid but, in addition, the fluid moves with respect to the chamber. This second effect is referred to as electroosmosis and is characterized by a movement of the fluid in one direction near the surface of the viewing chamber walls, accompanied by a return flow in the opposite direction in the center of the viewing chamber (26). There must be a surface where the fluid is stationary; this surface is called the stationary layer. All measurements were made at this stationary layer. A zeta potential measurement is made by adjusting the prism control until the apparent motion caused by the prism exactly cancels the particle velocity caused by the applied field. At this point, the particles appear stationary in the field of view, and the zeta potential is displayed on a digital readout on the front panel (11, 26).

At least two zeta potential measurements were made for each sample evaluated. Because of the complexity of PCS, these supports can not be evaluated for zeta potentials. Because zeta potentials can be dramatically affected by the pH of medium, measurements were made at several different pH values. Before and after the measurements, the pH of each sample was checked

for possible fluctuations during the testing. No change was observed. As can be seen from Figure 8, zeta potentials of positively charged support surfaces decreased with increasing pH. This is expected because the increasing hydroxyl ion concentration in the medium neutralizes the positively charged surfaces. The zeta potential of cells of P20 became increasingly negative with increasing pH. This is typical of the behavior of negatively charged surfaces, and supports the mechanism of attachment of the cells to the support materials as an interaction of oppositely charged surfaces.

The MATH test measures an interplay of hydrophobicity and electrostatic interactions. MATH measures solely hydrophobicity only when electrostatic interactions are absent, (i.e., close to the isoelectric point of the cells) (31). The MATH test was applied to *P. thoenii* strain P20, fire bricks, Type-Z, and Type-CZ support materials. Over the pH range 4 to 8, the $\log(A_f/A_o x100)$ value which indicates the normalized value of the difference in absorbance, did not change significantly. Larger changes were seen at pH 2 and 3, which is out of the range of typical fermentations. The tested materials and the cells did not prefer staying in the hydrophobic phase. Therefore, they can be categorized as hydrophilic in the pH range of a typical fermentation.

The kinetic MATH test as proposed by Lichtenberg et al. (20) was employed to find the initial removal rate, R_o (min^{-1}), of the cells and support materials as a measure of the adhesion of the materials to hexadecane. The R_o values suggested that adhesion of the materials to hexadecane also depends on

electrostatic characteristics of both cells and supports. Previous studies demonstrated a highly negative zeta potential for hexadecane droplets in aqueous suspension, although the source of the negative charge was not exactly known (10, 21). However, it was envisaged that the highly negative zeta potentials were due to oriented adsorption of water molecules to the hexadecane droplets by attractive Van der Waals forces as well as adsorption of miscellaneous anions (10). We speculate that when the pH of the medium is close to the isoelectric point of the cells, electrostatic interaction (repulsion in this case) is minimized. This allows the adhesion of the cells to the hydrocarbon droplets.

Ho et al. (13) discussed relative hydrophobicity of *Lactobacillus casei* subsp. *rhamnosus* and PCS by measuring contact angles by the sessile drop technique. They suggested that *L. casei* was hydrophilic while polypropylene and PCS discs possessed hydrophobic surfaces. They also suggested that addition of soybean hulls, yeast extract, and mineral salts decreased the contact angles (hydrophobicity) of the supports and made them more attractive to the organisms.

Attachment of propionibacteria on the surfaces of fire bricks and thimbles is shown in Figures 5 and 6. As can be seen from the cross section of the Type-Z biocarrier with biofilm in the SEM picture Figure 7b, most of the biofilm was formed on the surface with limited growth into the matrix. Possibly the first

generation settlers on the surface died, thus limiting further penetration into the matrix. It is also possible that the matrix might not attract the organisms.

It has also been suggested that bacterial activity in low-nutrient environments is enhanced at surfaces, (i.e., an environment where the carbon source is limited may stimulate cell attachment) (1, 3, 9, 34). Effects of various nutrients at low concentration on the adhesion mechanism should also be studied further with the propionibacteria.

Ho et al. (14) also studied the leachate bioavailability, leaching rate, and lactic acid accumulation properties of plastic-composite supports. They reported that PCS with only yeast extract as the minor agricultural ingredient leached out 51 to 60% of the total nitrogen during the first batch fermentation. Such leaching can be very useful for supplying nutrients, but may not be sustained after the first batch.

In summary, we have defined the problems of continuous propionic acid fermentation and sought alternatives to increase yield and productivity. Our overall results indicated that biofilm fermentations can be used as an alternative mode for biological propionic acid production. Biofilm-forming characteristics of the selected propionibacteria strains gave us an opportunity to test our organisms with various inert support materials. Reshaped fire bricks and PCSs are both suitable for microbial colonization on their surfaces. *P. thoenii* strain P20 was selected as one of the best candidates for biofilm formation. Biofilm systems increased retention time in the reactors. After

determining a good support-strain combination (*P. thoenii* and fire bricks), we examined the attachment process by measuring hydrophobicity and zeta potentials of cells and support materials, and observing attachment with the scanning electron microscope. Cells and support materials were found to be hydrophilic in the optimum pH range (5-8) of the propionic acid fermentation. Zeta potentials of support materials and cells had opposite signs over a wide pH range (4-8); this should encourage attachment.

Attachment undoubtedly is a multifactorial process that needs further investigation. Studies should be performed with much smaller reactors to be able to follow the system parameters more accurately. On the other hand, biofilm systems for propionic acid production should also be scaled up to determine the most effective design to eliminate problems seen in small reactors such as fluctuations in the gas flow that produce significant changes in flow patterns in the reactor.

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References

1. **Atkinson, B. and H. W. Fowler.** 1974. The significance of microbial film in fermenters. *In*: Ghose, T. K., A. Fiechter, N. Blakebrough (eds) Advances in biochemical engineering 3. Springer, Berlin Heidelberg, NY, pp 221-277.

2. **Babuchowski, A., E. G. Hammond, and B. A. Glatz.** 1993. Survey of propionibacteria for ability to produce propionic and acetic acids. J. Food Prot. **56**:493-496.

3. **Brown, C. M., D. C. Ellwood, J. R. Hunter.** 1977. Growth of bacteria at surfaces: influence of nutrient limitation. FEMS Microbiol. Lett. **1**:163-166.

4. **Busscher, H. J., J. Sjollema, and H. C. Van der Mei.** 1990. Relative importance of surface free energy as a measure of hydrophobicity in bacterial adhesion to solids, p. 335-360. *In* R. J. Doyle and M. Rosenberg (ed.), Microbial cell surface hydrophobicity. American Society for

Microbiology, Washington, D. C.

5. **Characklis, W. G. and K. C. Marshall.** 1990. Biofilms. John Wiley & Sons, Inc., New York.

6. **Chester, S. H.** 1986. An understanding of the forces in the adhesion of microorganisms to surfaces. Process Biochem. **10**:148-152.

7. **Demirci, A., A. L. Pometto III, and K. E. Johnson.** 1993a. Evaluation of biofilm reactor solid support for mixed-culture lactic acid production. Appl. Microbiol. Biotechnol. **38**:728-733.

8. **Demirci, A., A. L. Pometto III, and K. E. Johnson.** 1993b. Lactic acid production in a mixed-culture biofilm reactor. Appl. Environ. Microbiol. **59**:203-207.

9. **Fletcher, M.** 1979. The attachment of bacteria to surfaces in aquatic environments. *In*: Ellwood, D. C., J. Melling, P. Rutter (eds) Adhesion of microorganisms to surfaces. Academic Press, London, pp 87-108.

10. **Geertsema-Doornbusch, G. I., H. C. Van der Mei, and H. J. Busscher.** 1993. Microbial cell surface hydrophobicity. The involment of electrostatic interactions in microbial adhesion to hydrocarbons (MATH). J. Microbiol. Meth. **18**:61-68.

11. **Goetz, P. J. and J. G. Penniman, Jr.** 1976. A new technique for microelectrophoretic measurements. American Laboratory. October issue.

12. **Gu, Z.** 1997. Process development of propionic acid production by fermentation. Ph.D. dissertation. Iowa State University, Ames, IA.

13. **Ho, K-L. G., A. L. Pometto III, P. N. Hinz, J. S. Dickson, and A. Demirci.** 1997a. Ingredient selection for plastic-composite supports for L-(+)-lactic acid biofilm fermentation by *Lactobacillus casei* subsp. *rhamnosus*. Appl. Environ. Microbiol. **63**:2516-2523.

14. **Ho, K-L. G., A. L. Pometto III, P. N. Hinz, A. Demirci.** 1997b. Nutrient leaching and end product accumulation in plastic-composite supports for L-(+)-lactic acid biofilm fermentation. Appl. Environ. Microbiol. **63**:2524-2532.

15. **James, A. M.** 1991. Charge properties of microbial cell surfaces, p.221-262. *In* N. Mozes, P. S. Handley, H. J. Busscher, and P. G. Rouxhet (ed.), Microbial cell surface analysis-structural and physicochemical methods. VCH Publishers, Inc., New York.

16. **Jain, D. K., R. D. Tyagi, D. Kluepfel, and T. J. Agbebavi.** 1991. Production of propionic acid from whey ultrafiltrate by immobilized cells of *Propionibacterium shermanii* in batch process. Proc. Biochem. **26**:217-223.

17. **Kunduru, M. R. and A. L. Pometto III.** 1996a. Evaluation of plastic-composite-supports for enhanced ethanol production in biofilm reactors. J. Ind. Microbiol. **16**:241-248.

18. **Kunduru, M. R. and A. L. Pometto III.** 1996b. Continuous ethanol production *by Zymomonas mobilis* and *Saccharomyces cerevisiae* in biofilm reactors. J. Ind. Microbiol. **16**:249-256.

19. **Lewis, V. P. and S-T. Yang.** 1992. Continuous propionic acid fermentation by immobilized *Propionibacterium acidipropionici* in a novel packed-bed bioreactor. Biotechnol. Bioeng. **40**:465-474.

20. **Lichtenberg, D., M. Rosenberg, N. Sharfman, and I. Ofek.** 1985. A kinetic approach to bacterial adherence to hydrocarbon. J. Microbiol. Meth. **4**:141-146.

21. **Medrzycka, K. B.** 1991. The effect of particle concentration on zeta potential in extremely dilute solutions. Coll. Pol. Sci. **269**:85-90.

22. **Molin, G.** 1981. The impact of dilution rate and attached growth on steady-state characteristics of *Pseudomonas putida*. Eur. J. Appl. Microbiol. Biotechnol. **13**:102-106.

23. **Mozes, N., F. Marchal, M. P. Hermesse, J. L. Van Haecht, L. Reuliaux, A. J. Leonard, and P. G. Rouxhet.** 1987. Immobilization of microorganisms by adhesion: interplay of electrostatic and nonelectrostatic interactions. Biotechnol. Bioeng. **30**:439-450.

24. **Ozadali, F., B. A. Glatz, and C. E. Glatz.** 1996. Fed-batch fermentation with and without on-line extraction for propionic and acetic acid production by *Propionibacterium acidipropionici*. Appl. Microbiol. Biotechnol. **44**:710-716.

25. **Park, S-K.** 1991. Production of propionic acid by *Propionibacterium acidipropionici* in various fermentation processes. MS thesis, Iowa State University, Ames, IA.

26. PenKem, Inc. Technical Service and Engineering. 1981. User manual. Ossining, NY.

27. Playne, M. J. 1985. Propionic and butyric acids. *In*: Moo-Young, M (ed) Comprehensive biotechnology, vol. 3, Pergamon Press, Oxford, pp 737-759.

28. Rosenberg, M. 1984. Bacterial adherence to hydrocarbons: a usual technique for studying cell surface hydrophobicity. FEMS Microbiol. Lett. 22:289-295.

29. Solichien, M. S., D. O'Brien, E. G. Hammond, and C. E. Glatz. 1994. Membrane-based extractive fermentation to produce propionic and acetic acids: toxicity and mass transfer considerations. Enzyme Microb. Technol. 17:23-31.

30. Turakhia, M. H. and W. G. Characklis. 1989. Activity of *Pseudomonas aeruginosa* in biofilms: effect of calcium. Biotechnol. Bioeng. **33**:406-414.

31. Van der Mei, H. C., J. De Vries, and H. J. Busscher. 1993. Hydrophobic and electrostatic cell surface properties of thermophilic dairy streptococci. Appl. Environ. Microbiol. **59**:4305-4312.

32. Yang, S-T., Y. Huang, and G. Hong. 1995. A novel recycle batch immobilized cell bioreactor for propionate production from whey lactose. Biotechnol. Bioeng. **45**:379-386.

33. Woskow, S. A. and B. A. Glatz. 1991. Propionic acid production by a

propionic acid-tolerant strain of *Propionibacterium acidipropionici* in batch and semicontinuous fermentation. Appl. Environ. Microbiol. 57:2821-2828.

34. **ZoBell, C. E.** 1943. The effect of solid surfaces upon bacterial activity. J. Bacteriol. 46:39-43.

Table 1a. Production of acid by strains P20 and P127 with several support materials in the first three batches and in the last three batches in repetitive batch culture tube fermentations.

	P. thoenii strain P20				*P. thoenii* strain P127			
	Acetic acid (g/l)		Propionic acid (g/l)		Acetic acid (g/l)		Propionic acid (g/l)	
	I[a] Avg.	F[b] Avg.	I[a] Avg.	F[b] Avg.	I[a] Avg.	F[b] Avg.	I[a] Avg.	F[b] Avg.
Control[c]	1.80	1.73	4.52	5.86	1.42	1.72	4.42	4.81
Polypropylene	1.54	1.73	4.43	4.87	1.62	1.72	4.40	4.68
CS[d] + zein	1.52	1.84	4.30	4.95	1.17	1.72	4.07	4.74
CH[d] + zein	0.55	2.60	2.07	4.90	0.85	1.71	3.03	4.72
CH + SP[d]	1.49	1.93	4.19	4.86	1.36	1.82	4.38	4.81
CS + SP	1.09	1.85	3.39	5.04	0.95	1.70	3.32	4.77
SH[d] + SP	1.36	1.88	4.10	4.97	1.28	1.73	4.03	4.70
OH[d] + SP	1.42	1.87	3.87	4.95	1.39	1.72	4.02	4.67
Fire bricks	1.56	1.72	4.63	4.90	1.50	1.42	4.56	4.70

[a]I Avg.: Average final concentrations of the first three batches (second, third, and fourth day measurements; medium was changed every day).
[b]F Avg.: Average final concentrations of the last three batches (seventh, ninth, and eleventh day measurements; medium was changed every other day).
[c]Control is a cell-recycled free cell fermentation (cells are retained in the tube by centrifuging in between batches).
[d]CS: Corn starch; CH: Corn hull; SH: Soy hull; OH: Oat hull; SP: soy protein

Table 1b. Production of acid by strains P9 and P200910 with several support materials in the first three batches and in the last three batches in repetitive batch culture tube fermentations.

	P. acidipropionici strain P9				*P. acidipropionici* strain P200910			
	Acetic acid (g/l)		Propionic acid (g/l)		Acetic acid (g/l)		Propionic acid (g/l)	
	I[a] Avg.	F[b] Avg.	I[a] Avg.	F[b] Avg.	I[a] Avg.	F[b] Avg.	I[a] Avg.	F[b] Avg.
Control[c]	1.36	1.72	4.49	4.91	1.57	1.84	4.36	4.84
Polypropylene	1.49	1.57	4.26	4.60	1.23	1.62	3.67	4.22
CS[d] + zein	1.68	1.74	4.74	4.67	1.46	2.26	4.36	4.85
CH[d] + zein	1.33	1.76	4.10	4.63	0.71	1.38	2.73	4.47
CH + SP[d]	1.74	2.47	4.72	4.64	1.22	1.77	3.80	4.72
CS + SP	1.71	1.76	5.02	4.75	1.05	1.71	4.00	4.60
SH[d] + SP	1.79	1.69	4.95	4.55	1.25	1.61	3.97	4.35
OH[d] + SP	2.05	1.75	4.42	4.67	1.20	1.72	3.38	4.30
Fire bricks	1.45	1.73	4.46	4.62	1.44	1.84	4.30	4.85

[a]I Avg.: Average final concentrations of the first three batches (second, third, and fourth day measurements; medium was changed every day).
[b]F Avg.: Average final concentrations of the last three batches (seventh, ninth, and eleventh day measurements; medium was changed every other day).
[c]Control is a cell-recycled free cell fermentation (cells are retained in the tube by centrifuging in between batches).
[d]CS: Corn starch; CH: Corn hull; SH: Soy hull; OH: Oat hull; SP: soy protein

Table 1c. Production of acid by strains P38 and P4 with several support materials in the first three batches and in the last three batches in repetitive batch culture tube fermentations.

	P. thoenii strain P38				*P. thoenii* strain P4			
	Acetic acid (g/l)		Propionic acid (g/l)		Acetic acid (g/l)		Propionic acid (g/l)	
	I[a] Avg.	F[b] Avg.	I[a] Avg.	F[b] Avg.	I[a] Avg.	F[b] Avg.	I[a] Avg.	F[b] Avg.
Control[c]	ND	ND	0.34	0.48	1.66	1.69	4.39	4.84
Polypropylene	ND	ND	0.34	0.52	1.36	1.61	4.12	4.79
CS[d] + zein	ND	ND	0.32	0.53	0.19	0.64	1.04	1.58
CH[d] + zein	ND	ND	0.41	0.54	0.25	1.19	1.39	3.61
CH + SP[d]	ND	ND	0.27	0.46	1.13	1.70	2.85	4.82
CS + SP	ND	ND	0.45	0.49	1.03	1.61	2.58	4.92
SH[d] + SP	ND	ND	0.34	0.48	0.85	1.73	2.86	4.74
OH[d] + SP	ND	ND	0.35	0.96	0.89	1.74	2.47	4.76
Fire bricks	ND	ND	0.32	0.49	1.54	1.96	4.34	4.89

[a]I Avg.: Average final concentrations of the first three batches (second, third, and fourth day measurements; medium was changed every day).
[b]F Avg.: Average final concentrations of the last three batches (seventh, ninth, and eleventh day measurements; medium was changed every other day).
[c]Control is a cell-recycled free cell fermentation (cells are retained in the tube by centrifuging in between batches).
[d]CS: Corn starch; CH: Corn hull; SH: Soy hull; OH: Oat hull; SP: soy protein
ND: Not Detected.

Table 2. Gram staining results in repetitive batch experiments

	Color development with Propionibacteria strains				
SUPPORTS	P9	P200910	P127	P4	P20
Polypropylene	faded pink	light purple	light purple	–	light purple
Corn hull + zein	++	++	+++	+	+++
Corn starch + zein	+++	+++	+++	+	+++
Soy hull + zein	++	++	+++	+	+++
Oat hull + zein	++	++	+++	+	+++
Corn hull + soy protein	++	++	+++	+	+++
Corn starch + soy protein	++	++	+++	+	+++
Soy hull + soy protein	++	++	+++	+	+++
Oat hull + soy protein	++	++	+++	+	+++
Glass beads	–	–	light pink	–	light pink

\+ : slight blue color
++ : moderate blue color
+++ : dark blue color
– : no color change

Table 3. Acetic and propionic acid concentrations in continuous reactors with *P. thoenii* strain P127 and plastic-composites, fire bricks, and thimbles as supports.

	Free cells		PCS		Fire bricks		Thimbles	
D (h^{-1})	PA	AA	PA	AA	PA	AA	PA	AA
0.58	3.60	1.45	1.12	ND	4.17	1.64	3.90	1.66
1.15	ND	ND	1.48	ND	4.18	1.47	3.63	1.48
2.30	ND	ND	3.46	1.12	4.36	1.89	3.12	1.06
4.80	ND	ND	4.16	1.50	2.58	0.91	1.70	ND
7.20	ND	ND	3.15	2.45	1.94	ND	1.30	ND
8.64	ND	ND	ND	ND	1.81	ND	1.47	ND

D= Dilution rate, h^{-1}; PCS= Plastic-composite supports;

AA= Acetic acid concentration, g/l; PA= Propionic acid concentration, g/l;

ND: Not Detected

Table 4. Acetic and propionic acid concentrations in continuous reactors with *P. acidipropionici* strain P9 and plastic-composite supports (PCS) or fire bricks.

	PCS		Fire bricks	
D (h^{-1})	PA	AA	PA	AA
0.14	2.00	0.57	ND	ND
0.29	1.77	ND	ND	ND
0.58	1.70	1.40	2.26	ND
1.15	1.35	0.58	2.28	ND
2.30	1.10	0.49	2.68	0.85
4.80	ND	ND	3.70	1.43
7.20	ND	ND	3.16	1.44

D= Dilution rate, h^{-1}; PA= Propionic acid concentration, g/l; AA= Acetic acid concentration, g/l; PCS= Plastic-composite supports

ND: Not Detected

Table 5. Weight gain and clumping characteristics of various support materials with *P. thoenii* strain P127 in continuous fermentations.

SUPPORTS	WEIGHT GAIN (g) (Dry)	WEIGHT GAIN (g) (Wet)	CLUMPING
Polypropylene	0.06	4.30	–
Corn hull + zein	0.49	ND	+
Corn starch + zein	0.81	10.44	+++
Soy hull + zein	0.44	ND	++
Oat hull + zein	0.60	8.29	+
Corn hull + soy protein	0.40	8.40	+
Corn starch + soy protein	0.92	9.37	+++
Soy hull + soy protein	0.50	ND	+
Oat hull + soy protein	0.56	ND	++
Fire bricks	1.05	16.49	+++
Glass beads	0.10	2.20	–

ND : Not determined
\+ : slight clumping
++ : moderate clumping
+++ : strong clumping
– : no clumping

Table 6. Acetic and propionic acid concentrations in reactors with *P. thoenii* strain P20 and fire bricks and thimbles as supports.

	Fire bricks		Thimbles	
D (h^{-1})	PA	AA	PA	AA
0.58	3.83	1.45	3.93	1.59
1.15	3.81	1.36	3.01	1.03
2.30	3.29	1.23	3.33	1.29
4.80	2.48	0.86	2.33	0.89
7.20	2.16	ND	2.04	ND
8.64	2.55	ND	2.22	ND

D= Dilution rate, h^{-1}; PA= Propionic acid concentration, g/l; AA= Acetic acid concentration, g/l

ND: Not Detected

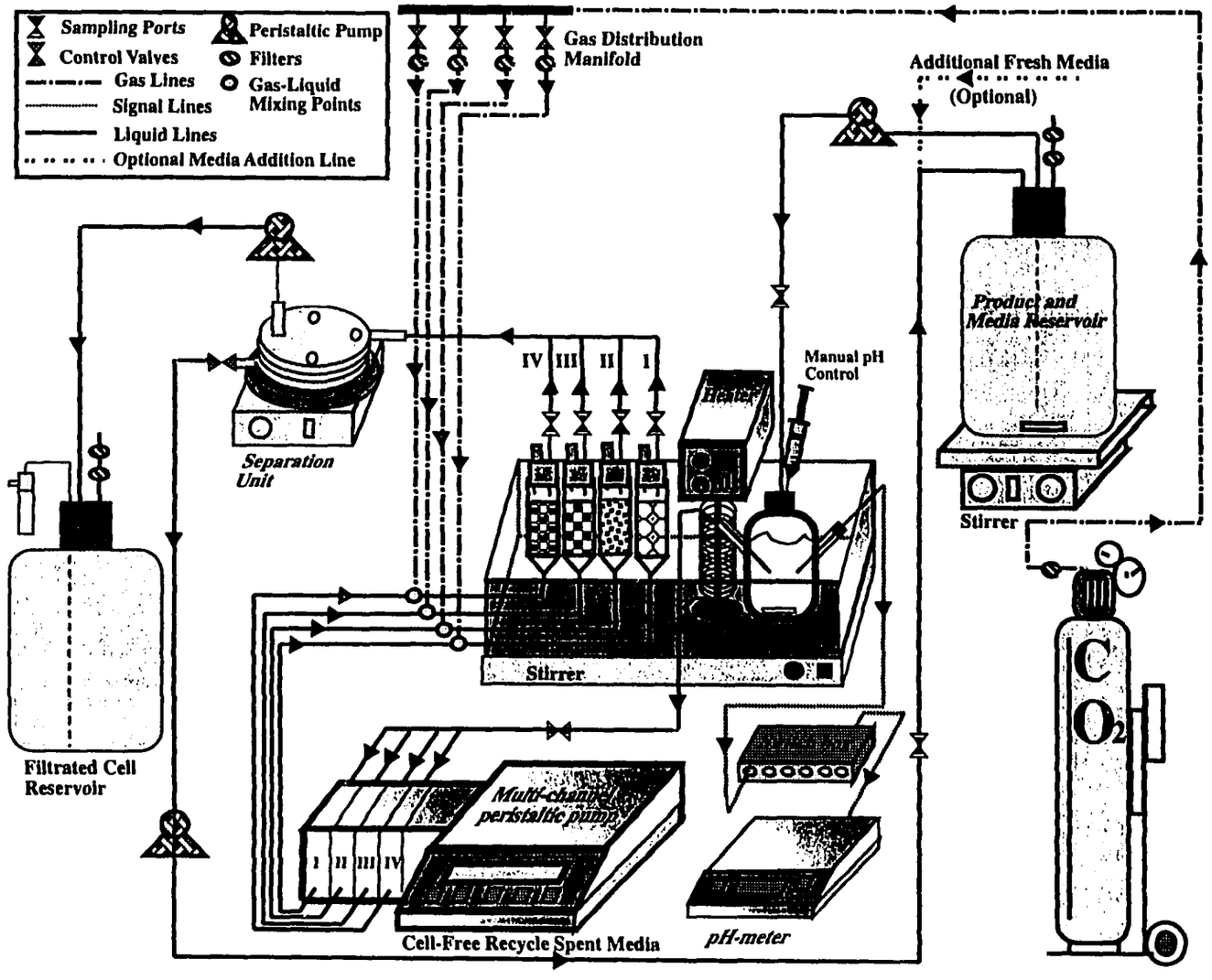


Figure 1. Schematic representation of continuous biofilm fermentations.

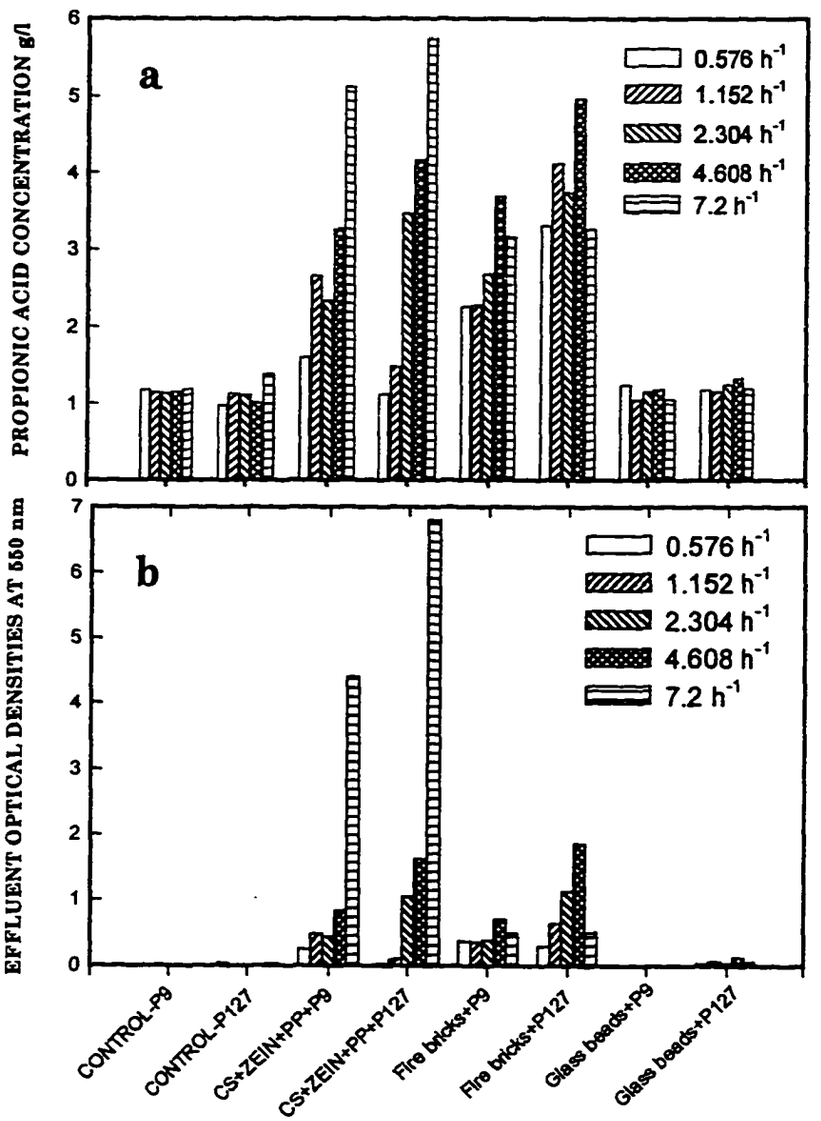


Figure 2. Comparison of *P. acidipropionici* P9 and *P. thoenii* P127 strains for (a) propionic acid production and (b) microbial growth in continuous biofilm reactors with several support materials. CS+ZEIN+PP: Support consists of corn starch, zein, and polypropylene.

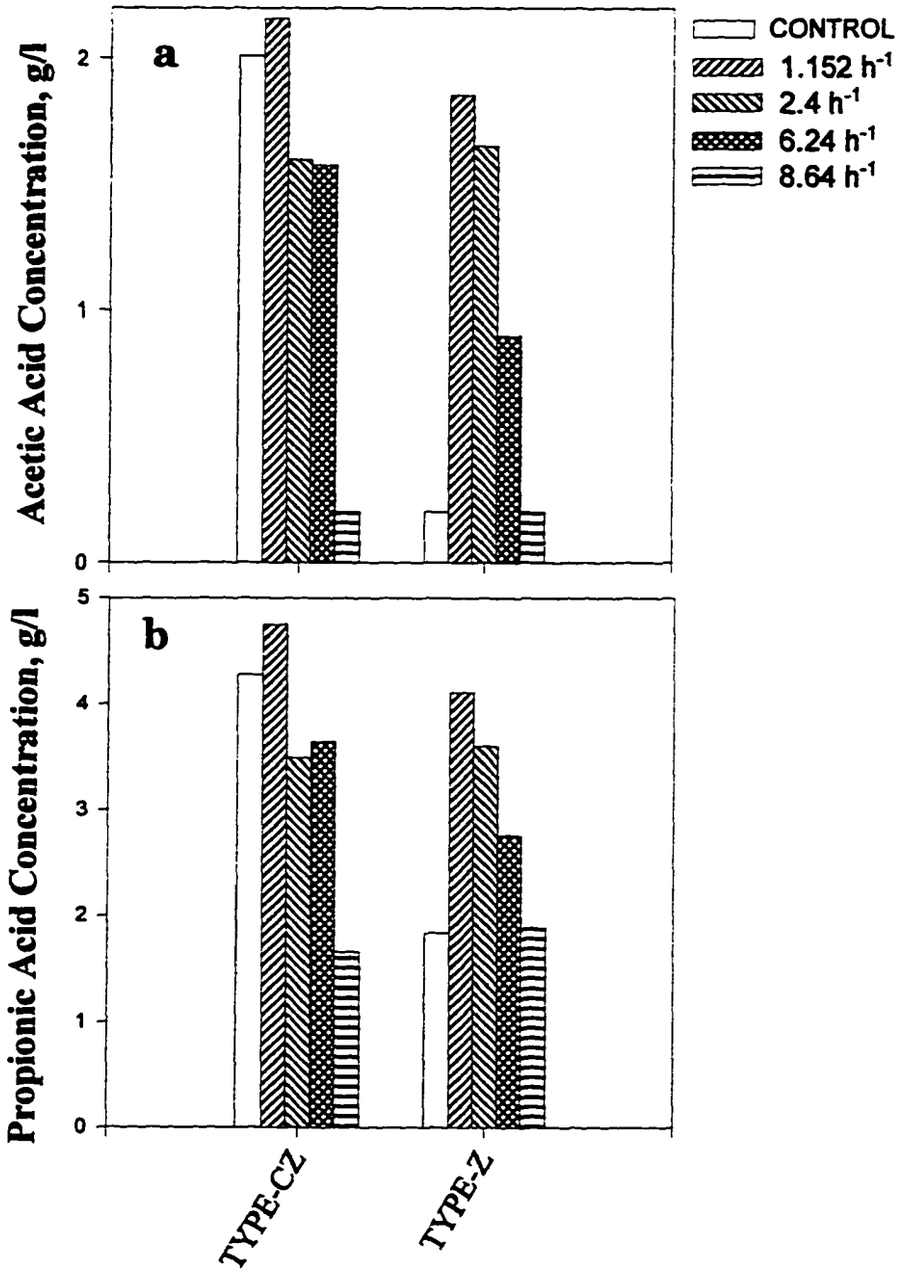


Figure 3. Commercial support materials, Type-Z and Type-CZ Grace biocarriers, for continuous (a) acetic and (b) propionic acid production.

Figure 4. Scanning Electron Microscopy (SEM) pictures for fire brick supports: A) Fire brick surfaces before biofilm formation. Magnification: X3,000; bar=5μm. B) Fire brick surface after 24-h biofilm formation. Magnification: X3,000; bar=5μm. C) Fire brick surfaces after full process of biofilm formation. Magnification: X470; 30μm. D) Fire brick surfaces after 24-h biofilm formation. Magnification: X1,000; bar=15μm.

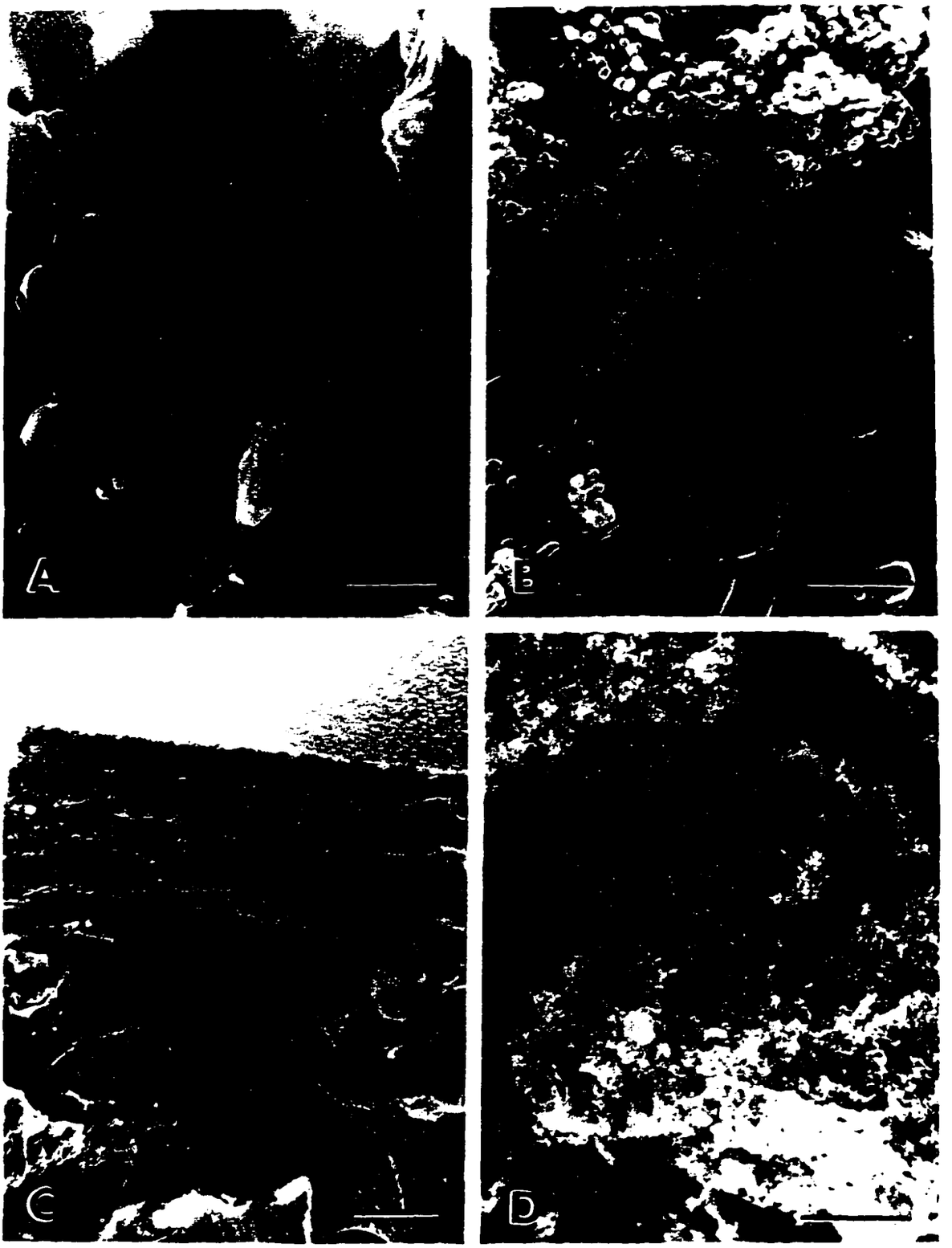

A
B
C
D

Figure 5. Scanning Electron Microscopy (SEM) pictures for thimbles: A) Thimbles before biofilm formation. Magnification: X440; bar=20μm. B) Thimbles after 24-h biofilm formation. Magnification: X4,000; bar=5μm. C) Thimbles after full process of biofilm formation. Magnification: X480; 20μm. D) Thimbles after 24-h biofilm formation. Magnification: X400; bar=30μm.

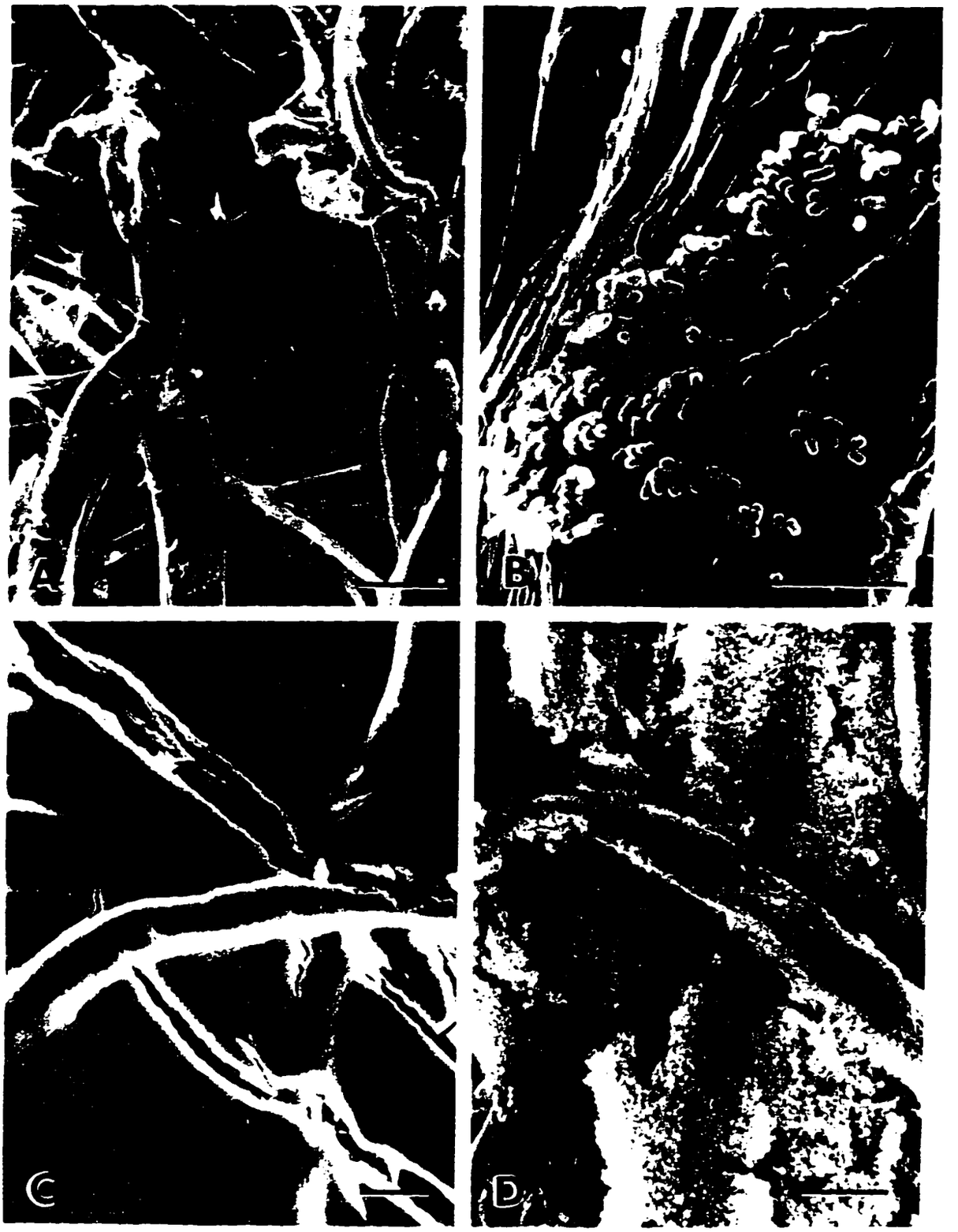
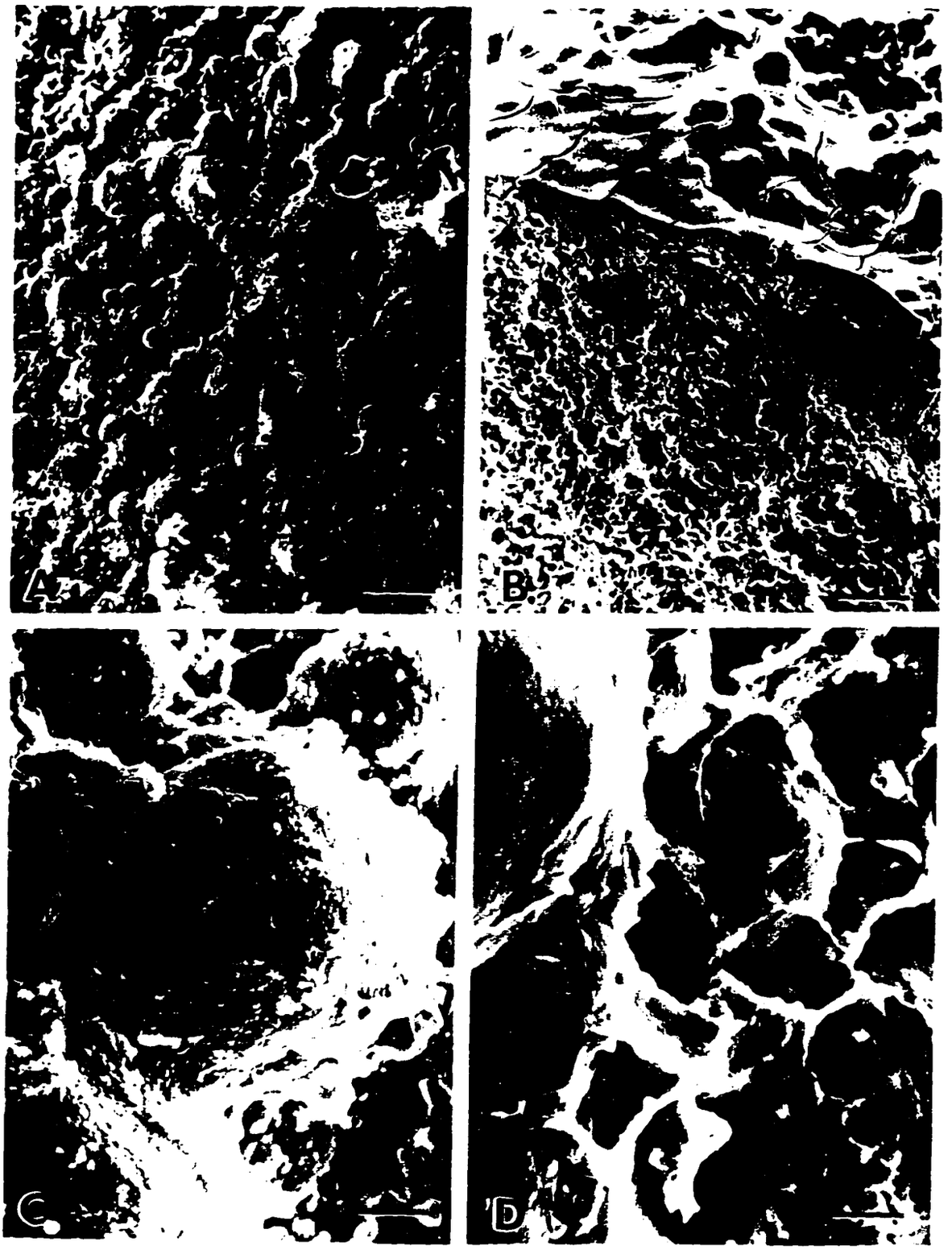

A
B
C
D

Figure 6. Scanning Electron Microscopy (SEM) pictures for Grace biocarriers, Type-Z and Type-CZ: A) Type-Z biocarrier before biofilm formation. Magnification: X100; bar=100μm. B) Cross-sectional view of the Type-Z biocarrier after 24-h biofilm formation. Magnification: X50; bar=200μm. C) Type-CZ biocarrier before biofilm formation. Magnification: X480; 20μm. D) Type-CZ biocarrier after 24-h biofilm formation. Magnification: X480; bar=20μm.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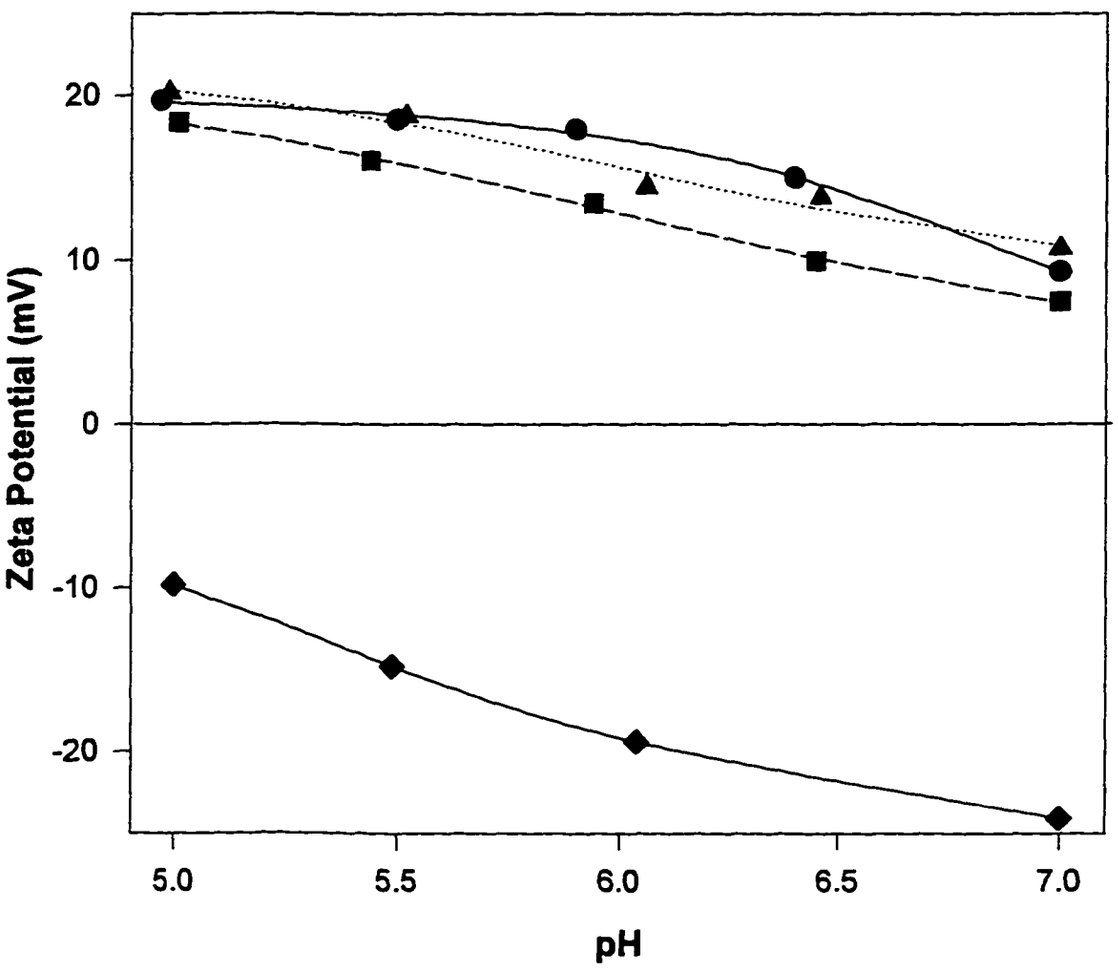

Figure 7. Effect of pH on zeta potentials of some support materials and *P. thoenii* P20. ● Fire bricks (before biofilm formation), ▲ Fire bricks after full process of biofilm formation and dehydration, ■ TYPE-Z Grace biocarriers, ◆ *P. thoenii* P20.

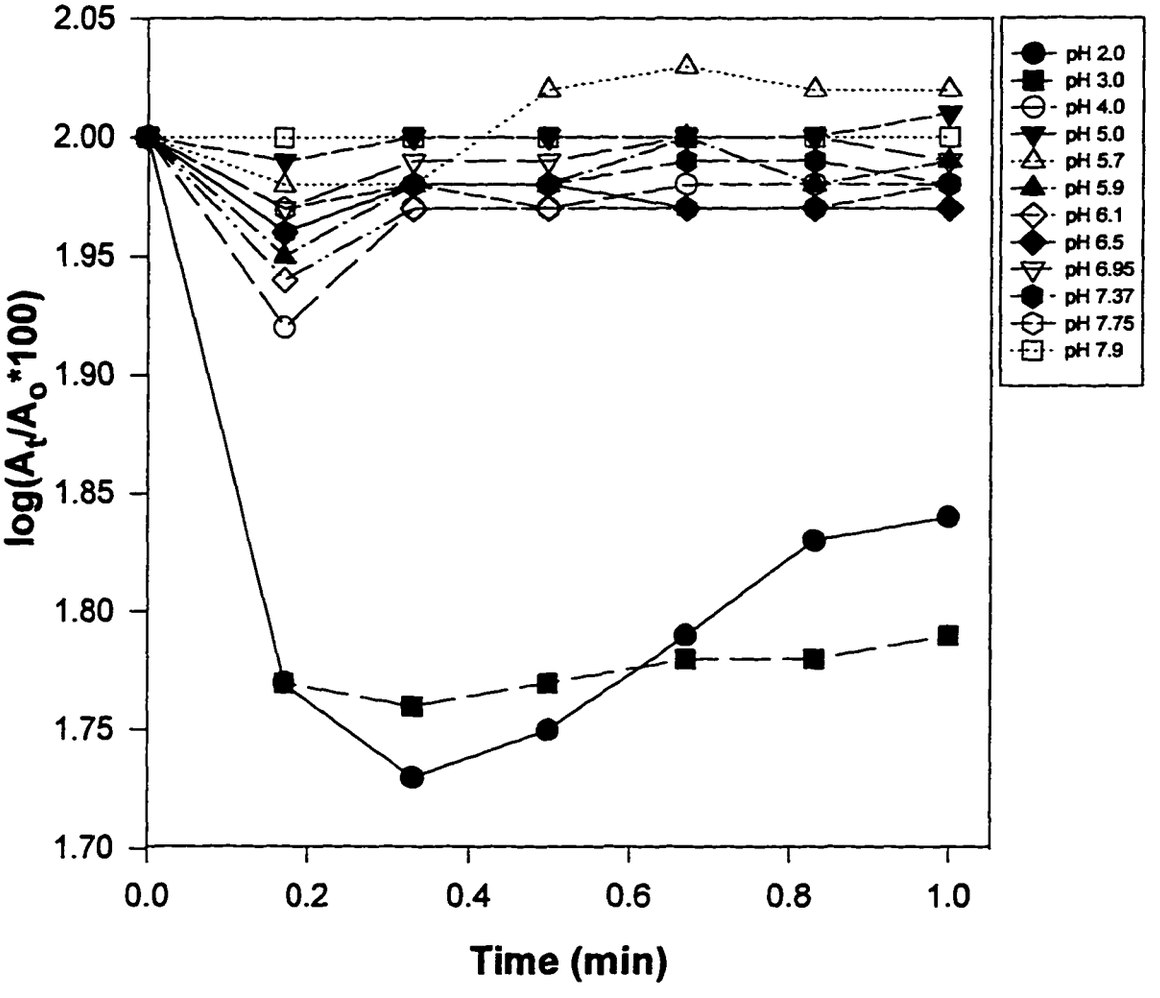

Figure 8. Application of MATH test on *P. thoenii* P20 at different pH levels. Symbols indicate the changes of optical density in time in the aqueous phase. A_t : absorbance of the aqueous phase at given time, A_o : Initial absorbance at zero time.

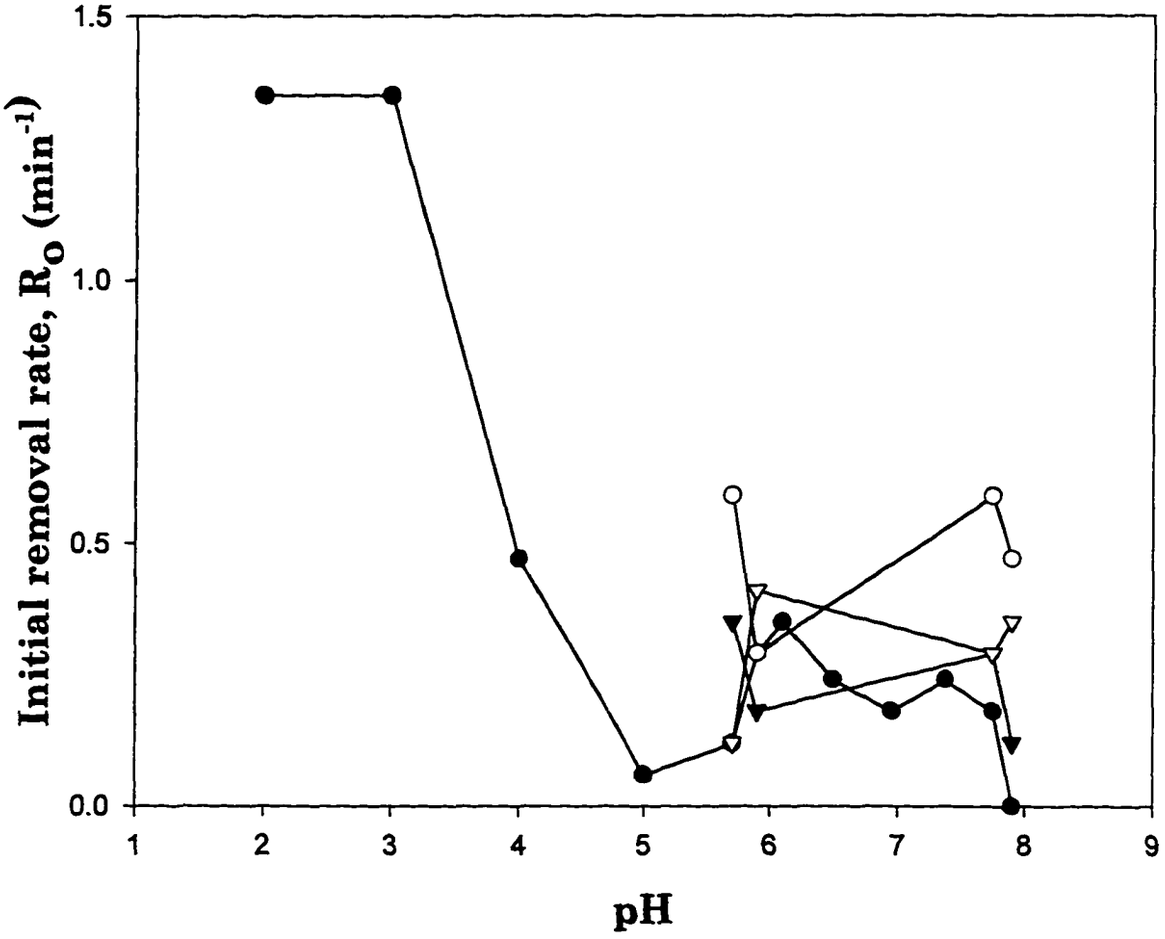

Figure 9. Initial removal rates (Ro) of MATH test for the supports and *P. thoenii* P20. ● *P. thoenii* P20; O Type-CZ biocarrier; ▼ Type-Z biocarrier; ∇ Fire bricks

CHAPTER 3. FED-BATCH PROPIONIC AND ACETIC ACID FERMENTATIONS IN NOVEL BASKET BIOFILM REACTORS WITH MODIFIED FIRE BRICK SUPPORT MATERIALS

A paper to be submitted to Biotechnolgy & Bioengineering

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Abstract

A biofilm is a form of natural cell immobilization on solid surfaces. Modified fire brick support materials (BioLifeSavers - patent pending) in novel basket bioreactors (BioCage - patent pending) were designed and tested specifically for the improvement of propionic and acetic acid production by immobilized propionibacteria. Problems of agitation, pH control, and homogeneous mixing were solved in the packed bed immobilized system. For all systems *Propionibacterium thoenii* strain P20 was used as the biofilm former and acid producer in a repetitive fed-batch fermentation system. The biofilm fermentations in the basket reactor were characterized by constant or increasing yield and productivity values in consecutive batches, resistance to process upsets, and long-term biofilm stability. Average yield and productivity values for acetic and propionic acid over four consecutive batches were 27%, 0.1 g/l/h and 71%, 0.26 g/l/h, respectively.

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Introduction

Propionic acid is a three-carbon fatty acid known as a natural mold inhibitor; it also has several uses as an industrial chemical. Propionic acid is made commercially by the oxidation of liquid phase propane or propionaldehyde, but acetic and propionic acids may also be produced biologically by the fermentation of sugars by various bacteria, especially the propionibacteria (1, 17).

The long-term goal of this study is to improve the economics of production of propionic acid by fermentation; one strategy is to develop appropriate platforms for downstream processing. The use of novel biofilm reactors to maintain high cell concentrations in the fermenter can help lower fermentation costs and increase productivity and yield. It may also help the recovery of the valuable acids (7).

Many microorganisms, primarily bacteria, tend to adsorb to and colonize surfaces submerged in aquatic environments. A biofilm is a natural form of cell immobilization that results from microbial attachment to solid support. Biofilms as a natural form of cell immobilization are dynamic micro-environments, encompassing processes such as metabolism, growth, and product formation, and finally detachment, erosion, or "sloughing" of the biofilm from the surface (4).

Fixed-film or biofilm systems, which are generally packed-bed systems filled with various support materials such as stoneware or plastic packing, are seeded once during their startup period and are generally operated upflow to increase contact time and to permit concurrent flow of liquids and gases. In a biofilm system, the film affords the bound organisms some protection from toxic materials and sudden changes in the feed (11).

A number of groups have investigated production of various organic acids and ethanol by immobilized cells (6, 8, 9, 12, 15, 16, 23, 27, 29, 30); some have explored the production of propionic acid by biofilms (7, 18, 33, 34).

Immobilization baskets have been designed and commercialized for mammalian cell systems (New Brunswick Scientific product catalog). The design criteria of a mammalian cell bioreactor include the efficient supply of sufficient oxygen to the culture for the survival and growth of the cells, and minimization of shear caused by agitation, sparging and bubble break-up (14, 21, 31).

One of the major problems of immobilized cell systems is the control of system parameters such as pH and agitation. Especially in packed-bed systems, uniform distribution and diffusion of the nutrient and the control of pH can be problematic. Another problem is the density differences between the reactor medium and the support materials. In some fluidized systems, floating supports may not only cause plugging problems but may also need additional agitation to suspend the supports uniformly in the medium.

To overcome some of the problems mentioned above, a novel bioreactor accessory, BioCage, was developed and tested along with fire brick supports (see Chapter 2 for the detailed explanation about fire brick supports) for propionic and acetic acid production by *P. thoenii* strain P20 in repetitive fed-batch biofilm fermentations.

Materials and Methods

Microorganisms and media

Propionibacterium thoenii strain P20 was obtained from the culture collection of the Department of Food Science and Human Nutrition, Iowa State University. Sodium lactate broth (NLB) contained 1% (v/v) sodium lactate 60% syrup (Fisher Scientific Co., Pittsburgh, PA), 1% (w/v) yeast extract (Difco Laboratories, Detroit, MI), and 1% (w/v) trypticase soy broth (Baltimore Biological Laboratories, BBL, Cockeysville, MD). Working cultures were maintained at 4°C on sodium lactate agar (NLA) plates as previously described by Woskow and Glatz (32).

When lactic acid consumption slowed, fermentation medium was supplemented with concentrated trypticase soy broth and yeast extract to reach a final concentration of 1% (w/v) of the total working volume for each to replenish the initial concentrations of these nutrients.

Support materials

Fire bricks (Al_2O_3) were obtained from the Department of Material Science and Engineering, Iowa State University and were first cut into 6x1.5-cm cylindrical pieces and then into 1.5-1.8-cm pieces with 6-7-mm central holes to make BioLifeSavers (patent pending).

Basket reactor insert

A multi-functional basket (BioCage - patent pending) was designed as a bioreactor insert (12 cm diameter) for immobilized cell systems and was constructed at ERI Technical Services, Iowa State University. The basket has four separate compartments each with its own lid (Figure 1A & 1B). Compartments were made by rolling and/or cutting from a plate of 304-stainless steel with 4.65 mm holes. Compartments were welded to a central stainless steel tube (made from the same material - 4 cm diameter) through which a shaft with 2 impellers was extended to achieve mixing of incoming acid/base and recycled broth. Control probes for pH and temperature were inserted into vertical slots (2.5 cm wide) between compartments. Agitation was achieved by the action of impellers at the bottom of the vessel, in the central tube, and by bubbling carbon dioxide gas into the bottom of the vessel.

Repetitive fed-batch fermentations with the basket bioreactor

Repetitive fed-batch fermentations were conducted in a 2-L bench-top fermenter model Biostat M (B. Braun Biotech. Inc., Allentown, PA). Working volume was 1700 ml with an empty basket present, and 1575 ml with the basket filled with fire brick supports. Figure 2 shows a schematic of the fermentation set-up.

Fed-batch fermentations were begun as in batch mode with medium containing 1% (v/v) of 60% sodium lactate syrup. Temperature, pH, and agitation set points were 32±0.17°C, 6.9±0.27, and 150±8 rpm, respectively. After 48 h of batch incubation, fed-batch operation was started. Approximately 20 ml of 60% sodium lactate syrup was added to the fermenter at about 12-h intervals. Samples of approximately 10-ml volume were taken before and after each substrate addition. Fermentation broth was continuously recycled at the rate of 16 ml/min through a peristaltic pump from two merged effluent streams through a small tube into the basket's central tube. Sampling and medium exchange ports were located on the recycling stream. When rate of lactic acid consumption slowed in the repetitive fed-batch fermentations, the spent medium was aseptically exchanged with fresh NLB.

Biofilm evaluations

Biofilm formation on the supports and the basket was evaluated by measuring weight change and plating for viable cells. Biofilm samples from

several locations on the surface of the basket were aseptically scraped with a spatula and weighed in sterile test tubes. Samples taken from the basket surfaces as well as free cells were plated onto NLA and incubated anaerobically for four days to obtain viable cell counts. Percent dry matter was calculated after drying biofilm samples overnight in a vacuum oven at 70°C.

Analytical methods and calculations

The suspended free cell density in the reactors was measured by absorbance at 550 nm (Spectronic 20 spectrophotometer, Milton Roy, Rochester, NY). Concentrations of glucose and lactic, acetic, and propionic acids were determined by high-performance liquid chromatography (HPLC) as previously described (22). Average percent deviations for glucose, lactate, acetate, and propionate over three sets of replicate injection data were 3.8, 2.4, 3.2, and 1.5%, respectively. L-lactic acid and D-glucose concentrations also were followed by means of a YSI Model 2700 Select Biochemistry Analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) with dextrose (D-glucose - glucose oxidase) and L-lactate (L-lactic acid - L-lac oxidase) membranes.

The percent yield, a measure of the conversion efficiency of lactic acid to propionic and acetic acids, was calculated as grams of propionic or acetic acid produced divided by grams of lactic acid consumed times 100. Based on the dicarboxylic acid pathway, the theoretical yield for propionic acid production from lactate is 55% (5, 25, 26), with a propionate to acetate ratio of 2:1. The

productivity (g/l/h) is a measure of propionic or acetic acid production per hour (calculated as propionic or acetic acid produced in g/l divided by the elapsed fermentation time). Nonlactate nutrients such as trypticase soy broth and yeast extract may also contribute to the overall productivity and yield, but were not included in these calculations.

Results and Discussion

After identification of the best support-strain combination for propionic and acetic acid production in biofilm systems (Chapter 2), the fermentation process was scaled up to 2 liters in fed-batch mode. In initial trials the packed supports in the reactor did not allow sufficient agitation, and problems with mixing and pH control resulted (data not shown).

Previously in our laboratory fed-batch fermentations were conducted for propionic and acetic acid production with free cells of strain P9 (22, 24), semi-continuous fermentations with free cells of strain P200910 (32), and batch, repeated-batch, and fed-batch fermentations with immobilized P200910, P127, and P20 cells in calcium alginate beads (23, 28). In the current study the performance of naturally immobilized (biofilm) cells of strain P20 in repetitive fed-batch fermentation in the basket reactor was tested.

The basket reactor was first tested in a fed-batch fermentation without any support materials in it (Figure 3). Broth was continuously circulated to achieve better mixing and to provide the type of flow across surfaces that

stimulates biofilm formation. As in previous studies, CO_2 gas was bubbled into the reactor to provide better flow regime and to increase agitation. After about 200 h of incubation, substrate consumption slowed. Even though acid production continued, substrate consumption dropped dramatically. This drop may be attributed to the accumulation of inhibitory propionic acid and other by-products in the fermentation medium, as well as to depletion of nutrients.

Propionic acid productivities throughout the process were calculated first by curve fitting propionic acid concentration versus time and then derivatizing this polynomial equation to find productivity values (Figure 4). A total of 145 g of lactic acid was fed over 14 feeding periods. Average yield and productivity values were 24% and 0.06 g/l/h for acetic acid and 59.5% and 0.15 g/l/h for propionic acid, respectively.

At the end of the experiment, the basket was covered with a very thick, red, hair-like biofilm which was much thicker at the lower sections. The amount of the biofilm accumulated on the surface of the basket was about 17 g. Biofilm formed only on the outside surface of the basket. This may be due to centrifugal forces created by the central impellers. The empty basket provided a good surface for biofilm formation.

To measure reproducibility of the HPLC method, values for two samples taken at the same time (before and after each feeding) were compared. The average deviation for propionate was about 1.8% over thirteen data sets.

Next, the basket (empty weight = 585.5 g) was filled with fire brick support materials (total of 160 g in four compartments) and sterilized before filling with sterile media. Fermentation was started with a 3% (v/v) inoculum and incubated as a batch for 48 h before lactate feedings were started. After about 170 h of incubation, substrate consumption slowed. At this point, concentrated trypticase soy broth and yeast extract were added to a final concentration of 1% (w/v) of the working volume in the fermenter. After this nutrient addition, substrate consumption rates returned to higher levels.

Rather than supplementing with the basal medium to replenish other nutrients, a repetitive fed-batch system was used for the following batches. When the rate of acid production was seen to slow, the entire volume of medium was removed and replaced with fresh medium. Since the biofilm was already established in the system, lactate feeding was continued with the same frequency in each new batch. However, carry-over acid amounts appeared in consecutive batches due to physical entrapment of acids in the wet biofilm and leftover broth in the curved-bottom reactor. In calculations for yield and productivity values, the carry-over amounts were corrected for. Patterns of substrate utilization and acid production over six repeated batches are shown in Figure 5.

At the end of the first batch, acetic and propionic acid amounts in 1.575 liter working volume were about 31.5 g and 80 g, respectively. Total lactic acid used in the first batch as carbon source was 163 g over 14 feeding periods. The

accumulated propionic and acetic acids were 194 g and 76 g, respectively, over six repetitive batches; a total of 372 g of lactic acid was consumed. Final acetic and propionic acid concentrations in successive batches were lower, because of shorter fermentation time, less substrate added per batch, and pH change from 7.0 to 5.5 after the first three batches. Yields and productivity values over the repeated batch fermentations are presented in Table 1. These values were constant or even increased in each pH group (5.5 and 7.0), even when the pH of the last 3 batches was reduced to 5.5. This pH was tested because the extractants used to recover the product acids are most effective for acids in the undissociated form (22).

The first batch at pH 5.5 had low acid productivities, probably because of the sudden change in medium acidity. However, reactor performance returned to previous values in the next batch at pH 5.5. Even though the third batch in this pH group had to be terminated early because of other technical difficulties, it showed similar trends to the second batch at pH 5.5.

Upon termination of the sixth batch, the basket with supports was dried and weighed. The accumulated biofilm weighed 35.4 g. Some of the biofilm formed on the basket itself; the rest formed on the surfaces of the fire brick supports. Given the amount of biofilm that developed on the empty basket in the previous experiment, it is estimated that about half of the weight gain was from the biofilm formed on the support materials. Fire brick support materials were also tested for their clumping characteristics. After drying they seemed

glued to each other and could be separated only if vigorous external force was applied. They did separate if soaked in water.

Viable cell determinations were made on the fermentation medium, to estimate free cell concentrations, and on the biofilm. Biofilm samples were taken from eight different locations on the basket. Free cell concentrations were between 1.2 x 10^9 and 4.6x10^9 cells/ml. Viable cells per g of biofilm (wet weight) ranged between 1.9 x 10^9 and 4.6 x 10^9. The question can be asked reasons why free cell and immobilized cell concentrations were so similar, when it would be expected that immobilized cells would be more concentrated. Since these measurements were made at the end of the process, the biofilm mass may contain large numbers of injured and/or dead cells. A large percentage of the biofilm mass also could be extracellular polysaccharide.

Different layers of the biofilm might have different microbial composition through formation of new layers during the fermentation process. Biofilm layers were thicker at the bottom of the basket than at the top. This may be due to differences in mixing at different locations and/or to gravitational forces. Biofilm samples were dried overnight to estimate the dry weight, which would include cells plus other biofilm components such as extracellular polysaccharides. This was found to be 23% (w/v) of the original wet weight.

Stability of the biofilm and consistency of performance were tested over four consecutive fed-batches with no environmental changes (Figure 6). Rates and amounts of acid production were very consistent. Yields and productivities

for acetic and propionic acid were also consistent (Table 2). The basket was covered with a thick, red, hair-like biofilm, which seemed to be very stable to the disruption caused by medium exchanges, gas bubbling, agitation, and prolonged incubation.

When propionic acid production in the first 400 h of experiments are compared (see Figures 5 and 6), some major differences can be observed. Eighty grams propionic acid were produced after a single batch in 370 h (Figure 5), whereas 178 g propionic acid were produced after three batches in 423 h (Figure 6). Immobilized cells were retained in the fermenter from one batch to another and resumed acid production with little or no lag phase, while culture medium exchange solved problems of nutrient depletion and accumulation of waste products.

Others have reported immobilized-cell fermentations for propionic acid production. Vorob'eva et al. (30) immobilized *Propionibacterium shermanii*, *P. technicum*, and *P. arabinosum* in polyacrylamide gels; they did not report individual acid concentrations. However, Paik and Glatz (23) estimated the highest concentration of volatile acids in this study to be 7.9 g/l in 200 h batch culture. In another study Champagne et al. (3) entrapped *P. shermanii* in alginate for 24 h in neutralized lactobacilli-fermented whey, and reported propionic acid concentration and volumetric productivity values of 8 g/l and 0.23 g/l/h, respectively.

Jain et al. (12) immobilized *P. shermanii* on an inert support to produce propionic acid from cheese whey in batch process. They obtained 11.5 g/l propionic acid in 161 h with 2% (w/v) $CaCO_3$ addition. However, propionic acid concentration in 48 h was only about 8 g/l.

The closest study to the current one is probably that of Dr. Yang's group at The Ohio State University. They have immobilized *P. acidipropionici* in spiral-wound fibrous bed bioreactors in continuous and recycle batch fermentations and have used whey (lactate) as the substrate (18, 33, 34). They obtained about 20 g/l propionic acid from 40 g/l lactate (concentration in the feeding stream) at a dilution rate of 1 day^{-1}. The reactor was stable to low-pH conditions without much loss in reactor productivity. In recycled batch fermentation with immobilized cells of *P. acidipropionici*, they obtained 65 g/l (ca. 224 g) propionate from 195 g/l (ca. 673 g) initial lactose concentration in 285 h (33). Propionate yield and productivity were 40% and 0.23 g/l/h, respectively. However, average propionate yield and productivity over nine recycle batch whey fermentations were found as 49.5% and 0.3 g/l/h, respectively. When we compare our results with their best batch results among nine batches (33) after 280 h fermentation, our results show higher yield (avg. 71%) and propionate production (ca. 120 g) by consuming 169 g lactic acid.

Boyaval et al. (2) used a continuous bioreactor coupled with a UF membrane unit for the fermentation of glycerol by *P. thoenii*. They also performed fed-batch experiments with highest reported propionate productivity

of 0.3 g/l/h and average volumetric productivity of 0.12 g/l/h over four glycerol addition cycles. Over more than 300 h of fed-batch fermentation, maximum propionic acid concentration reached was about 38 g/l (ca. 46 g) at the end of the third addition of glycerol. Total glycerol used was 91 g/l (ca. 109 g).

The basket insert for bioreactors should be useful for cultivation of microbial and mammalian cells. Addition of this stainless steel module to the bioreactor gives more flexibility for controlling system parameters such as pH and agitation for biofilm or anchorage-dependent cells. Any amount of support material can be added to the four compartments; mixing of incoming (or circulated) feed, acid, or base in the central tube reduces fluctuations experienced by biofilm or attached cells. Inserts with walls of smaller mesh size can be placed into compartments, to hold smaller supports or calcium alginate beads. The biofilm formed only on the outside of the empty basket.

For biofilm formation a key concept is "stress." Under stress conditions cells develop special characteristics such as formation of secondary metabolites. Increased stress level on the cells is thought to trigger chemical communication among bacteria and eventually induce formation of the biofilm (19, 20). Flow can be one of the factors causing stress on the organisms. In nature, organisms form biofilms on rocks in rivers to protect themselves from turbulent flow. Biofilms also show improved resistance, compared to free cells, to stress conditions such as sanitizers, sudden temperature or pH changes (10, 13). Organisms in the biofilm matrix influence each other, and extracellular

materials that contribute to biofilm formation can serve as protection for the cells.

In summary, the novel basket aids immobilized cell fermentations by providing easier control of parameters such as pH and agitation. Current design of this basket can be used for both natural (biofilm) or artificial (entrapped) immobilization techniques. Modified fire bricks as support materials for microbial colonization have the advantages of inexpensive source, efficient shape, durability, and suitable surface characteristics.

Comparisons of biofilms with cells immobilized by other methods (e.g. calcium-alginate entrapment) for rate of acetic and propionic acid production and response to changing acid concentrations should be made in small reactors, to determine which immobilization method should be preferred.

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References

1. **Babuchowski, A., E. G. Hammond, and B. A. Glatz.** 1993. Survey of propionibacteria for ability to produce propionic and acetic acids. J. Food Prot. **56**:493-496.

2. **Boyaval, P., C. Corre, and M-N. Madec.** 1994. Propionic acid production in a membrane bioreactor. Enzyme Microb. Technol. **16**:883-886.

3. **Champagne, C. P., C. Baillargeon-Cote, and J. Goulet.** 1989. Whey fermentation by immobilized cells of *Propionibacterium shermanii*. J. Appl. Bacteriol. **66**:175-184.

4. **Characklis, W. G. and K. C. Marshall.** 1990. Biofilms. John Wiley & Sons, Inc., New York.

5. **Clausen, E. C.** 1978. The kinetic and economic effects of separating the stages of the anaerobic digestion process. Ph.D. dissertation, University of Missouri-Rolla.

6. **Clausen, E. C. and J. L. Gaddy.** 1984. Organic acids from biomass by continuous fermentation. Chem. Eng. Prog. 12:59-63.

7. **Crespo, J. P. S. G., J. S. Almeida, M-J. Moura, and M. J. T. Corrondo.** 1990. Modelling of immobilized cell reactor for propionic acid fermentation. Biotechnol. Bioeng. **36**:705-716.

8. **Demirci, A., A. L. Pometto III, and K. E. Johnson.** 1993a. Evaluation of

biofilm reactor solid support for mixed-culture lactic acid production. Appl. Microbiol. Biotechnol. 38:728-733.

9. **Demirci, A., A. L. Pometto III, and K. E. Johnson.** 1993b. Lactic acid production in a mixed-culture biofilm reactor. Appl. Environ. Microbiol. 59:203-207.

10. **Frank, J. F. and R. A. Koffi.** 1990. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. J. Food Protection. 53:550-554.

11. **Griffith, W. L. and A. L. Compere.** 1974. A new method for coating fermentation tower packing so as to facilitate microorganism attachment. Dev. Ind. Microbiol. 17:241-246.

12. **Jain, D. K., R. D. Tyagi, D. Kluepfel, and T. J. Agbebavi.** 1991. Production of propionic acid from whey ultrafiltrate by immobilized cells of *Propionibacterium shermanii* in batch process. Process Biochem. 26:217-223.

13. **Krysinski, E. P., L. J. Brown, and T. J. Marchisella.** 1992. Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. J. Food Prot. 55:246-251.

14. **Kunas, K. T. and E. T. Papoutsakis.** 1990. Damage mechanisms of suspended animal cells in agitated bioreactors with and without bubble entrainment. Biotechnol. Bioeng. 36:476-483.

15. **Kunduru, M. R. and A. L. Pometto III.** 1996a. Evaluation of plastic-composite supports for enhanced ethanol production in biofilm reactors. J. Ind. Microbiol. **16**:241-248.

16. **Kunduru, M. R. and A. L. Pometto III.** 1996b. Continuous ethanol production by *Zymomonas mobilis* and *Saccharomyces cerevisiae* in biofilm reactors. J. Ind. Microbiol. **16**:249-256.

17. **Lee, I. H., A. G. Fredrickson, and H. M. Tsuchiya.** 1974. Diauxic growth of *Propionibacterium shermanii*. Appl. Microbiol. **28**:831-835.

18. **Lewis, V. P. and S-T. Yang.** 1992. Continuous propionic acid fermentation by immobilized *Propionibacterium acidipropionici* in a novel packed-bed bioreactor. Biotechnol. Bioeng. **40**:465-474.

19. **Lipkin, R.** 1995a. How patterns reveal clues about bacteria's chemical communication. Science News. **147**:136-137, 142.

20. **Lipkin, R.** 1995b. Stressed bacteria spawn elegant colonies. Science News. **145**:167.

21. **Oh, S. K. W., A. W. Nienow, M. Al-Rubeai, and M. Emery.** 1989. The effects of agitation intensity with and without continuous sparging on the growth and antibody production of hybridoma cells. J. Biotechnol. **12**:45-60.

22. **Ozadali, F., B. A. Glatz, and C. E. Glatz.** 1996. Fed-batch fermentation

with and without on-line extraction for propionic and acetic acid production by *Propionibacterium acidipropionici*. Appl. Microbiol. Biotechnol. 44:710-716.

23. **Paik, H-D. and B. A. Glatz.** 1994. Propionic acid production by immobilized cells of a propionate-tolerant strain of *Propionibacterium acidipropionici*. Appl. Microbiol. Biotechnol. 42:22-27.

24. **Park, S-K.** 1991. Production of propionic acid by *Propionibacterium acidipropionici* in various fermentation processes. M.S. thesis, Iowa State University, Ames, IA.

25. **Playne, M. J.** 1985. Propionic and butyric acids. *In*: Murray, Moo-Young (ed.) Comprehensive biotechnology: the principles, application and regulations of biotechnology in industry, agriculture, and medicine. Pergamon Press, Oxford, pp 731-759.

26. **Prescott, S. C. and C. G. Dunn.** 1949. Industrial microbiology, 2nd ed. McGraw-Hill, New York.

27. **Qureshi, N. and I. S. Maddox.** 1995. Continuous production of acetone-butanol-ethanol using immobilized cells of *Clostridium acetobutylicum* and integration with product removal by liquid-liquid extraction. J. Ferm. Bioeng. 80:185-189.

28. **Rickert, D. A.** 1996. Evaluation of efforts to improve organic acid production by calcium alginate-immobilized propionibacteria. M.S. thesis. Iowa State University, Ames, IA.

29. **Sitton, O. C. and J. L. Gaddy.** 1980. Ethanol production in an immobilized-cell reactor. Biotechnol. Bioeng. **22**:1735-1748.

30. **Vorob'eva, L. I., M. A. Alekseeva, I. G. Surkhova, and V. I. Gaitan.** 1977. Formation of volatile acids by immobilized cells of propionic acid bacteria. Appl. Biochem. Microbiol. **13**:416-421.

31. **Wang, S. J., J. J. Zhong, Y. L. Chen, and J. T. Yu.** 1995. Characterization and modeling of oxygen transfer in a 20-l modified cell-lift bioreactor with a double-screen cage. J. Ferm. Bioeng. **80**:71-77.

32. **Woskow, S. A. and B. A. Glatz.** 1991. Propionic acid production by a propionic acid-tolerant strain of *Propionibacterium acidipropionici* in batch and semicontinuous fermentation. Appl. Environ. Microbiol. **57**:2821-2828.

33. **Yang, S-T., Y. Huang, and G. Hong.** 1995. A novel recycle batch immobilized cell bioreactor for propionate production from whey lactose. Biotechnol. Bioeng. **45**:379-386.

34. **Yang, S-T., H. Zhu, Y. Li, and G. Hong.** 1994. Continuous propionate production from whey permeate using novel fibrous bed bioreactor. Biotechnol. Bioeng. **43**:1124-1130.

Table 1. Productivity and yield coefficient values for acetic and propionic acid production in repetitive fed-batch biofilm fermentations with the basket and fire brick support materials at different pH values.

				Acetic acid			Propionic acid		
Batch #	Time (h)	pH	LA	AA	Y_{AA}	P_{AA}	PA	Y_{PA}	P_{PA}
1	367.0	6.9	103.6	19.2	18.5	0.05	50.5	48.7	0.14
2	155.0	6.9	41.4	9.3	22.4	0.04	23.0	55.6	0.15
3	75.0	6.9	35.6	6.7	18.8	0.03	18.2	51.0	0.24
4	85.5	5.5	20.1	3.4	16.7	0.02	8.2	40.8	0.10
5	95.5	5.5	26.3	6.7	25.6	0.03	17.0	64.4	0.18
6	43.5	5.5	9.0	3.1	34.3	0.02	6.4	71.0	0.15

LA : Lactic acid consumed (g/l)

AA and PA: Acetic and propionic acid produced (g/l), respectively.

Y_{AA} and Y_{PA} : Yield coefficients (%) for acetic and propionic acid production, respectively.

P_{AA} and P_{PA} : Volumetric productivities (g/l/h) for acetic and propionic acids, respectively.

Table 2. Productivity and yield coefficient values for acetic and propionic acid production in repetitive fed-batch biofilm fermentations with the basket and fire brick support materials at pH 7.0.

			Acetic acid			Propionic acid		
Batch #	Time (h)	LA	AA	Y_{AA}	P_{AA}	PA	Y_{PA}	P_{PA}
1	161.0	52.9	15.0	28.4	0.09	42.0	79.4	0.26
2	120.0	54.6	13.1	24.1	0.11	34.3	62.8	0.29
3	141.5	49.4	13.9	28.1	0.10	36.8	74.6	0.26
4	212.5	65.9	17.4	26.4	0.08	45.5	69.0	0.22

LA : Lactic acid consumed (g/l)

AA and PA: Acetic and propionic acid produced (g/l), respectively.

Y_{AA} and Y_{PA} : Yield coefficients (%) for acetic and propionic acid production, respectively.

P_{AA} and P_{PA} : Volumetric productivities (g/l/h) for acetic and propionic acids, respectively.

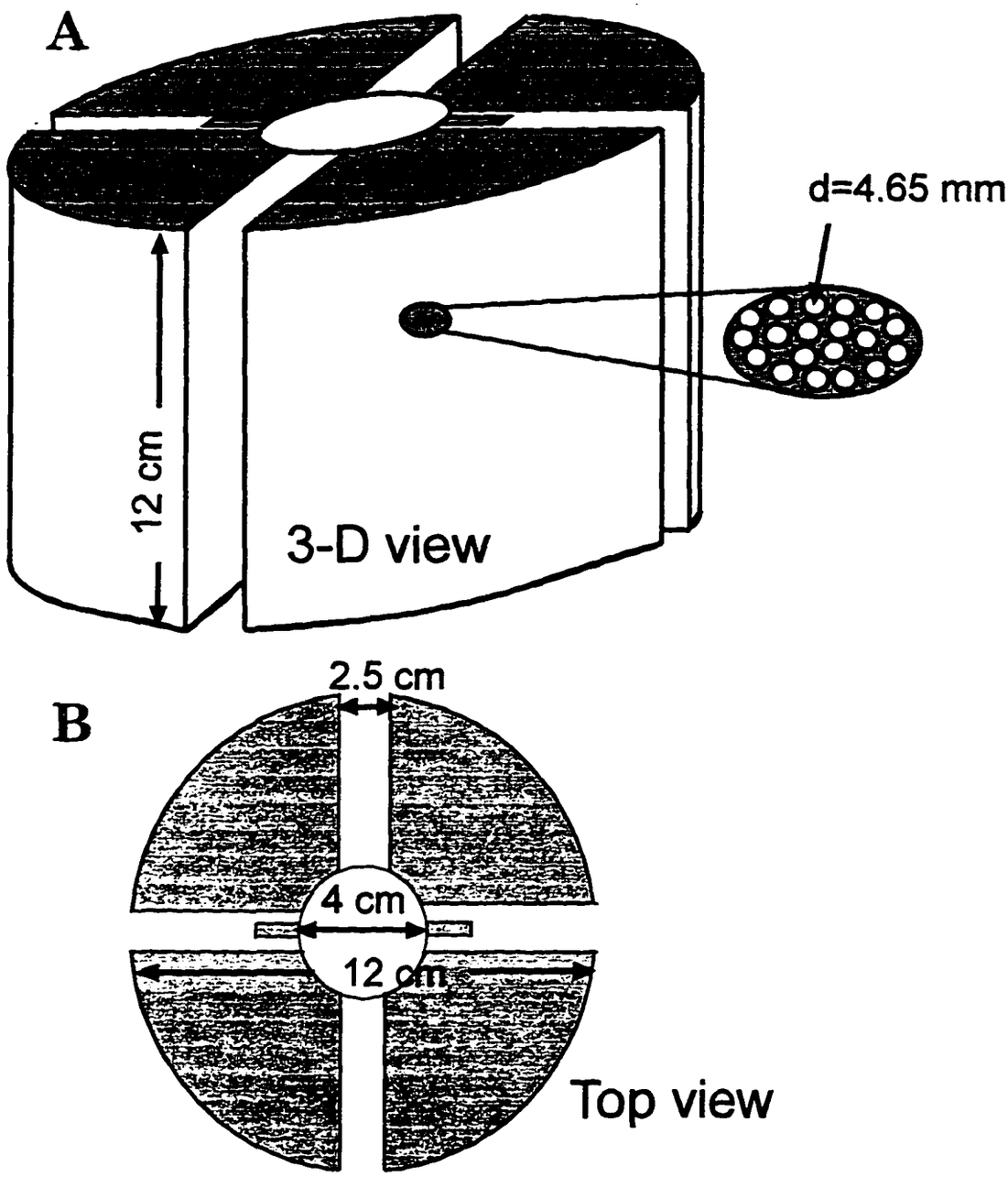
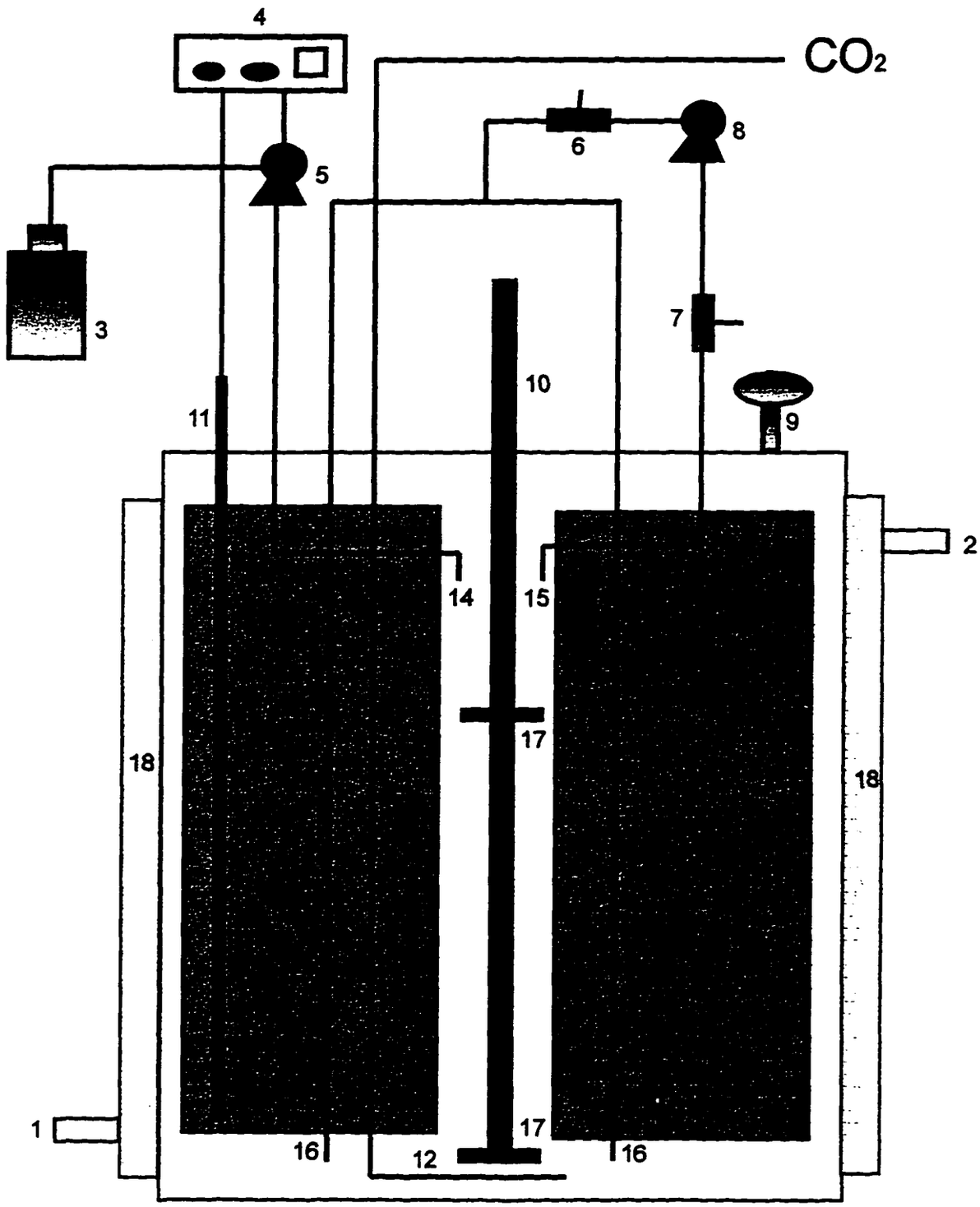


Figure 1. Different views of the bioreactor insert. A) 3-D view. Magnified wall design shows the mesh structure of the reactor walls. Whole reactor is made of the same stainless steel material. B) top view.

Figure 2. Schematic representation of the fermentation setup with the basket bioreactor insert. 1: water in (jacket), 2: water out (jacket), 3: acid and/or base reservoir, 4: pH controller, 5: peristaltic pump, 6: medium exchange port, 7: sampling port, 8: peristaltic pump, 9: air vent, 10: agitation shaft, 11: pH probe, 12: CO2 gas sparging arm, 13: basket, 14: acid or base addition line, 15: medium circulation or new medium addition line, 16: medium drawing line for circulating and sampling purposes, 17: impellers, 18: temperature-controlled heating jacket.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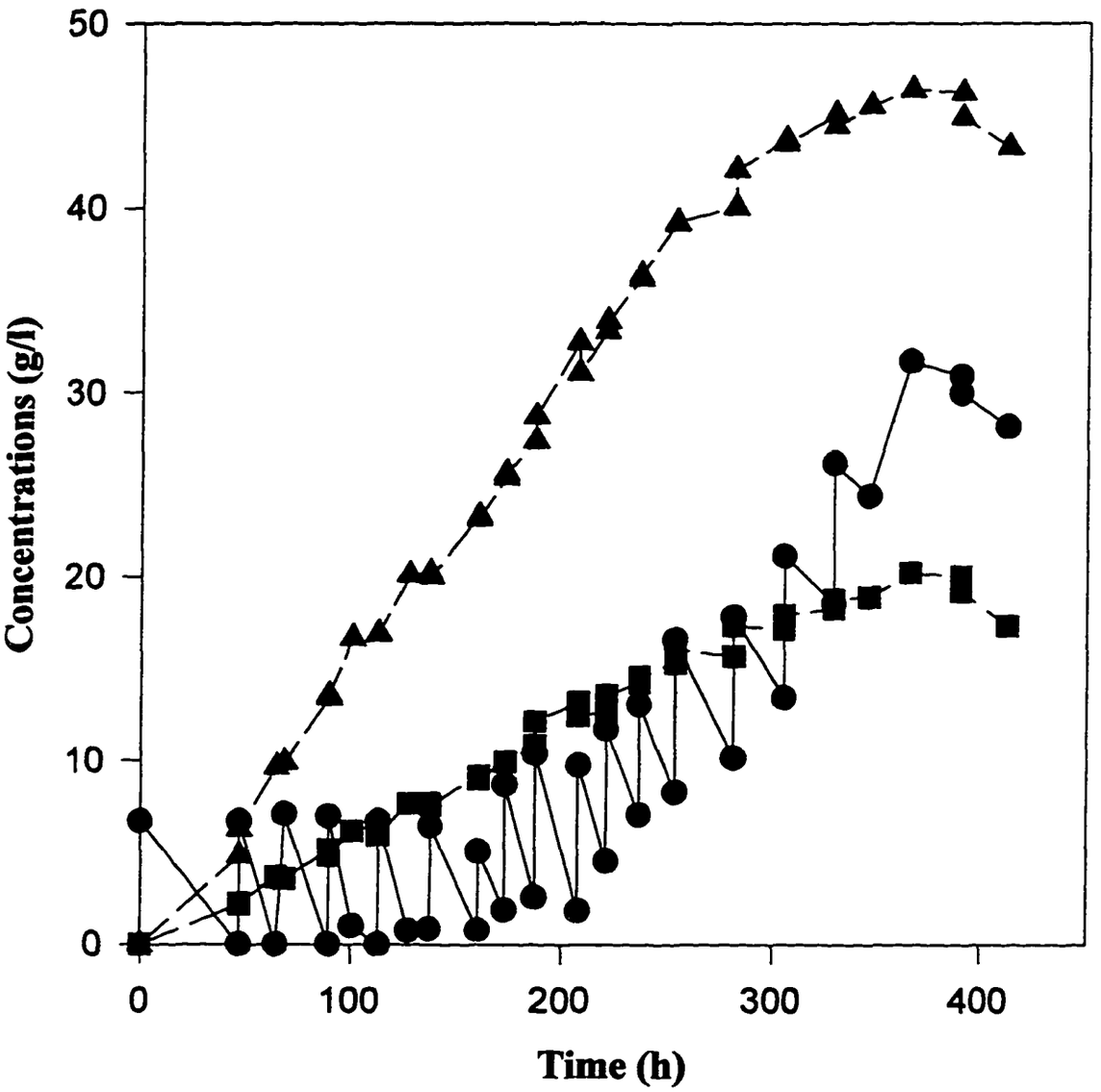

Figure 3. Fed-batch fermentation with basket reactor insert and *P. thoenii* P20. ● Lactic acid; ■ Acetic acid; ▲ Propionic acid.

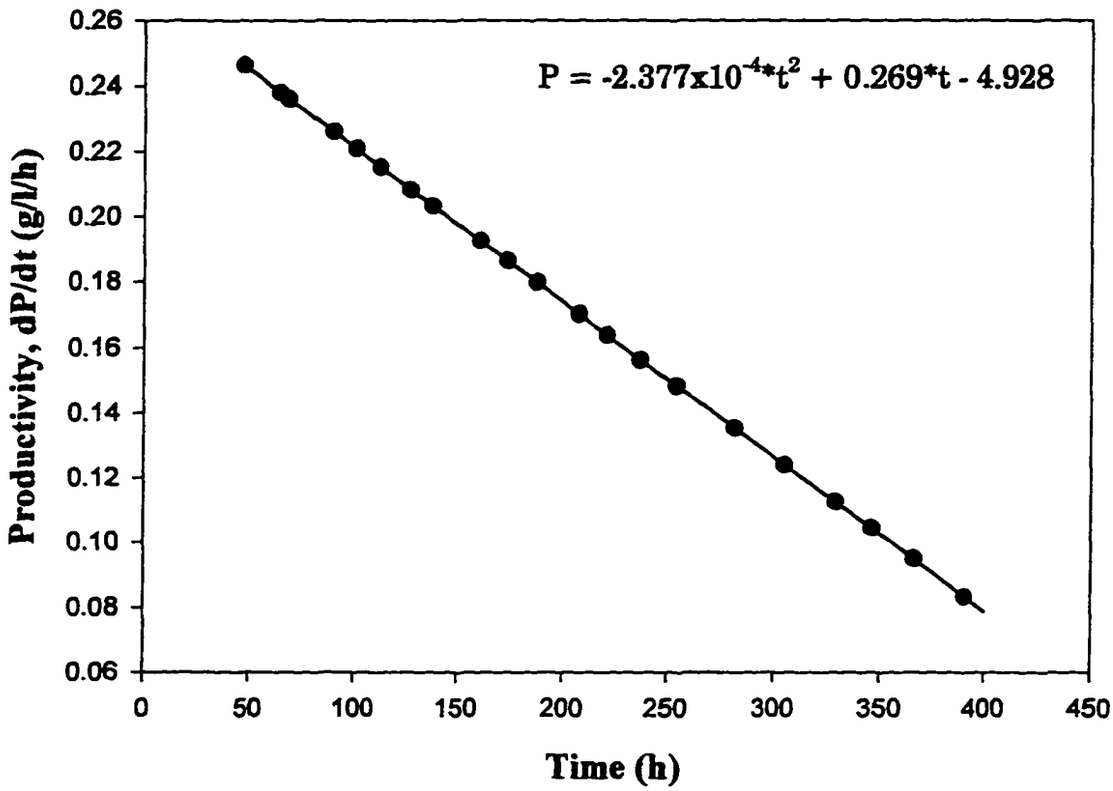

Figure 4. Propionic acid productivity trend throughout the fed-batch fermentation with basket reactor insert. Productivity values calculated by using the derivative of a mathematical model which was used to fit product concentration versus time.

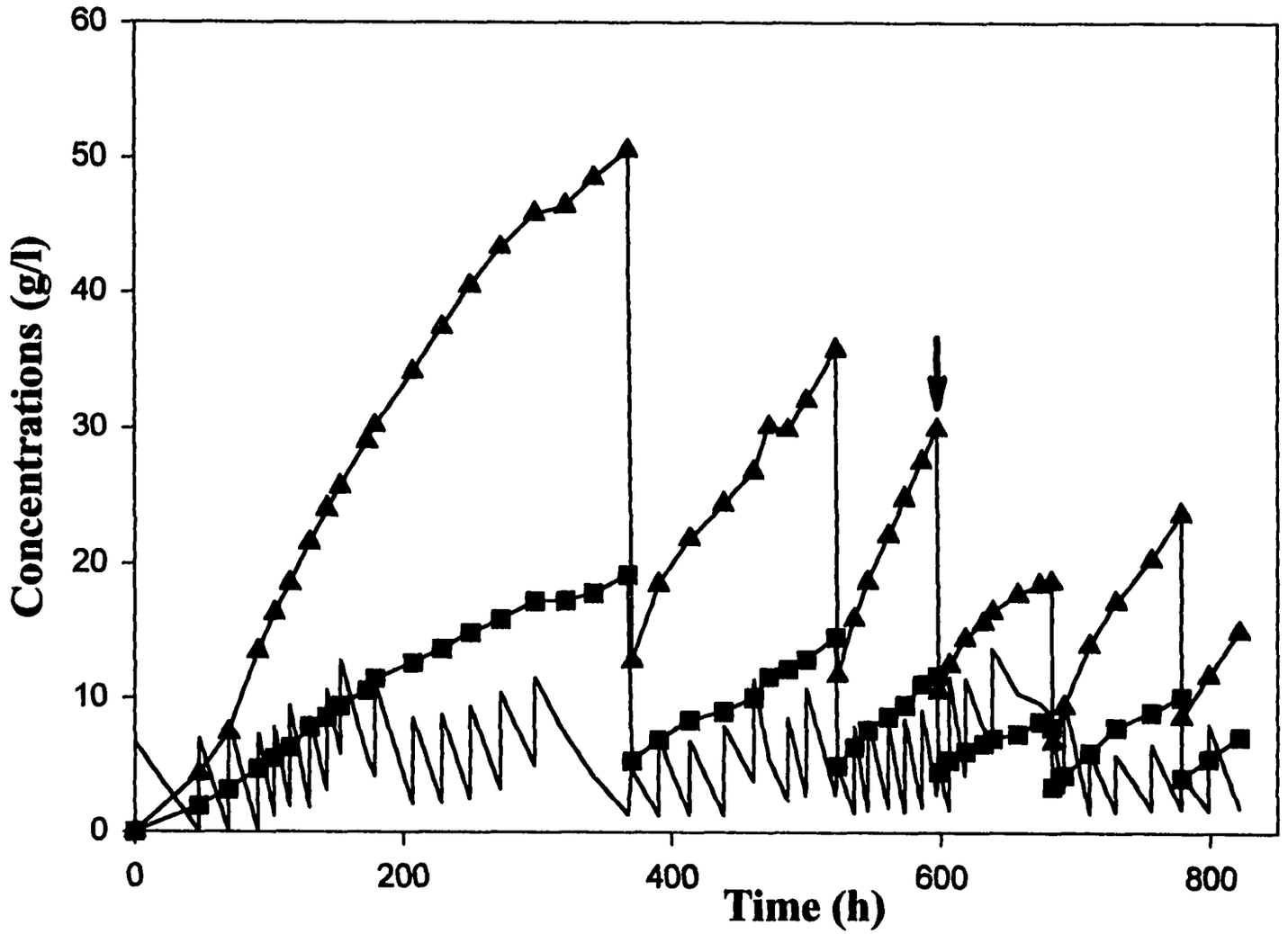

Figure 5. Repetitive fed-batch fermentation for propionic and acetic acid production by biofilm formed on the basket and fire bricks. pH shifted from 7.0 to 5.5 at arrow. — Lactate; ■ Acetate; ▲ Propionate.

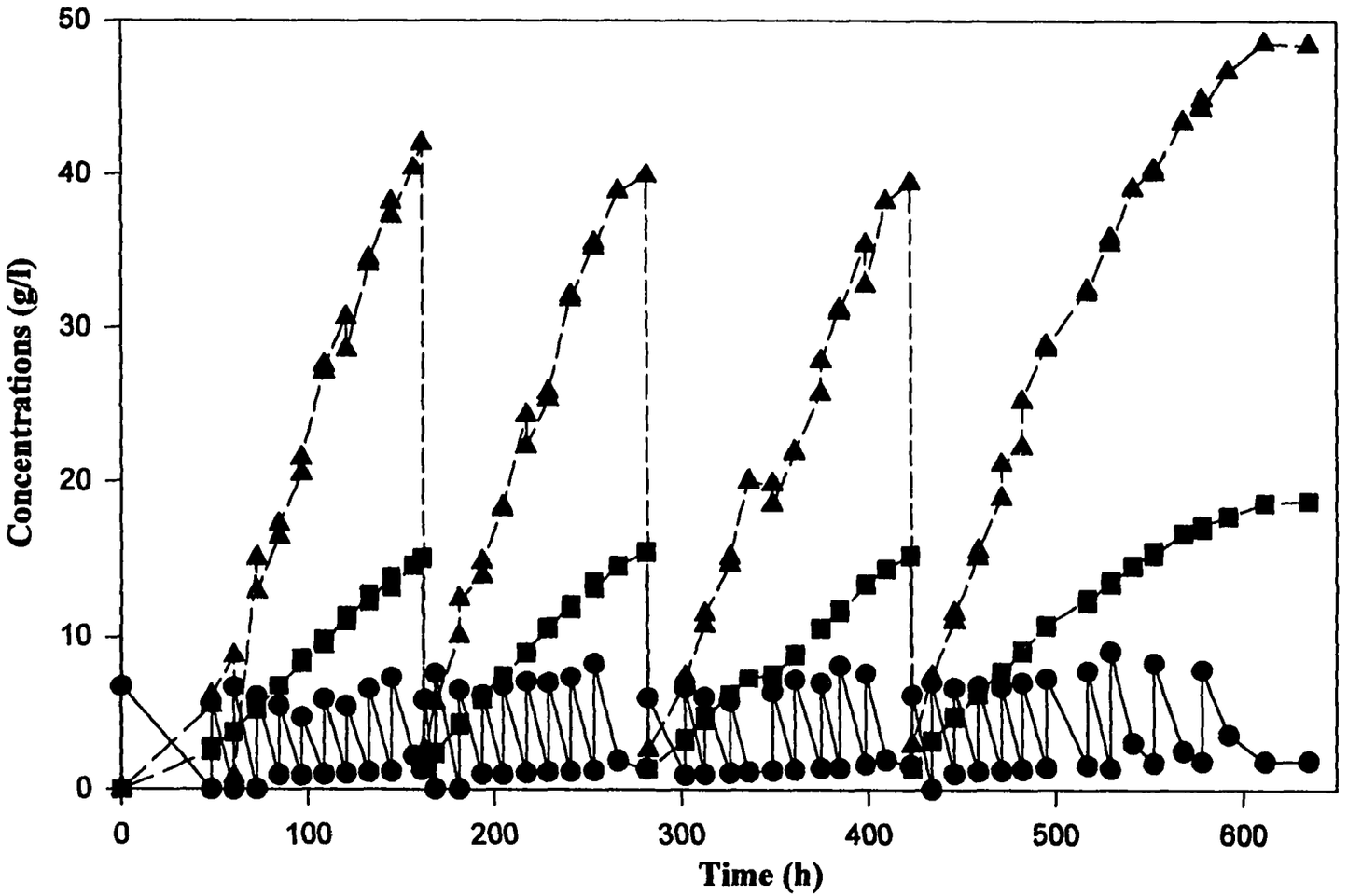

Figure 6. Acid production by biofilm in repetitive fed-batch fermentation over four consecutive batches. ● Lactate, ■ Acetate, ▲ Propionate.

CHAPTER 4. EVALUATION OF BIOFILM AND CELL-LOADED ALGINATE BEAD SYSTEMS IN CELL-FREE CIRCULATED MINI REACTOR FOR PROPIONIC ACID PRODUCTION

A paper to be submitted to Journal of Agricultural and Food Chemistry

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Abstract

Previously designed and tested biofilm and cell-loaded calcium alginate bead systems were evaluated in a miniaturized system for acetic and propionic acid production and substrate utilization rates. A jacketed mini reactor was used to hold the support materials and the beads with a coupled hollow-fiber cell separation unit. Main goals of this study are to follow differential acid production and substrate consumption rates in a relatively small system compared to the cell-free medium reservoir and to evaluate the effects of accumulated propionic acid on acid production.

Average propionic acid production and lactate consumption rates for biofilm and cell-loaded bead systems were 0.06 g/l/h, 0.085 g/l/h and 0.09 g/l/h, 0.14 g/l/h, respectively. In the biofilm system, calculated average yield coefficients were about 86.5 and 37.5% for propionic and acetic acid, respectively. For the bead system,

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yield coefficients were 84.4 and 39% for propionic and acetic acids, respectively. Beads disintegrated after the first batch of the fermentations.

Introduction

Propionic acid has many and varied uses as an antifungal agent in foods and feeds and as an ingredient in thermoplastics, antiarthritic drugs, perfumes, flavors, and solvents (5). Propionic acid is produced by chemical synthesis from petroleum; production of propionic acid via fermentation processes has also received attention (3).

The most popular methods to increase the productivity of the propionic acid fermentation have been immobilization of propionibacteria by entrapment or adsorption. An example of entrapment is immobilization of propionibacteria in calcium alginate beads. Cell-loaded beads have been tested for propionic and acetic acid production in semidefined laboratory medium, whey, and corn steep liquor in batch, fed-batch, and continuous fermentation (1, 5, 6, 7). Biofilm systems employing natural immobilization by means of adsorption were also tested for propionic and acetic acid production (Chapter 2 and 3).

Since it is hard to evaluate or follow the production and consumption rates in highly concentrated immobilized-cell systems, a miniature reactor with a relatively small amount of cells compared to the volume of the cell-free reservoir was used to analyze trends of substrate utilization and acid production. Propionic acid has been shown to be inhibitory to microbial growth

at levels above 2% (9). Acid production and substrate utilization per unit basis of immobilized cells, as affected by product concentration, were also studied.

Materials and Methods

Microorganism and media

A strain of propionibacteria, *P. thoenii* strain P20, was obtained from the culture collection of the Department of Food Science and Human Nutrition at Iowa State University. Sodium lactate broth (NLB) contained 1% (v/v) sodium lactate 60% syrup (Fisher Scientific Co., Pittsburgh, PA), 1% (w/v) yeast extract (Difco Laboratories, Detroit, MI), and 1% (w/v) Trypticase soy broth (Baltimore Biological Laboratories, BBL, Cockeysville, MD) and was used as medium in all experiments. Propionibacteria were maintained at 4°C on sodium lactate agar (NLA) plates as previously described by Woskow and Glatz (8).

Support material

Fire bricks (Al_2O_3) were used as the support materials (BioLifeSaver - patent pending). Fire bricks were cut into 6 x 1.5-cm cylindrical pieces and into 1.5-1.7-cm pieces with 6-7-mm hole to give the life-saver shape.

Cell immobilization

Natural attachment: Biofilm

Seven fire brick supports were incubated in the mini reactor for 48 h at 32°C to allow batch biofilm formation due to natural cell attachment. Inoculum size was 25% (v/v), taken from a 24-h culture.

Immobilization by entrapment

Cells were immobilized in calcium alginate beads as described by Rickert (6), Paik and Glatz (5), and Yongsmith and Chutima (10). Detailed explanation of this procedure can be found in Rickert (6). However, a brief procedure is given in Appendix B.

Mini reactor

A small glass reactor with a water jacket was built at the Glass Blowing Shop, Iowa State University (Figure 1). The overflow line, which passed through the rubber stopper, was covered with stainless steel mesh to prevent the beads from escaping into the effluent line. The dimensions of the mini reactor were 17 mm (inner diameter) and 48 mm (length of the jacketed part). The total volume of the mini reactor was 10 ml. At the unjacketed conical bottom of the reactor was an inlet with a 4 mm (outer diameter) tube. The reactor was sterilized either with fire brick supports when it is used for biofilm fermentations or empty for fermentations with calcium alginate beads.

Fermentation setup and conditions

A 1.2-liter Fleaker beaker (Cole Parmer Instrument Co., Niles, IL), controlled at 32°C in a water bath, was used as a medium reservoir (Figure 2). The reservoir was filled with 700 ml of separately autoclaved medium. Medium was exchanged when the substrate level in the reservoir reached about 0.2% (w/v). A hollow-fiber microfiltration unit (A/G Technology Corporation, Needham, MA) was connected to the mini reactor to remove any free cells from the effluent stream. Filtrate (cells and detached biofilm) was collected in a graduate cylinder and the cell-free permeate was recycled back into the reservoir where the medium was maintained at pH 6.9 and 32°C. Two-ml samples were taken every 3 to 4 hours from three sampling ports.

Preparation of mini reactor with fire brick supports

Seven fire brick supports (3.73 g total, 0.53 g each) were stacked into the mini reactor. The holes in the supports were aligned with the feed stream for better flow characteristics. Mini reactor with supports was autoclaved at 121°C for 45 min.

Preparation of mini reactor with beads

Six ml of the cell-loaded beads (285 beads) were aseptically transferred to the sterile mini reactor. Targeted bead load was set as 0.5% (w/v). Wet weight of 6 ml beads was 5.7 g. See Appendix B for the bead load determination.

Hollow-fiber cell separation unit

A hollow-fiber microfiltration (0.2 mm) cartridge was used to separate the free cells from the effluent stream. The membrane cartridge was operated in a horizontal position. It was preferred to recycle the permeate from both ports to minimize permeate-side back pressure which could contribute to permeate back-flow through the membrane portion at the end of the cartridge. See Appendix B for preparing and cleaning procedures for the microfiltration unit.

Biofilm evaluations

Extent of biofilm formation on the supports was quantitatively and qualitatively evaluated by measuring weight change of the supports, by determining extent of clumping among fire brick pieces after drying at 70°C (2), and by plating and enumerating colony formation units (CFU). The CFUs were determined by removing some of the supports with biofilm from the mini reactor and aseptically transferring with a spatula into sterile sample vials containing peptone water, (0.1% peptone, Difco Laboratories), and vigorously vortexing them to detach all the biofilm from the support surfaces. Those samples were then plated for viable cell counts. Similarly taken biofilm samples were dried overnight in a vacuum oven at 70°C to determine dry weight.

Analytical methods

Concentrations of glucose and lactic, acetic, and propionic acids were determined by high-performance liquid chromatography (HPLC) (4).

L-lactic acid and D-glucose concentrations were followed with YSI Model 2700 Select Biochemistry Analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) with dextrose (D-glucose - Glucose oxidase) and L-lactate (L-lactic acid - L-lac oxidase) membranes.

Viable cells were enumerated by standard plate counting procedures on duplicate sodium lactate agar plates incubated anaerobically for 4 days at 32°C. Immobilized cells were released from beads by dissolving two beads in a known volume of sterile 10 g/l sodium citrate for 3 h at room temperature. After dissolving, the resulting slurry was serially diluted and plated on NLA.

Results and Discussion

After establishment of biofilms on inert support materials (Chapter 2), it was important to evaluate the acid production per unit basis of the biofilm. Trends of substrate utilization and acid production by small amounts of immobilized cells were analyzed in a large-volume cell-free circulated system. The system is specifically designed to produce acetic and propionic acids in the biofilm reactor and accumulate products in the cell-free medium reservoir where the pH and the temperature are controlled. The medium was continuously

circulated through the mini reactor. To prevent free cells and/or detached biofilm cells from the reactor from entering the reservoir through the circulation loop and starting free-cell fermentation there, the reactor effluent was passed through a hollow-fiber membrane module to remove cells.

The mini reactor system was also a good platform to compare natural and entrapped cell immobilization techniques. The size of the reactor was minimized to be able to follow the performance of the immobilized cells in a considerably larger reservoir. Depletion of the lactic acid as a substrate was followed in short time intervals for both systems. For the biofilm system, after the substrate had been depleted, medium in the reservoir was changed and a second batch was run. Only a single batch was tested with alginate-immobilized cells.

Comparison of the two immobilization techniques has been made based on lactic acid consumption, acid production rates, stability of the system for repetitive use, and product yields (Table 1). The biofilm system in the mini reactor demonstrated improvement in terms of acid production and substrate consumption rates from the first to the second batch (Figures 3-5). The biofilm had been well established by the start of the second batch. The lactic acid consumption rate increased from 0.07 to 0.1 g/l/h. Propionic acid production rate increased from 0.05 to 0.07 g/l/h between batches, but acetic acid production rate stayed at 0.02 g/l/h. The straight section of the experimental data fit a straight regression line equation for all cases (r^2=0.99). In the second batch acetic acid production did not lag, while there was a 30-h lag in the first batch.

After the second batch, extensive biofilm formation and sloughing caused clogging of the exit lines. Elevated pressure developed inside the reactor which eventually led to leakage in the system. The pressure build-up was partly due to cell accumulation in the hollow fiber separation module. This problem has been overcome by adding a bypass route to clean the separation module. When clogging occurred, sterile distilled water was pumped through the module at a high flow rate to remove any built up biofilm in the hollow fiber tubes. In preliminary experiments CO_2 gas had been used to help the flow and keep the air out of the system. However, this seemed to increase the clogging problem. Technical service personnel of the membrane companies speculated that CO_2 might stimulate membrane pore clogging (personal communication).

Substrate consumption and acid production rates for the cell-loaded calcium alginate bead system were slightly higher than for the biofilm system: 0.14 g/l/h for lactate use, 0.09 g/l/h for propionate production, and 0.03 g/l/h for acetate production (Figures 6-8). One advantage of this system is that there is not any lag phase necessary to establish immobilization, so production started right at the beginning of the fermentation process. Still, a 15-hour lag was observed for acetic acid production (Figure 8).

The major disadvantage of this system was the disintegration of the beads even before the end of the first batch. It is likely that the sodium ions in the medium replaced the calcium ions in the bead matrix. This situation could be avoided by adding calcium chloride to the medium. By the end of the

experiment dissolved beads started to clog the lines. Before their disintegration beads swelled to twice their original size. Such swelling enlarges the void volume of the beads and makes the structure more vulnerable to physical disruption. A decline in acid production and substrate consumption toward the end of the batch could be one indication of performance failure.

In yield of acids from substrate, the biofilm system performed competitively compared to the bead system. The calculated average yield coefficients were about 86.5 and 37.5% for propionic and acetic acids, respectively, in the biofilm versus 84.4 and 39%, respectively, for the bead system (Table 1). Because biofilm was being formed in batch I, some of the substrate was used for biomass production in this batch. The greater than 100% yield of propionic acid from lactate on batch II of the biofilm system is likely due to the fact that other nutrients in yeast extract and Trypticase soy broth contributed to acid production.

In summary, differential acid production and substrate utilization were evaluated in a small immobilized cell system. Cell-loaded alginate beads and biofilm were used as the immobilization methods. Beads disintegrated after the first batch of the fermentation. In terms of yield and productivity values, biofilm system showed competitive performance compared to cell-loaded calcium alginate beads.

Acknowledgments

This work was supported in whole or part by the Iowa Corn Promotion Board, the Consortium for Plant Biotechnology Research, Inc. by DOE cooperative agreement no. DE-FC05-920R22072, New Energy Company of Indiana, and the United States Department of Agriculture through the Biotechnology Byproducts Consortium. This support does not constitute an endorsement by any of these groups of the views expressed in this article.

References

1. **Champagne, C. P., C. Baillargeon-Cote, and J. Goulet.** 1989. Whey fermentation by immobilized cells of *Propionibacterium shermanii*. J. Appl. Bacteriol. **66**:175-184.
2. **Demirci, A., A. L. Pometto III, and K. E. Johnson.** 1993. Evaluation of biofilm reactor solid support for mixed-culture lactic acid production. Appl. Microbiol. Biotechnol. **38**:728-733.
3. **Lee, I. H., A. G. Fredrickson, and H. M. Tsuchiva.** 1974. Diauxic growth of *Propionibacterium shermanii*. Appl. Microbiol. **28**:831-835.
4. **Ozadali, F., B. A. Glatz, and C. E. Glatz.** 1996. Fed-batch fermentation with and without on-line extraction for propionic and acetic acid production by *Propionibacterium acidipropionici*. Appl. Microbiol. Biotechnol. **44**:710-716.

5. **Paik, H-D and B. A. Glatz.** 1994. Propionic acid production by immobilized cells of a propionate-tolerant strain of Propionibacterium acidipropionici. Appl. Microbiol. Biotechnol. **42**:22-27.

6. **Rickert, D. A.** 1996. Evaluation of efforts to improve organic acid production by calcium alginate-immobilized propionibacteria. M.S. thesis. Iowa State University, Ames, IA.

7. **Vorob'eva, L. I., M. A. Alekseeva, I. G. Surkhova, and V. I. Gaitan.** 1977. Formation of volatile acids by immobilized cells of propionic acid bacteria. Appl. Biochem. Microbiol. **13**:416-421.

8. **Woskow, S. A. and B. A. Glatz.** 1991. Propionic acid production by a propionic acid-tolerant strain of *Propionibacterium acidipropionici* in batch and semicontinuous fermentation. Appl. Environ. Microbiol. **57**:2821-2828.

9. **Yang, S-T., Y. Huang, and G. Hong.** 1995. A novel recycle batch immobilized cell bioreactor for propionate production from whey lactose. Biotechnol. Bioeng. **45**:379-386.

10. **Yongsmith, B. and K. Chutima.** 1983. Production of vitamin B_{12} by living bacterial cells immobilized in calcium alginate gels. J. Ferment. Technol. **61**:593-598.

Table 1. Comparison of biofilm and cell-loaded bead systems in terms of yield and rates of lactate consumption and acid production

	Biofilm system					Cell-loaded beads				
Batch #	Y_{AA} (%)	P_{AA}[a] (g/l/h)	Y_{PA} (%)	P_{PA}[a] (g/l/h)	K_{LA}[b] (g/l/h)	Y_{AA} (%)	P_{AA}[a] (g/l/h)	Y_{PA} (%)	P_{PA}[a] (g/l/h)	K_{LA}[b] (g/l/h)
I	31.0	0.018 (0.02)	69.0	0.035 (0.05)	0.07	39.0	0.03	84.4	0.09	0.14
II	44.0	0.018 (0.02)	104.0	0.044 (0.07)	0.10	-	-	-	-	-
Avg.	37.5	0.018 (0.02)	86.5	0.040 (0.06)	0.085	-	-	-	-	-

[a] Volumetric productivities. Productivity values in the parenthesis are maximum productivities calculated by linear regression analysis.

[b] K_{LA}: Substrate consumption rates (lactic acid).

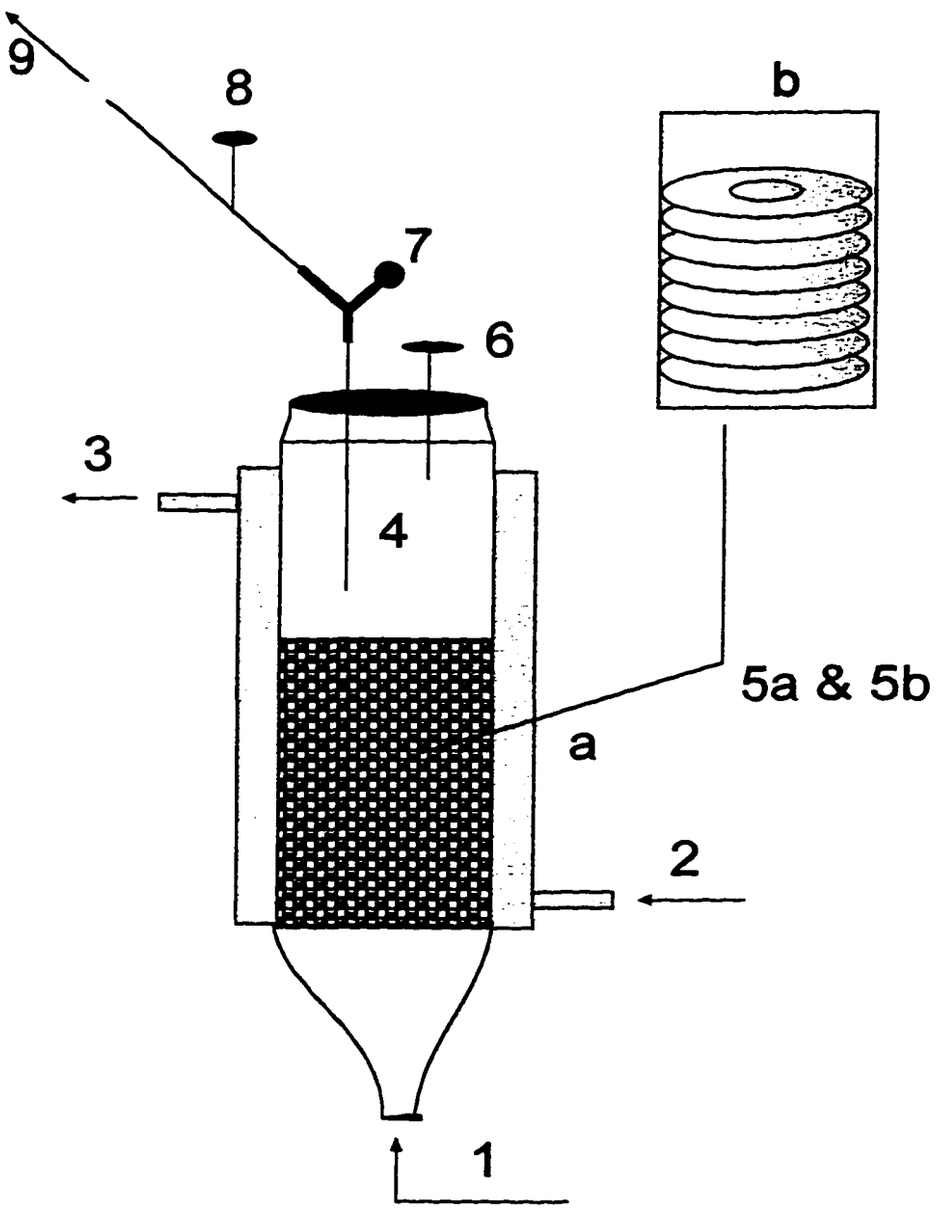

Figure 1. Schematic representation of the mini reactor. 1: medium inlet, 2: water inlet (32°C), 3: water outlet, 4: effluent and level control tube, 5a: reactor loaded with immobilized-cell beads, 5b: reactor loaded with fire brick supports, 6: vent, 7: inoculation port, 8: vent, 9: spent medium to the microfiltration unit.

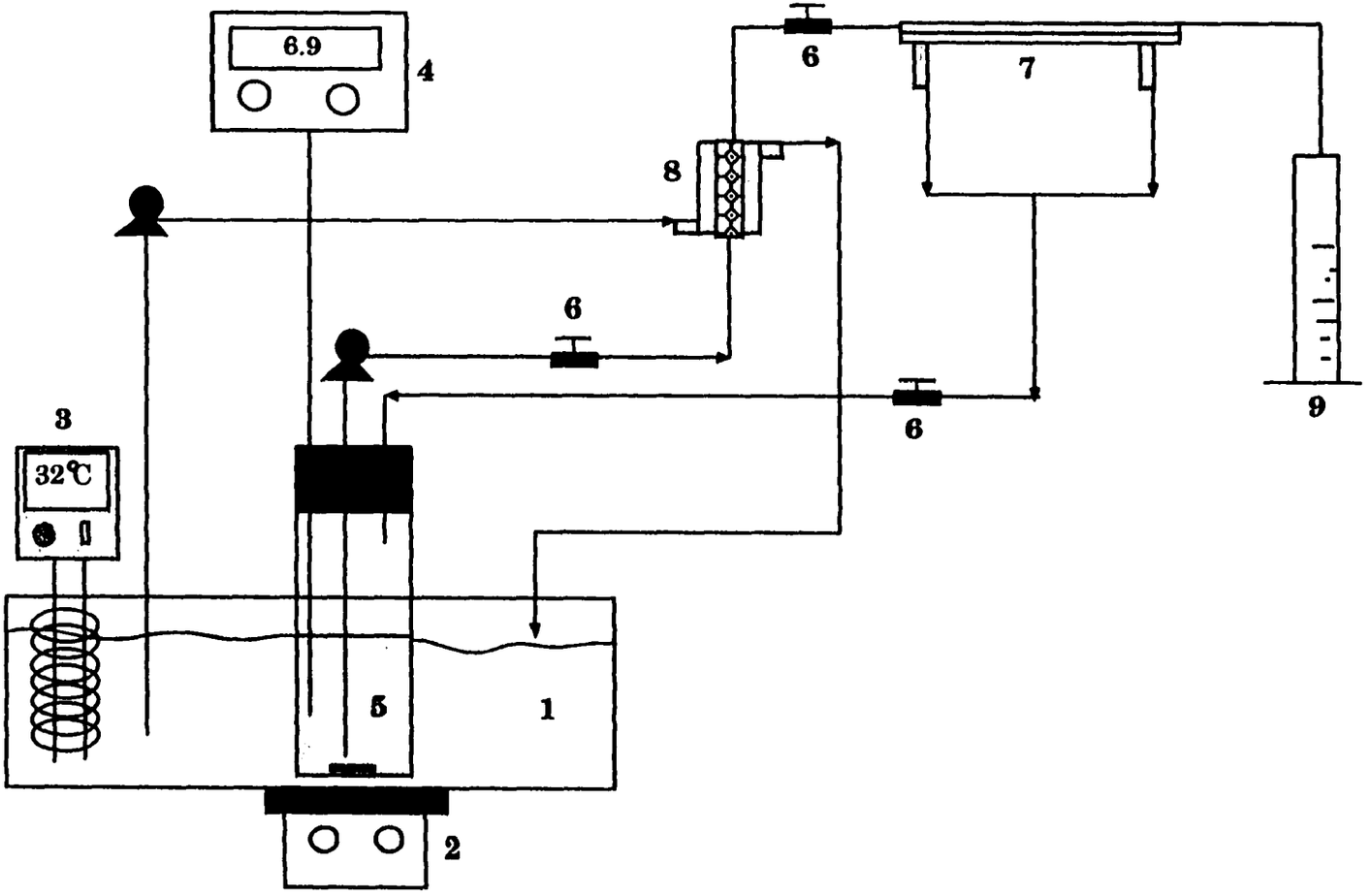

Figure 2. Fermentation setup of integrated mini immobilized-cell reactor and cell separation unit. 1: water bath at 32°C, 2: magnetic stirrer, 3: temperature controller, 4: pH-controller, 5: cell-free medium reservoir, 6: sampling ports, 7: hollow fiber microfiltration unit (0.2 μm), 8: mini reactor, 9: retentate collector.

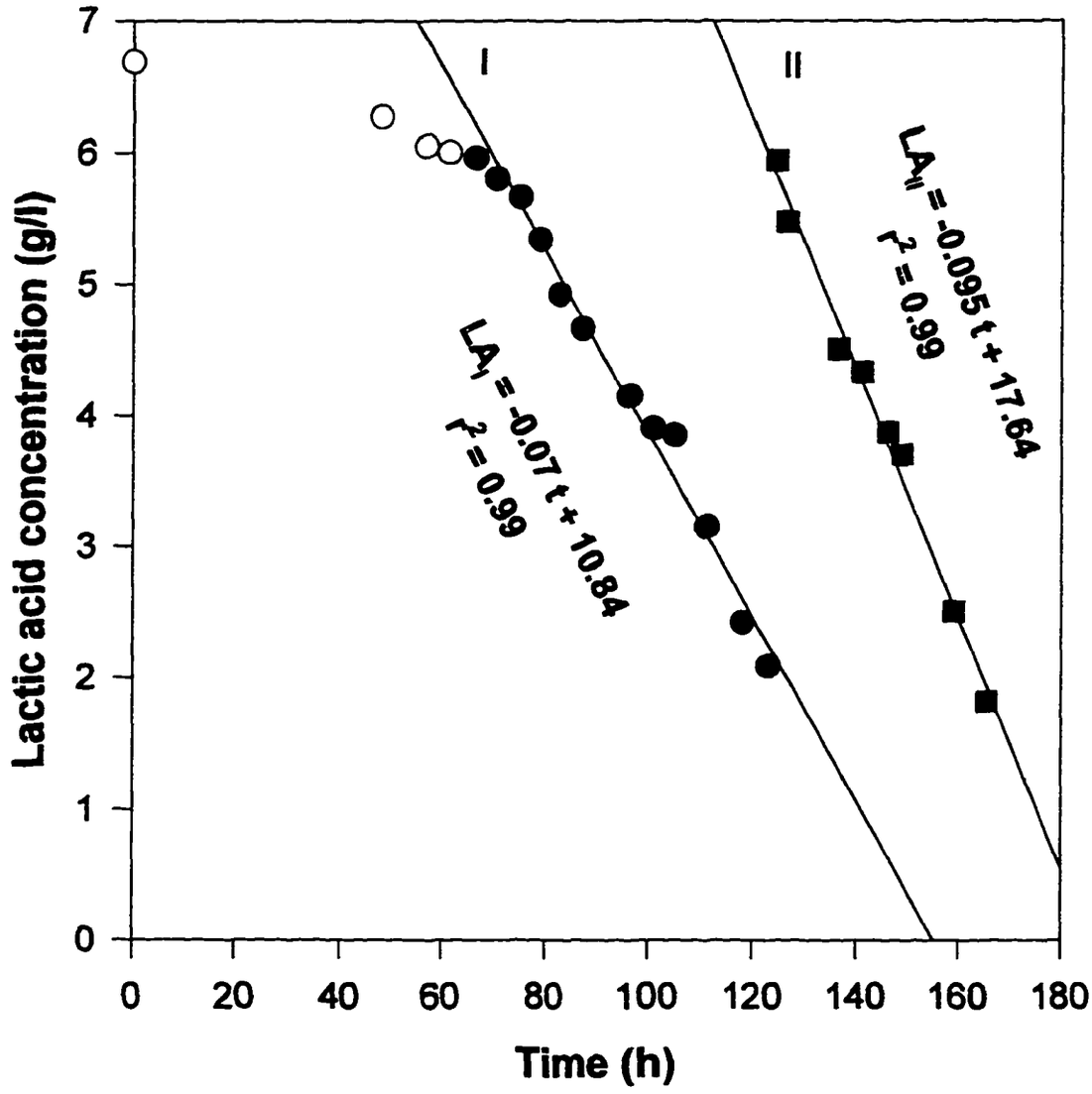

Figure 3. Lactic acid consumption rates of two consecutive batches in mini biofilm reactor. Lactic acid concentration in batch I (● O). Lactic acid concentration in batch II (■). Data shown by filled symbols are used in regression (—).

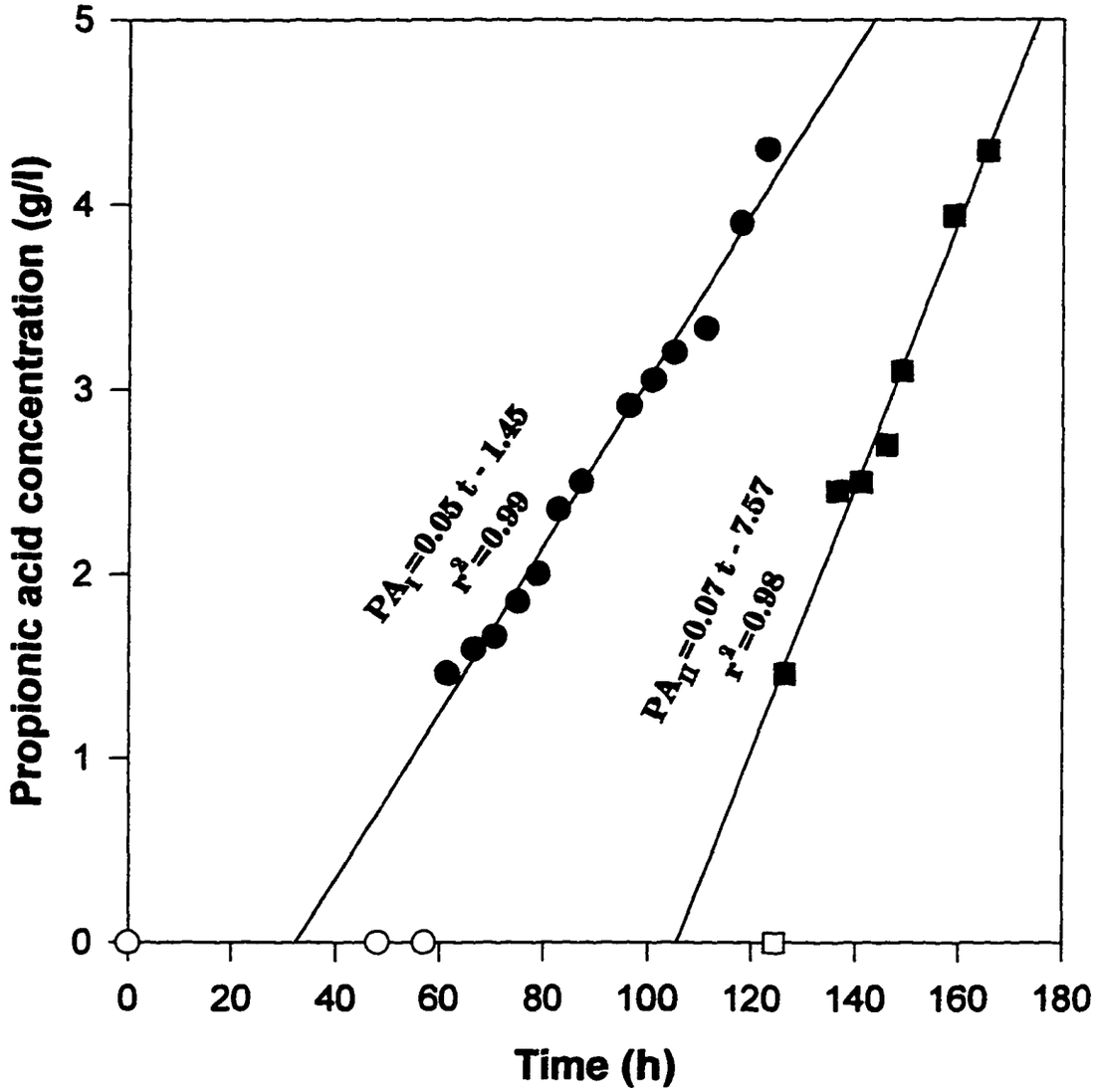

Figure 4. Propionic acid production rates of two consecutive batches in mini biofilm reactor. Propionic acid concentration in batch I (● O). Propionic acid concentration in batch II (■□). Data shown by filled symbols are used in regression (—).

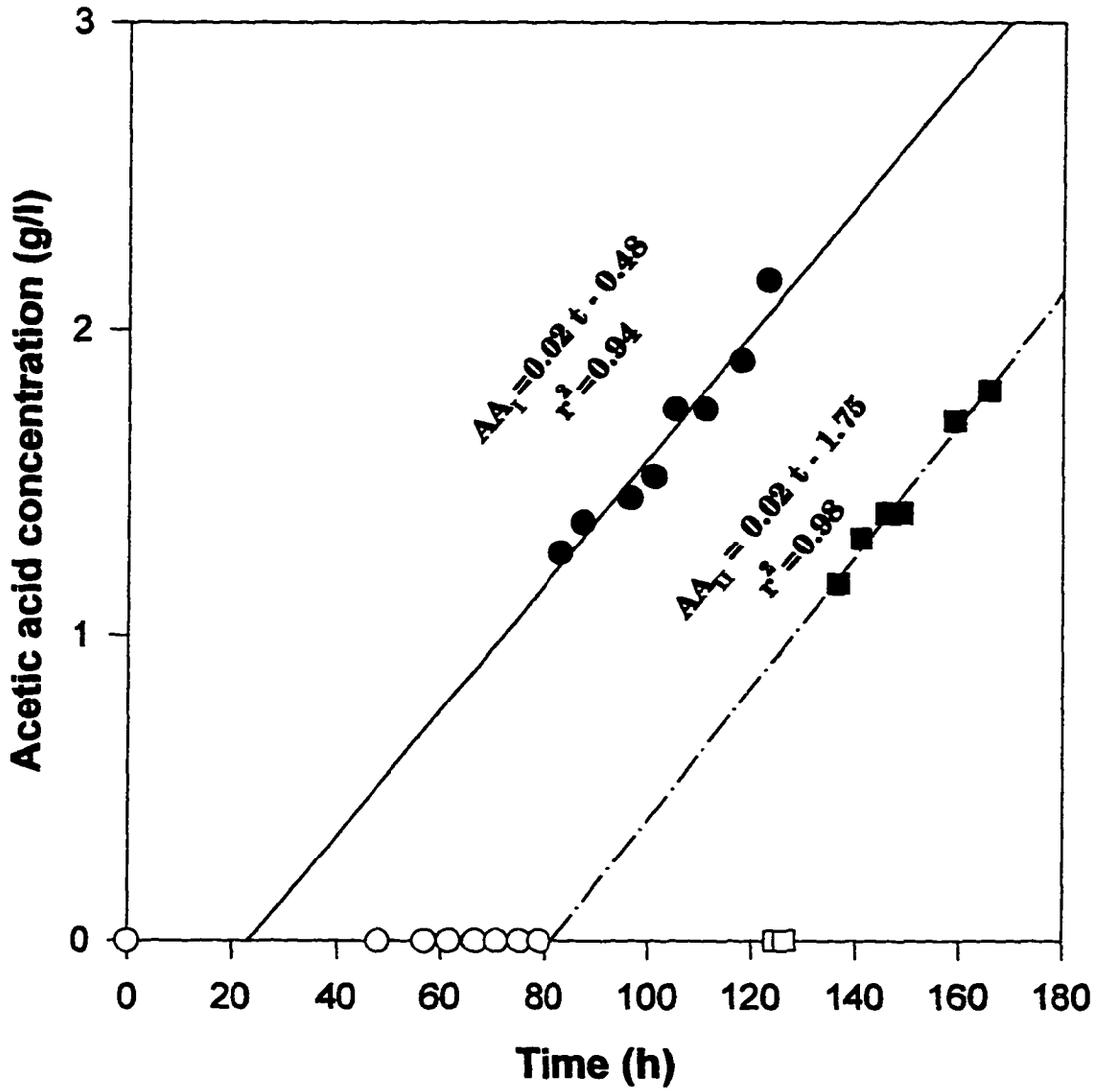

Figure 5. Acetic acid production rates of two consecutive batches in mini biofilm reactor. Acetic acid concentration in batch I (● O). Acetic acid concentration in batch II (■□). Data shown by filled symbols are used in regression (—).

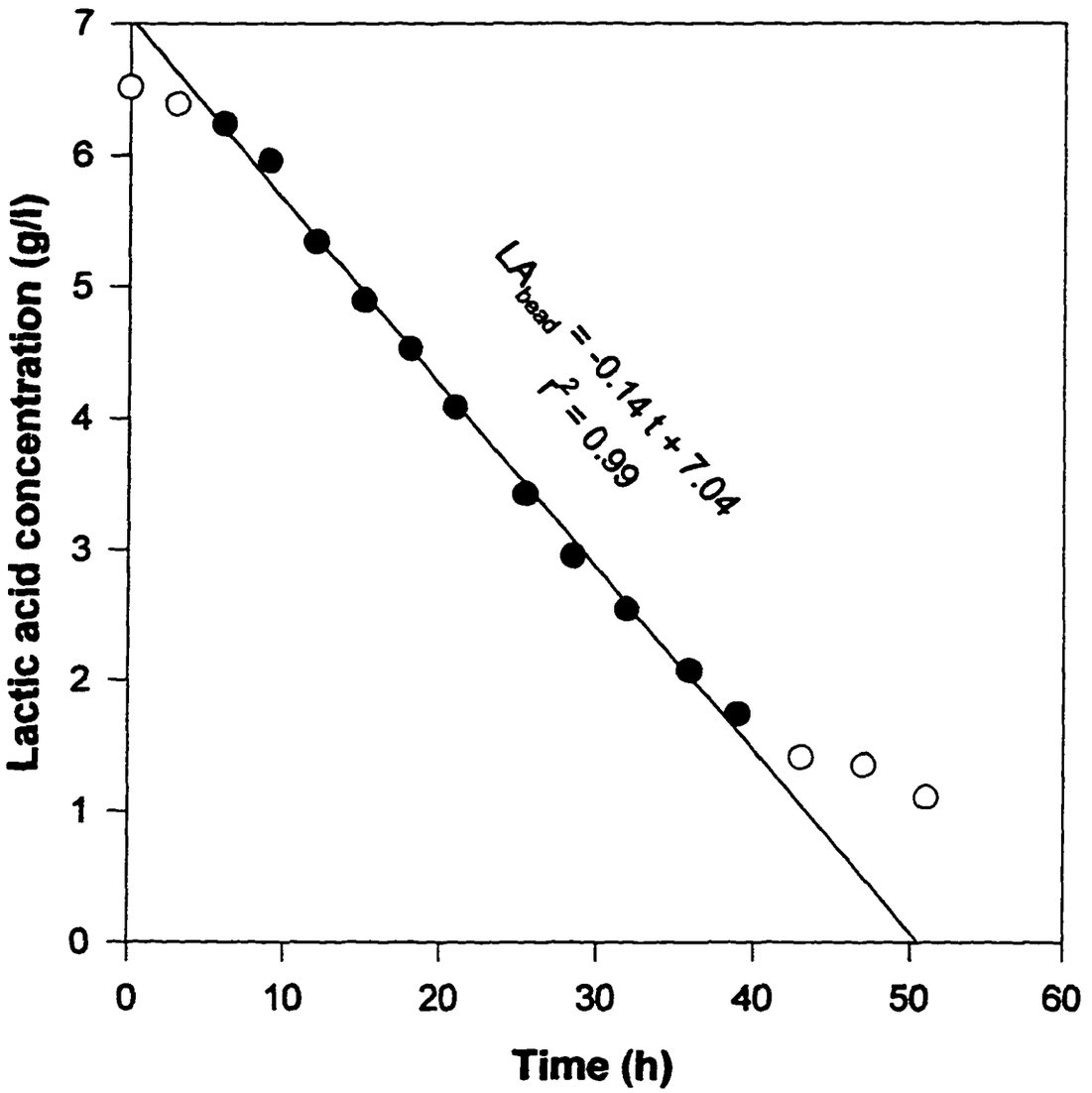

Figure 6. Lactic acid consumption rate in mini reactor loaded with calcium alginate-immobilized cell beads. Lactic acid concentration (● O). Data shown by filled symbols are used in regression (—).

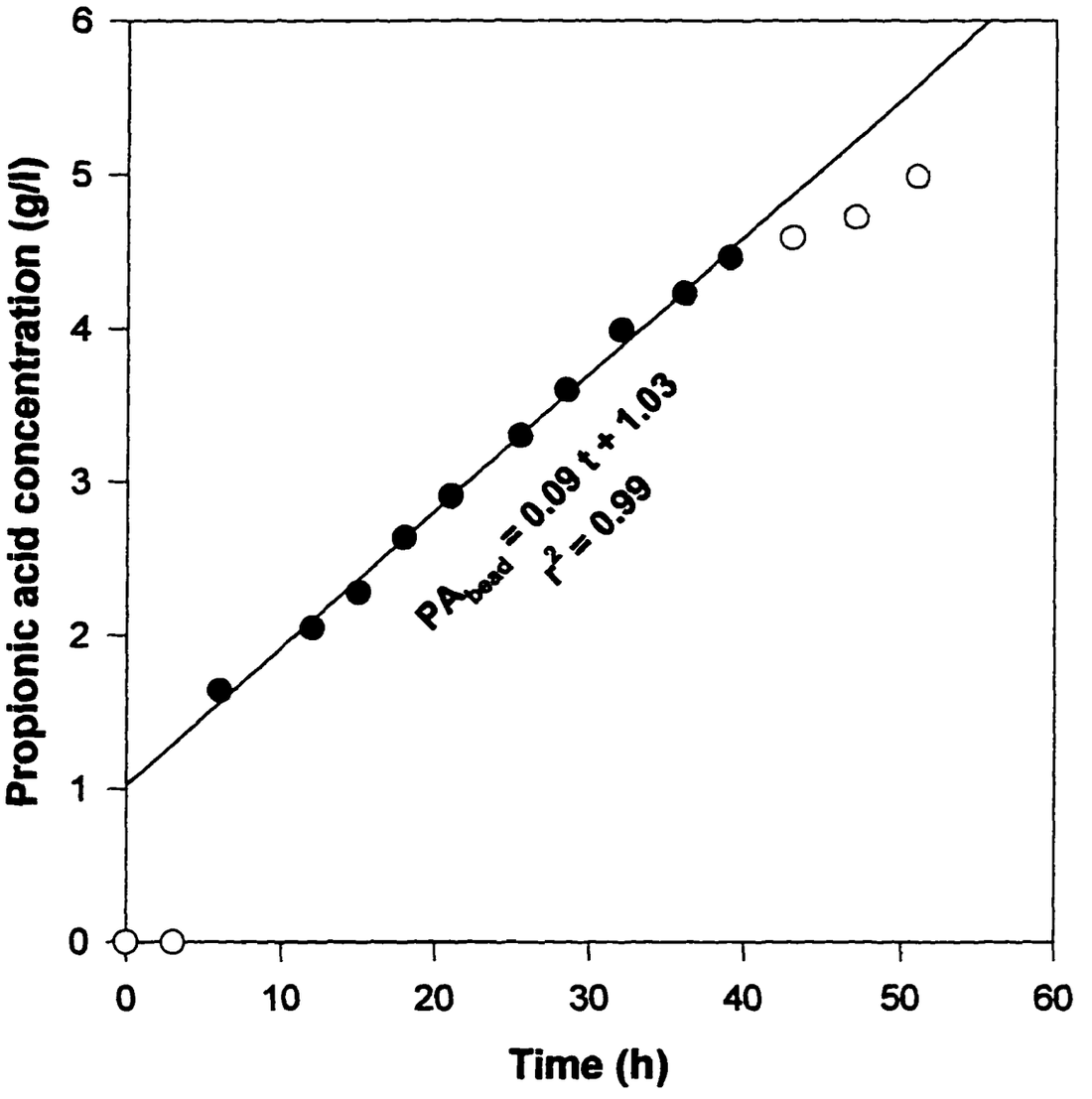

Figure 7. Propionic acid production rate in mini reactor loaded with calcium alginate-immobilized cell beads. Propionic acid concentration (● O). Data shown by filled symbols are used in regression (—).

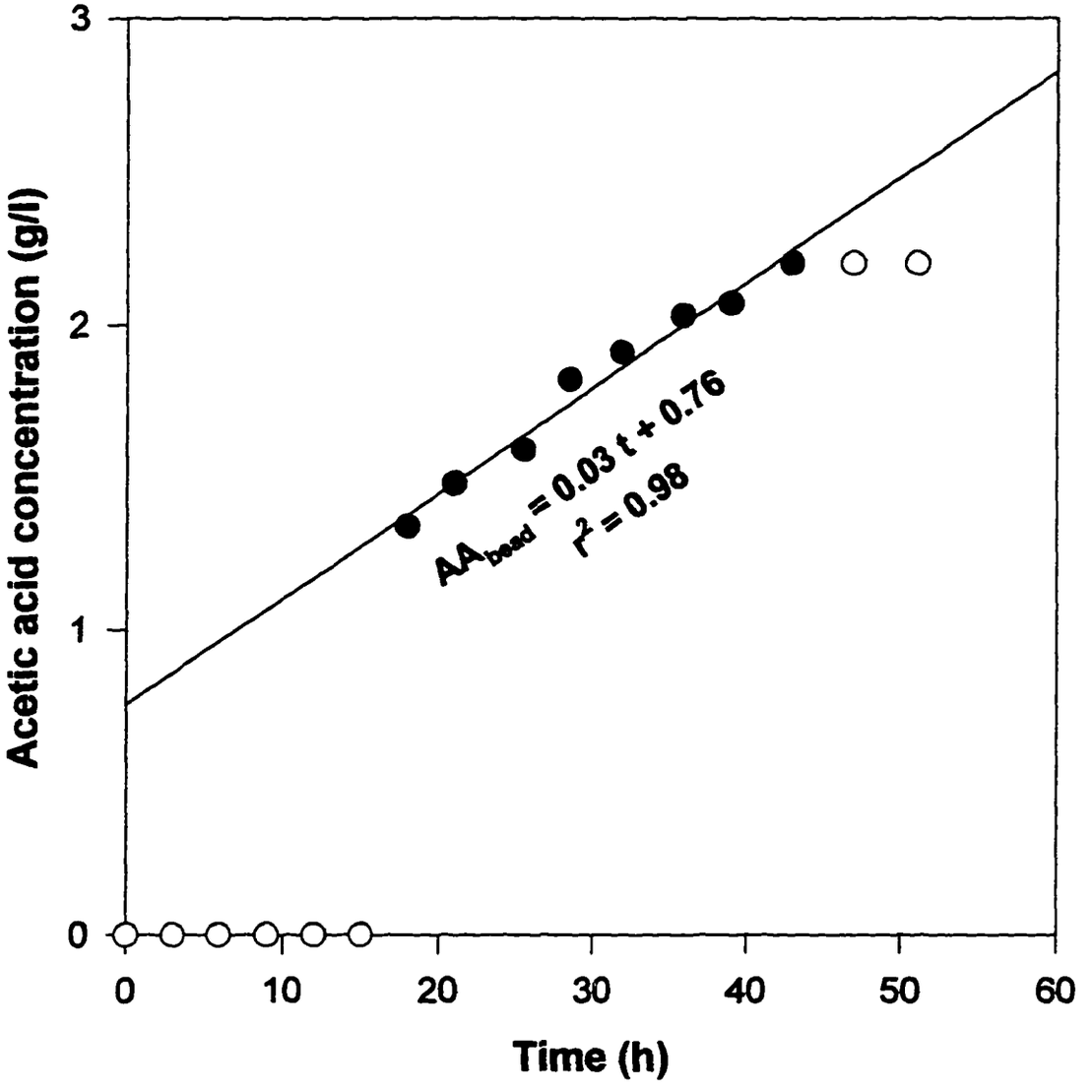

Figure 8. Acetic acid production rate in mini reactor loaded with calcium alginate-immobilized cell beads. Acetic acid concentration (● O). Data shown by filled symbols are used in regression (—).

CHAPTER 5. GENERAL CONCLUSIONS

Summary

A biofilm is a natural form of cell immobilization that results from microbial attachment to solid supports. Ten support materials including plastic-composite supports and six strains of propionibacteria were tested for their possible use in biofilm systems for enhanced production of propionic and acetic acid by fermentation.

In this project the problems of continuous propionic acid fermentation have been defined and alternatives to increase yield and productivity have been sought. Biofilm-forming characteristics of selected strains propionibacteria were tested with various inert support materials. The best support-strain combination was found to be *P. thoenii* P20 and fire bricks.

Propionibacterium thoenii P20 resists low-pH conditions, produces acid rapidly, forms luxuriant biofilms, and resists solvent inhibition better than other strains. Fire bricks are inexpensive, reusable, and compare favorably to commercial supports in ease of use and structural stability. A modified "lifesaver" shape for the individual fire brick particles was found to provide increased available surface area for biofilm formation and better flow patterns of the medium through and around supports.

The attachment process was examined by measuring hydrophobicity and zeta potentials of cells and support materials, and observing attachment with the scanning electron microscope. Cells and support materials were found to be hydrophilic in the optimum pH range (5-8) of the propionic acid fermentation. Zeta potentials of support materials and cells had opposite signs over a wide pH range (4-8); this should encourage attachment.

To hold support materials and to provide better flow of medium through and around the supports, a novel stainless-steel basket was designed to fit into the fermenter. The basket, called the BioCage, holds support materials in four separate compartments, with provision for introduction of acid or base for pH control through a central channel, and with agitation at the base and at the center of the basket. Current design of the basket can be used for both natural (biofilm) or artificial (entrapped) immobilization techniques.

When repetitive fed-batch fermentations were performed with the empty basket in the fermenter, a hairy biofilm covered the outside of the empty basket. With fire brick supports in the basket, the bacterial biofilm formed preferentially on the fire bricks inside the basket.

In repetitive fed-batch fermentations, yields of propionic acid and acetic acid from substrate lactate have ranged from 63 to 79% and from 24 to 28%, respectively, with the higher yields obtained when biofilms were formed. Productivities for propionic and acetic acids have been relatively consistent at about 0.26 and 0.1 g/l/h, respectively.

Two immobilization methods, biofilm formation and calcium alginate entrapment, were compared in a mini reactor to determine the rates of substrate consumption and acid production per unit of the immobilized systems. Yield and productivity were similar in the biofilm and bead systems. Beads disintegrated after the first batch of the fermentation, most likely because sodium ions replaced calcium in the alginate matrix. Average substrate consumption and propionic acid production rates for reactors with biofilm and calcium alginate beads were 0.085 and 0.06 g/l/h, and 0.14 and 0.09 g/l/h, respectively. Acid production rate increased in sequential batches in the biofilm system, as the biofilm became better established.

Overall results indicate that *P. thoenii* P20 is an excellent biofilm former, and that biofilm fermentations can maintain high acid productivities even at low pH values.

Recommendations for future research

1. Attachment undoubtedly is a multifactorial process that needs further investigation. Studies should be performed with much smaller reactors to be able to follow the system parameters more accurately. On the other hand, biofilm systems for propionic acid production should also be scaled up to determine the most effective design to eliminate problems seen in

small reactors such as fluctuations in the gas flow that produce significant changes in flow patterns in the reactor.

2. As alternative substrates, inexpensive by-products such as whey, corn steep liquor, and glycerol should be investigated.
3. Surface of the fire bricks can be treated and/or coated with special chemicals (i.e., positively charged Cytodex III) to make the surfaces more attractive for cell attachment.
4. Genetic alteration of propionibacteria strains should be studied to increase propionic to acetic acid ratio and extracellular polysaccharide production. Production of some enzymes in the metabolic pathway might be altered by means of mutation or by changing the substrate composition to shift acid production in favor of propionic acid.
5. Biofilm formation by propionibacteria should be studied by means of confocal microscopy to analyze the three dimensional structure of the biofilm.
6. Regeneration of the cells in the biofilm should be studied to find the rates of detachment and attachment of the cells. Age of the biofilm at various locations or depths on the supports should be determined.
7. Extracellular polysaccharide (EPS) in the biofilm should be analyzed for complete component profile.
8. A cost analysis for support preparation is essential for further discussion.

APPENDIX A. SEM PHOTOGRAPHS

Figure 1. Scanning Electron Microscopy (SEM) pictures for thimbles as supports: A) Thimble surface before biofilm formation. Magnification: X100; bar=100μm. B) Thimble surface after full process of biofilm formation. Magnification: X100; bar=100μm. C) Thimble surface after full process of biofilm formation (cross-section view). Magnification: X480; 20μm. D) Thimbles after 24-h biofilm formation. Magnification: X4,000; bar=5μm. E) Thimbles after 24-h biofilm formation. Magnification: X10,000; bar=1.5μm. F) Thimbles after 24-h biofilm formation. Magnification: X20,000; bar=0.5μm.

A
B
C
D
E
F

Figure 2. Scanning Electron Microscopy (SEM) pictures for PCS (polypropylene + Corn starch + zein) and fire bricks: A) PCS surface before biofilm formation. Magnification: X100; bar=100μm. B) PCS surface after 24-h biofilm formation. Magnification: X4,000; bar=5μm. C) Fire brick surface before biofilm formation. Magnification: X480; 20μm. D) Fire bricks after full biofilm formation. Magnification: X470; bar=30μm. E) Fire bricks after 24-h biofilm formation. Magnification: X6,000; bar=1.5μm. F) Fire bricks after 24-h biofilm formation. Magnification: X6,000; bar=1.5μm.

A
B
D
E
F

Figure 3. Scanning Electron Microscopy (SEM) pictures for commercial supports, Type-Z and Type-CZ and PCS (Polypropylene + corn starch + zein): A) Type-Z biocarrier surface before biofilm formation. Magnification: X480; bar=20μm. B) Type-Z biocarrier after full process of biofilm formation (cross-section view). Magnification: X400; bar=30μm. C) Type-CZ biocarrier surface before biofilm formation. Magnification: X100; 100μm. D) Type-CZ biocarriers after 24-h biofilm formation. Magnification: X100; bar=100μm. E) PCS before biofilm formation. Magnification: X480; bar=20μm. F) PCS after 24-h biofilm formation. Magnification: X4,000; bar=5μm.

A
B
C
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APPENDIX B. ADDITIONAL PROCEDURES AND CALCULATIONS

Preparation of the microfiltration cartridge (Chapter 4)

Microfiltration cartridge was flushed with deionized distilled water (Milli-Q Reagent Water System, Millipore Corp., Bedford, Mass.) until about one liter of permeate had been collected for each square foot of membrane area. The permeate solution was not recycled, but discharged to the drain. To enhance the flushing process, water at 50°C with 100 ppm chlorine was used. The chlorine solution was prepared by adding 2 ml of household bleach, e.g., Clorox, which contains 5% chlorine per liter of feed water.

Special consideration was given to startup of high flux microfiltration membranes to avoid rapid gel layer formation and its associated flux decline. To achieve this, permeate ports were blocked during startup, so that the cross-flow velocity could be fully established. After opening permeate ports, the cross-flow port was gradually closed.

Cleaning of the microfiltration unit (Chapter 4)

Cleaning of the hollow-fiber cartridge was performed at low pressure and high velocity, at about 50°C. In an initial cleaning step, residual feed was flushed (one-pass) from the cartridge with clean, warm water (50°C). After the initial flushing, 0.2% Terg-A-Zyme® (Alconox, Inc., New York, NY) solution at 50°C, pH 9-10, was pumped through the cartridge for 1 h. Finally, clean water was pumped through the cartridge to remove any remaining Terg-A-Zyme. The

filtration module and fittings were then autoclaved at 121°C for 30 min. After cleaning, cartridge was stored dry when not in use. Prior to reuse, however, it was cleaned and conditioned. If the cartridge was stored for an extended time, the membrane (inside and outside) was exposed to 70% ethanol for one hour, drained, and flushed with water.

Determination of mini-reactor bead load (Chapter 4)

Previously in our laboratory, beads were put into a graduated cylinder up to 6.6 ml and the void volume was filled with water without changing the final total volume of 6.6 ml. The amount of water added (3 ml in this case) gave the void volume, the rest (3.6 ml) is registered as the bead volume.

Based on the diameter of the spherical beads (2.5 mm - volume = 0.00818 m^3/bead) and weight of one bead (0.0093 g), 1 g of mass corresponds to 0.88 ml beads and 1 ml of bead volume to 1.14 g beads. With this conversion factor, 3.6 ml bead corresponds to 4.1 g of beads. Initially we made our calculations based on 1% (w/v) of 700 ml reservoir as the amount of beads. If 4.1 g of beads corresponds to 3.6 ml beads, 7 g (1% (w/v) of the reservoir) of beads corresponds to 6.15 ml of beads and based on this value, the void volume is 5.16 ml. So, the total volume is 11.3 ml. Since this amount exceeded the volume of the mini reactor, we cut the volume in half to get approximately 0.5% (w/v) instead of 1% (w/v). Our final bead load was 5.7 ml with 3.1 ml of beads and 2.6 ml of void volume.

Immobilization by entrapment (Chapter 4)

Cells were immobilized in calcium alginate beads as described by Rickert (69) and Paik and Glatz (65). After several consecutive transfers, cells were grown for 48 h in sodium lactate broth. Harvest was accomplished by centrifugation of the culture at 9954 x *g* for 15 min. Harvested cells were resuspended in sterile 0.85% (w/v) NaCl solution to remove any nutrients present and again centrifuged at 9954 x *g* for 20 min. Pelleted cells were removed from the centrifuge bottles and weighed. For 1:1:6 (cells:saline:alginate) slurry mixtures, equivalent volumes of 0.85% sterile saline solution and pelleted cells were combined. The appropriate volume of sodium alginate solution (~2.5%) (medium gel strength, Sigma Chemical Co., St. Louis, MO) was slowly blended into the cell/saline mixture. Twenty-five ml of this slurry were extruded through a 22-gauge needle into 150 ml of 0.1 M $CaCl_2$ solution. The spherical, cell-loaded beads were incubated at 37°C for 90 min in 0.1 M $CaCl_2$ solution. After incubation the beads were transferred to 0.05 M $CaCl_2$ solution and stored at 4°C for 1-7 d before use.

APPENDIX C. MEDIA COMPARISONS

Problem and rationale

Fermentation processes depend on medium composition. Since the cost of the ingredients is a major factor in fermentation economics, optimum broth composition is a concern for a process designer.

Propionibacteria are fastidious about the feed and some essential growth factors are necessary (18, 19, 79, 80). Two defined medium recipes have been used in our lab for propionic acid fermentations: Fermentation broth (FB) and sodium lactate broth (NLB). To be able to compare the fermentation results, it was deemed necessary to perform a media comparison study. Even though this is not a comprehensive study, it provides an overall idea about the differences.

Approach

The FB and NLB recipes were tested at different levels of ingredients. Compositions of the media are given in Table 1. Sodium lactate and glucose concentrations were kept constant at 1% (v/v) and 2% (w/v), respectively.

Each combination was tested in triplicate in 50 ml volumes in screw-cap tubes. Tubes were inoculated with 2 ml of 24-h cultures of *P. theonii* and incubated at 32°C. Samples were taken at 12, 24, 36, and 48 hours of the fermentation to follow the microbial growth. Acid concentrations at the end of

fermentation were also determined by using HPLC. Results were statistically analyzed.

Table 1. Recipes for media comparisons

2% Constant Glucose				1% Constant Sodium Lactate			
1G	1% YE	2G	0.6% YE	1L	1% YE	2L	0.6% YE
	0.3% TSB		1% TSB		0.3% TSB		1% TSB
3G	0.6% YE	4G	1% YE	3L	0.6% YE	4L	1% YE
	0.3% TSB		1% TSB		0.3% TSB		1% TSB

G: 2% glucose, L: 1% lactate

Results and Discussion

Eight different medium compositions were compared by analyzing the averages of triplicate growth and acid production data (Figure 1). Glucose with 1% (w/v) TSB and 1% (w/v) YE gave the best microbial growth for both groups (Figure 1a).

Changes in biomass throughout the 48-h fermentation clearly demonstrated the differences in utilization of medium components. Even though microbial growth was the same for all tubes at 12 h, by 48 h growth in tubes with glucose had almost doubled and reached higher levels than did growth in tubes with lactate. However, acid production followed a different pattern. At 48 h acid concentrations were equal or greater in the tubes with

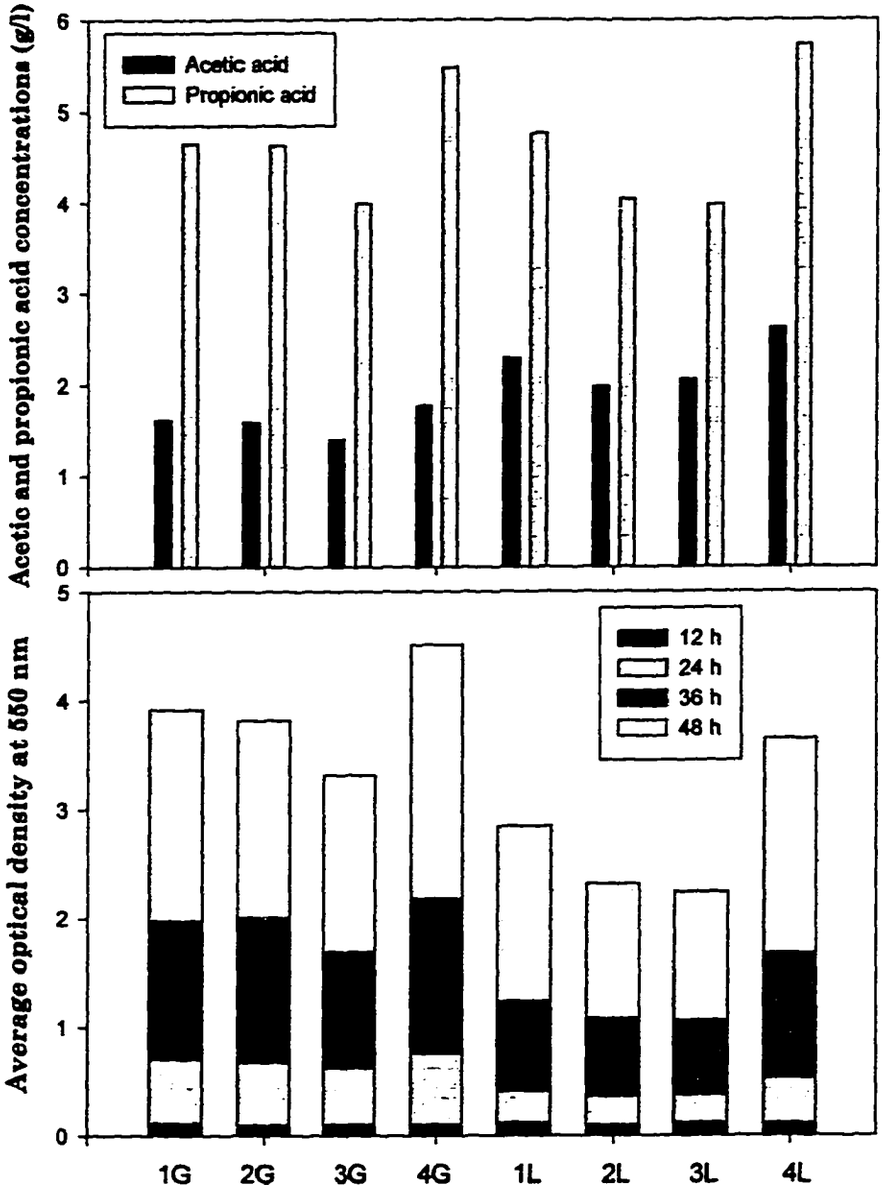

Figure 1. Comparison of different media composition in terms of (a) final acid concentrations and (b) microbial growth. G: 2% glucose, L: 1% lactate, 1: 1% YE & 0.3% TSB, 2:1% TSB & 0.6% YE, 3: 0.6% YE & 0.3% TSB, 4: 1% TSB & 1% YE.

lactate as a carbon source as in the tubes with glucose. This suggests that more glucose goes toward cell growth and maintenance while more lactate is converted to acetic and propionic acids.

The effect of trypticase soy broth can be seen by comparing 1G to 4G, 1L to 4L, 2G to 3G, and 2L to 3L. The effect of yeast extract can be seen by comparing 1G to 3G, 1L to 3L, 2G to 4G, and 2L to 4L.

To determine if statistically significant differences existed among these trials, contrasts were created and all data were analyzed by the same procedure (3 replicates were used for each treatment). Overall differences between two groups (L & G) and between sampling times were significantly different at α=0.05.

Differences between 1 and 2 and 2 and 3 were not significant for glucose and lactate, respectively. The results were significantly different for the rest of the combinations with an exception of 2 (glucose) and 4 (lactate) cross-comparison at α=0.05 level (Table 2).

Conclusions

Results showed that the amount of yeast extract and trypticase soy broth in the media have significant effects on cell growth. With glucose as carbon source, lowering yeast extract from 1 to 0.6% (w/v) and increasing TSB from 0.3 to 1% (w/v) did not significantly affect growth.

Table 2. Statistical comparison of the microbial growth between different medium compositions.

Contrasts	Contrast SS	Mean Square	Probability	Significance at α=0.05
between groups[a]	1.9057570	1.9057570	0.0001	S
among times[b]	35.0503443	35.0503443	0.0001	S
G1 VS G2	0.0038760	0.0038760	0.2899	NS
G1 VS G3	0.1380167	0.1380167	0.0001	S
G1 VS G4	0.1320167	0.1320167	0.0001	S
G1 VS L1	0.4301404	0.4301404	0.0001	S
G1 VS L2	0.9660094	0.9660094	0.0001	S
G1 VS L3	1.0542042	1.0542042	0.0001	S
G1 VS L4	0.0275404	0.0275404	0.0060	S
G2 VS G3	0.0956344	0.0956344	0.0001	S
G2 VS G4	0.1811344	0.1811344	0.0001	S
G2 VS L1	0.3523527	0.3523527	0.0001	S
G2 VS L2	0.8475042	0.8475042	0.0001	S
G2 VS L3	0.9302344	0.9302344	0.0001	S
G2 VS L4	0.0107527	0.0107527	0.0802	NS
G3 VS G4	0.5400000	0.5400000	0.0001	S
G3 VS L1	0.0808520	0.0808520	0.0001	S
G3 VS L2	0.3737510	0.3737510	0.0001	S
G3 VS L3	0.4293375	0.4293375	0.0001	S
G3 VS L4	0.0422520	0.0422520	0.0008	S
G4 VS L1	1.0387520	1.0387520	0.0001	S
G4 VS L2	1.8122510	1.8122510	0.0001	S
G4 VS L3	1.9323375	1.9323375	0.0001	S
G4 VS L4	0.2801520	0.2801520	0.0001	S
L1 VS L2	0.1069335	0.1069335	0.0001	S
L1 VS L3	0.1375620	0.1375620	0.0001	S
L1 VS L4	0.2400000	0.2400000	0.0001	S
L2 VS L3	0.0019260	0.0019260	0.4546	NS
L2 VS L4	0.6673335	0.6673335	0.0001	S
L3 VS L4	0.7409620	0.7409620	0.0001	S

S : Difference is significant
NS : Difference is not significant
[a] Comparison of two groups with lactate and glucose (L vs G)
[b] Comparison of growth in time

Since propionic and acetic acid concentrations were determined only at the end of the fermentation, it was not possible to analyze acid production throughout the fermentation.

REFERENCES

1. **Atkinson, B. and A. J. Knights.** 1975. Microbial film fermenters: their present and future applications. Biotechnol. Bioeng. 17:1245-1267.

2. **Balaguer, M. D., M. T. Vicent, and J. M. Paris.** 1992. Anaerobic fluidized bed reactor with sepiolite as support for anaerobic treatment of vinasse. Biotechnol. Lett. 14:433-438.

3. **Bitton, G. and K. C. Marshall.** 1980. Adsorption of microorganisms to surfaces. Wiley-Interscience, New York.

4. **Boyaval, P., C. Corre, and M-N. Madec.** 1994. Propionic acid production in a membrane bioreactor. Enzyme Microb. Technol. 16:883-886.

5. **Bryers, J. D. and W. G. Characklis.** 1982. Process governing primary biofilm formation. Biotechnol. Bioeng. 24:2451-2476.

6. **Characklis, W. G.** 1981. Fouling biofilm development: a process analysis. Biotechnol. Bioeng. 23:1923-1960.

7. **Characklis, W. G. and K. C. Marshall.** 1990. Biofilms. John Wiley & Sons, Inc., New York.

8. **Cheetam, P. S. J., K. W. Blunt, and C. Bucke.** 1979. Physical studies on cell immobilization using calcium alginate gels. Biotechnol. Bioeng. 21:2155-2168.

9. **Clausen, E. C.** 1978. The kinetic and economic effects of separating the

stages of the anaerobic digestion process. Ph.D. dissertation, University of Missouri, Rolla.

10. **Clausen, E. C. and J. L. Gaddy.** 1984. Organic acids from biomass by continuous fermentation. Chem. Eng. Prog. **80**:59-63.

11. **CMR, Chemical Marketing Reporter.** 1994. Chemical profiles: propionic and acetic acids. Schnell Publishing Company, Inc.

12. **CMR, Chemical Marketing Reporter.** 1996. Chemical prices. September issue. Schnell Publishing Company, Inc.

13. **Colomban, A., L. Roger, and P. Boyaval.** 1993. Production of propionic acid from whey permeate by sequential fermentation, ultrafiltration, and cell recycling. Biotechnol. Bioeng. **42**:1091-1098.

14. **Costerton, J. W., G. G. Geesey, and K. J. Cheng.** 1978. How bacteria stick. Sci. Amer. **238**:86.

15. **Crueger, W. and A. Crueger.** 1984. *In* T. D. Brock (ed.), Biotechnology: a textbook of industrial microbiology. Science Tech, Inc., Madison, WI.

16. **Das, D., N. R. Gaidhani, K. Murari, and P. S. Gupta.** 1993. Ethanol production by whole cell immobilization using lignocellulosic materials as solid matrix. J. Ferment. Bioeng. **75**:132-137.

17. **Dean, J. A. (ed.).** 1979. Lange's handbook of chemistry, 12th ed. McGraw-Hill Book Co, New York.

18. **Delwiche, E. A.** 1949. Vitamin requirements of the genus

Propionibacterium. J. Bacteriol. **58**:395-398.

19. Delwiche, E. A. 1950. A biotin function in succinic acid decarboxylation by *Propionibacterium pentosaceum*. J. Bacteriol. **59**:439-442.

20. Demirci, A., A. L. Pometto III, and K. E. Johnson. 1993a. Lactic acid production in a mixed-culture biofilm reactor. Appl. Environ. Microbiol. **59**:203-207.

21. Demirci, A., A. L. Pometto III, and K. E. Johnson. 1993b. Evaluation of biofilm reactor solid support for mixed-culture lactic acid production. Appl. Environ. Microbiol. **38**:728-733.

22. Demirci, A. and A. L. Pometto III. 1995. Repeated-batch fermentation in biofilm reactors with plastic-composite supports for lactic acid production. Appl. Microbiol. Biotechnol. **43**:585-589.

23. Durham, D. R., L. C. Marshall, J. G. Miller, and A. B. Chmurny. 1994. Characterization of inorganic biocarriers that moderate system upsets during fixed-film biotreatment processes. Appl. Environ. Microbiol. **60**:3329-3335.

24. Eikmeier, H. R. and H. J. Rehm. 1987. Stability of calcium-alginate during citric acid production of immobilized *Aspergillus niger*. Appl. Microbiol. Biotechnol. **26**:105-111.

25. El-Hagarawy, I. S., W. L. Slatter, and W. J. Harper. 1957. Organic acid production by propionibacteria. I. Effect of strains, pH, carbon source, and intermediate fermentation products. J. Dairy Sci. **40**:579-587.

26. Ernst, C. and H-J. Rehm. 1995. Development of a continuous system for the degradation of a cyanuric acid by adsorbed *Pseudomonas sp.* NRRL B-12228. Appl. Microbiol. Biotechnol. **43**:150-155.

27. Fletcher, M. and K. C. Marshall. 1982. Are solid surfaces of ecological significance to aquatic bacteria? pp. 199-236. *In* K. C. Marshall (ed.), Advances in microbial ecology, Vol. 6. Plenum Press, New York.

28. Geertsema-Doornbusch, G. I., H. C. Van der Mei, and H. J. Busscher. 1993. Microbial cell surface hydrophobicity. The involment of electrostatic interactions in microbial adhesion to hydrocarbons (MATH). J. Microbiol. Meth. **18**:61-68.

29. Geldreich, E. E. 1988. Coliform noncompliance nightmares in water supply distribution systems, Chapter 3. *In* Water quality: a realistic perspective. U. of Michigan, College of Engineering; Michigan Section, American Water Works Association; Michigan Water Pollution Control Association; Michigan Department of Public Health, Lansing, MI.

30. Glatz, B. A. 1992. The classical propionibacteria: their past, present, and future as industrial organisms. ASM News. **58**(4):197-201.

31. Goncalves, L. M. D., M. T. O. Barreto, A. M. B. R. Xavier, M. J. T. Carrondo, J. Klein. 1992. Inert supports for lactic acid fermentation - a technology assesment. Appl. Microbiol. Biotechnol. **38**:305-311.

32. Grote, W. and P. L. Rogers. 1985. The susceptibility to contamination of *Zymomonas mobilis* process for ethanol production. J. Ferment. Technol.

63:287-290.

33. **Hahn-Hagerdal, B. and B. Mattiasson.** 1982. Azide sterilization of fermentation media, ethanol production from glucose using immobilized *Saccharomyces cerevisiae*. Eur. J. Appl. Microbiol. Biotechnol. **14**:140-143.

34. **Heitkamp, M. A., W. J. Adams, and V. Camel.** 1993. Evaluation of five biocarriers as supports for immobilized bacteria: comparative performance during high chemical loading, acid shocking, drying and heat shocking. Environ. Toxicol. Chem. **12**:1-11.

35. **Heitkamp, M. A., W. J. Adams, and L. E. Hallas.** 1992. Glyphosate degradation by immobilized bacteria: laboratory studies showing the feasibility for glyphosate removal from wastewater. Can. J. Microbiol. **38**:921-928.

36. **Heitkamp, M. A., V. Camel, T. J. Reuter, and W. J. Adams.** 1990. Biodegradation of p-nitrophenol in an aqueous waste stream by immobilized bacteria. Appl. Environ. Microbiol. **56**:2967-2973.

37. **Hettinga, D. H. and G. W. Reinbold.** 1972. The propionic acid bacteria - a review, II. Metabolism. J. Milk Food Tech. **35**:358-372.

38. **Ho, K-L. G, A. L. Pometto III, and P. N. Hinz.** 1997. Optimization of L-(+)-lactic acid production by ring and disc plastic-composite supports through repeated-batch biofilm fermentation. Appl. Environ. Microbiol. **63**:2533-2542.

39. Ho, K-L. G, A. L. Pometto III, P. N. Hinz, and A. Demirci. 1997. Nutrient leaching and end product accumulation in plastic-composite supports for L-(+)-lactic acid biofilm fermentation. Appl. Environ. Microbiol.. **63**:2524-2532.

40. Ho, K-L. G, A. L. Pometto III, P. N. Hinz, J. S. Dickson, and A. Demirci. 1997. Ingredient selection for plastic-composite supports for L-(+)-lactic acid biofilm fermentation by *Lactobacillus casei* subsp. *rhamnosus*. Appl. Environ. Microbiol. **63**:2516-2523.

41. Hobson, P. N. 1988. Problems and solutions in full-scale biogas plants concerned with feedstocks and effluents. pp. 425-433. *In*: E. R. Hall and P. N. Hobson (eds.), Anaerobic digestion. Pergamon, Oxford.

42. Hsu, S. T. and S-T. Yang. 1991. Propionic acid fermentation of lactose by *Propionibacterium acidipropionici*: effects of pH. Biotechnol. Bioeng. **38**:571-578.

43. Hsu, Y. and W. K. Shieh. 1993. Startup of anaerobic fluidized bed reactors with acetic acid as the substrate. Biotechnol. Bioeng. **41**:347-353.

44. Huag, A., B. Larsen, and O. Smidsrod. 1974. Uronic acid sequence in alginate from different sources. Carbohydrate Res. **32**:217-225.

45. Jack, T. R. and J. E. Zajic. 1977. The immobilization of whole cells. Adv. Biochem. Eng. **5**:125-145.

46. Kawabata, N., H. Hatanaka, and H. Odaka. 1995. Continuous production

of L-aspartic acid from ammonium fumarate using immobilized cells by capture on the surface of nonwoven cloth coated with a pyridinium-type polymer. J. Ferm. Bioeng. **79**:317-322.

47. Kida, K., S. Morimura, Y. Sonoda, M. Obe, and T. Kondo. 1990. Support media for microbial adhesion in an anaerobic fluidized-bed reactor. J. Ferm. Bioeng. **69**:354-359.

48. Kolot, F. B. 1984. Immobilized cells for solvent production. Process Biochem. **19**:7-13.

49. Kunduru, M. R. and A. L. Pometto III. 1996a. Evaluation of plastic-composite supports for enhanced ethanol production in biofilm reactors. J. Ind. Microbiol. **16**:241-248.

50. Kunduru, M. R. and A. L. Pometto III. 1996b. Continuous ethanol production by *Zymomonas mobilis* and *Saccharomyces cerevisiae* in biofilm reactors. J. Ind. Microbiol. **16**:249-256.

50a. Kunduru, M. R. 1994. Development of a biofilm bioreactor for enhanced ethanol production. Ph.D. dissertation. Iowa State University, Ames, IA.

51. Kurt, M., I. J. Dunn, and J. R. Bourne. 1987. Biological denitrification of drinking water using autotrophic organisms with H_2 in a fluidized-bed biofilm reactor. Biotechnol. Bioeng. **29**:493-501.

52. Lee, I. H., A. G. Fredericksen, and H. M. Tsuchiya. 1974. Diauxic growth of *Propionibacterium shermanii*. Appl. Microbiol. **28**:831-835.

53. Lee, I. H., A. G. Fredericksen, and H. M. Tsuchiya. 1976. Dynamics of mixed cultures of *Lactobacillus plantarum* and *Propionibacterium shermanii*. Biotechnol. Bioeng. 18:513-526.

54. Lewis, V. P. and S-T. Yang. 1992. A novel extractive fermentation process for propionic acid production from whey lactose. Biotechnol. Bioeng. 23:1517-1525.

55. Lewis, V. P. and S-T. Yang. 1992. Continuous propionic acid fermentation by immobilized *Propionibacterium acidipropionici* in novel packed-bed bioreactors. Biotechnol. Bioeng. 40:465-474.

56. Meta-Alvarez, J. and P. Llabres. 1988. A kinetic study of the anaerobic digestion of piggery waste using down-flow stationary fixed film reactors. Appl. Microbiol. Biotechnol. 28:311-315.

57. McGhee, J. E., G. St. Julian, R. W. Detroy, and R. J. Bothast. 1982. Ethanol production by immobilized *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, and *Zymomonas mobilis*. Biotechnol. Bioeng. 24:1155-1163.

58. Molin, G. 1981. The impact of dilution rate and attached growth on steady-state characteristics of *Pseudomonas putida*. Eur J. Appl. Microbiol. Biotechnol. 13:102-106.

59. Mozes, N., F. Marchal, M. P. Hermesse, J. L. Van Haecht, L. Reuliaux, A. J. Leonard, and P. G. Rouxhet. 1987. Immobilization of

microorganisms by adhesion: interplay of electrostatic and nonelectrostatic interactions. Biotechnol. Bioeng. **30**:439-450.

60. Nagashima, M., M. Azuma, S. Noguchi, K. Inuzuka, and B. Samejima. 1984. Continuous ethanol fermentation using immobilized yeast cells. Biotechnol. Bioeng. **26**:992-997.

61. Nishizawa, K., M. Toriyama, T. Suzuki, Y. Kawamoto, Y. Yokogawa, and H. Nagae. 1993. Effects of the surface wettability and zeta potential of bioceramics on the adhesiveness of anchorage-dependent animal cells. J. Ferm. Bioeng. **75**:435-437.

62. Ohta, T., J. C. Ogbonna, H. Tanaka, and M. Yajima. 1994. Development of a fermentation method using immobilized cells under unsterile conditions. 2. Ethanol and L-lactic acid production without heat and filter sterilization. Appl. Microbiol. Biotechnol. **42**:246-250.

63. Ozadali, F., B. A. Glatz, and C. E. Glatz. 1996. Fed-batch fermentation with and without on-line extraction for propionic and acetic acid production by *Propionibacterium acidipropionici*. Appl. Microbiol. Biotechnol. **44**:710-716.

64. Paik, H-D. 1992. Immobilization of cells of *Propionibacterium* strains and production of propionic acid by immobilized cells. M.S. thesis, Iowa State University, Ames, IA.

65. Paik, H-D. and B. A. Glatz. 1994. Propionic acid production by

immobilized cells of a propionate-tolerant strain of *Propionibacterium acidipropionici*. Appl. Microbiol. Biotechnol. **42**:22-27.

66. Park, S-K. 1991. Production of propionic acid by *Propionibacterium acidipropionici* in various fermentation processes. M.S. thesis, Ames, Iowa State University, Ames, IA.

67. Parker, J. A. and N. J. Moon. 1982. Interaction of *Lactobacillus* and *Propionibacterium* in mixed culture. J. Food Protect. **45**:326-330.

68. Playne, M. J. 1985. Propionic and butyric acids, p.731-759. *In* Comprehensive biotechnology, Vol. 3 (ed. Murray Moo-Young), Pergamon Press, Oxford.

69. Rickert, D. A. 1996. Evaluation of efforts to improve organic acid production by calcium alginate-immobilized propionibacteria. M.S. thesis, Iowa State University, Ames, IA.

70. Rose, A. H. 1961. Industrial microbiology, Butterworths, London, England.

71. Roy, D., J. Goulet, and A. LeDuy. 1987. Continuous production of lactic acid from whey permeate by free and calcium alginate entrapped *Lactobacillus helveticus*. J. Dairy Sci. **70**:506-513.

72. Scott, C. D. 1987. Immobilized cells: a review of recent literature. Enzyme Microb. Technol. **9**:66-73.

73. Sherman, J. M. and R. H. Shaw. 1923. The propionic acid fermentation of lactose. J. Biol. Chem. **56**:695-700.

74. Shieh, W. K. and J. D. Keenan. 1986. Fluidized bed biofilm reactor for wastewater treatment. *In* pp 132-169, A. Fiechter (Ed.), Advances in Biochemical Engineering/Biotechnology. Springer-Verlag, Berlin.

75. Solichien M. S., D. O'Brien, E. G. Hammond, and C. E. Glatz. 1994. Membrane-based extractive fermentation to produce propionic and acetic acids: toxicity and mass transfer considerations. Enz. Microbiol. Technol. 17:23-31.

76. Stiles, H. R. and P. W. Wilson. 1933. Production of propionic acid. US Pat. 1,932,755 (Chem. Abst., 28:565).

77. Strenrous, S. L., Y. Y. Linko, and P. Linko. 1982. Production of L-lactic acid with immobilized *Lactobacillus delbrueckii*. Biotechnol. Lett. 4:159-164.

78. Tanaka, H., T. Ohta, S. Harada, J.C. Ogbonna, and M. Yajima. 1994. Development of a fermentation method using immobilized cells under unsterile conditions. 1. Protection of immobilized cells against anti-microbial substances. Appl. Microbiol. Biotechnol. 41:544-550.

79. Tatum, E. L., W. H. Peterson, and E. B. Fred. 1936. Essential growth factors for propionic acid bacteria: I. Sources and fractionation. J. Bacteriol. 32:157-166.

80. Tatum, E. L., H. G. Wood, and W. H. Peterson. 1936. Essential growth

factors for propionic acid bacteria: II. Nature of the Neuberg precipitate fraction of potato: replacement of ammonium sulfate or certain amino acids. J. Bacteriol. **32**:167-174.

81. Thonart P., M. Custine, and M. Paguot. 1982. Zeta potential of yeast cells: application in cell immobilization. Enzyme Microb. Technol. **4**:191-194.

82. Tramper, J. 1990. Conversion by immobilized cells versus traditional fermentations. *In* pp 1-23, J. A. M. de Bont, J. Visser, B. Mattiasson, and J. Tamper (Eds.), physiology of immobilized cells, proceedings of an international symposium held at Wageningen, The Netherlands, Elsevier Science Publishers B. V., Amsterdam.

83. Trevan, M. D. 1980. Immobilized enzymes. Wiley, Chichester, UK.

84. Turakhia, M. H. and W. G. Characklis. 1989. Activity of *Pseudomonas aeruginosa* in biofilms: effect of calcium. Biotechnol. Bioeng. **33**:406-414.

85. Van Oss, C. J. 1991. Interaction forces between biological and other polar entities in water: how many different primary forces are there? J. Dis. Sci. Tech. **12**:201-219.

86. Wayman, M., M. E. Martin, and G. Graf. 1962. Propionic acid fermentation. US Pat. 3,067,107 (Chem Abst., 58:7337).

87. Weuster-Botz, D. A. Aivasidis, and C. Wandrey. 1993. Continuous

ethanol production by *Zymomonas mobilis* in a fluidized bed reactor. Part II: Process development for the fermentation of hydrolysed B-starch without sterilization. Appl. Microbiol. Biotechnol. **39**:685-690.

88. **Windholz, M.** (ed.) 1976. The Merck Index, 9th edition. Merck & Co., Inc., Rahway, NJ.

89. **Wood, H. G.** 1981. Metabolic cycles in the fermentation by propionic acid bacteria. Curr. Top. Cell Regul. **18**:255-287.

90. **Wood, H. G., R. W. Stone, and C. H. Werkman.** 1937. The intermediate metabolism of the propionic acid bacteria. Biochem. J. **31**:349-359.

91. **Zellner, G., E. Feuerhake, H-J. Jordening, A. J. L. Macario, E. Conway de Macario.** 1995. Denitrifying and methanogenic bacteria in the biofilm of a fixed-film reactor operated with methanol/nitrate demonstrated by immunofluorecence and microscopy. Appl. Microbiol. Biotechnol. **43**:566-571.

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