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Characterization, selection, and verification of plastic composite-supports for L(+)-lactic acid biofilm-fermentation

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Characterization, selection, and verification of plastic composite-supports for L(+)-lactic acid biofilm-fermentation

by

Kai-Lai Grace Ho

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Major Professor: Anthony L. Pometto III

Iowa State University

Ames, Iowa

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Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Kai-Lai Grace Ho

has met the dissertation requirements of Iowa State University

Committee Member

Committee Member

Committee Member

Committee Member

Major Professor

For the Major Program

For the Graduate College

To my parents, Tak-Him Ho and Hoi-Man Pang,

husband, Wai-Ki Frankie Lam, and

daughter, Yat-Yee Jacqueline Lam.

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ABSTRACT

Discs plastic composite-supports (PCS) containing 50% agricultural products and 50% polypropylene (w/w) were produced by twin-screw high-temperature extrusion. PCS properties and ingredients (oat hulls, soybean hulls, yeast extract, soybean flour, dried red blood cells, bovine albumen, and/or salts) for *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443) biofilm formation and L(+)-lactic production was evaluated by two replications of a 2^{5-1} design.

Soybean hulls, and salts decreased the hydrophobicity of PCS ($P < 0.0001$) and enhanced cells attachment ($P < 0.03$). Yeast extract enhanced the growth of free and attached cells in minimal medium ($P < 0.0001$). Bovine albumen blended with soybean hulls, yeast extract, soybean flour, and salts had the highest lactic acid concentration in the first (7.6 g/L) and twentieth (1.4 g/L) simulated repeated-batch fermentation. Under all conditions, suspended cells and polypropylene discs control gave negligible lactic-acid production and cell density.

PCS blended with bovine albumen, red blood cells, and soybean flour-leached nutrients gradually (20 - 30% initial leached nitrogen) and could still maintain 1 g/L lactic acid and cell density (absorbance at 620 nm:0.4-0.6) after the twentieth 20-mL simulated-repeated-batch fermentation. Polypropylene discs under all circumstances gave negligible lactic acid production and cell density. Lactic acid accumulation in PCS was shown to be mainly due to absorption and had no correlation with lactic acid production or biofilm formation.

PCS rings and discs with 35% soybean hulls, 5% yeast extract, 5% soybean flour, 5% dried bovine albumen, and 50% polypropylene were selected for long-term biofilm repeated-batch fermentation at controlled pH (5) and temperature (37°C). The viable cells count on the PCS surface in 0.2, 0.4, and 0.8% yeast extract (YE) Lactic-Acid-Fermentation (LAF) medium (8% glucose) was 7.1×10^9, 8.5×10^9, and 2.4×10^{10} cfu/g PCS, respectively. PCS-bioreactors in 0.4 and 0.8% YE LAF medium shortened the lag time by 3-fold and 6-fold, respectively. PCS-bioreactors, at all YE concentration, increased lactic acid productivity by 40-70 %. PCS-bioreactors' total fermentation time with 0.2, 0.4, and 0.8% YE LAF medium were 1.4, 2.1, and 2.6 times faster than that of the control (suspended cells bioreactor), respectively. PCS-bioreactors had its fastest productivity (4.26 g/L/h) at 10% starting glucose, whereas the control (2.78 g/L/h) was at 8%. PCS biofilm lactic-acid fermentation can drastically improve fermentation rate under reduced complex nutrient addition.

GENERAL INTRODUCTION

Introduction

The global market of lactic acid has been estimated to be growing at about 3 - 5 %

annually, and lactic acid has been called a 'commodity chemical sleeping giant' due to its

large potential market in the production of biodiegradable polylacticde polymers for the

packaging industry [43]. Microbial fermentation is the only source for producing optically-

pure lactic acid isomers. Hence, in order for lactic acid to be competitive in the plastic

commodity market, there is a need to develop advanced fermentation processes which yield a

relatively pure and high lactic-acid concentration solution.

Lactic acid production rates and concentration can be increased by increasing cell

density in the fermentor. Cell immobilization is a common way to increase cell density,

although industrial application of cell immobilization with calcium alginate beads and

polyacrylamide gels are few. This is mainly due to the high cost of immobilization, mass-

transfer limitations, lack of stability of the biocatalysts, and changes in product pattern of

reactions catalyzed by certain immobilized cells [43].

Biofilms are a natural form of cell immobililzation that results from microbial

attachment to solid supports in submerged environment [18]. This increases the cell density

and enables the biofilm population to withstand stresses such as pH change and starvation.

Attachment of cells on supports to form biofilm depends largely on the formation of

extracellular polysaccharides and surface charge between the solid surface and the microorganisms [19]. Studies by Van Loosdrecht et al. [80] demonstrated that measurement of hydrophobicity of cell surfaces by contact-angle method gave more consistent results than methods such as the hexadecane test and partitioning of cells in the two-phase polyethylene glycol and dextran system.

Previous studies in our laboratory had successfully proved that biofilm fermentation with plastic-composite supports (PCS) chips containing 75% polypropylene (PP) and 25% agricultural material (w/w) benefitted lactic acid production. In pure- and mixed-culture continuous fermentation, 30 and 35 g/L/h productivity was achieved, respectively [25]. In addition, the PCS chips were shown to be effective in long-term (more than 2 months) repeated-batch lactic acid biofilm fermentation with both pure and mixed cultures [24]. However, medium channelling and clumping of cells among the PCS chips interfered with medium mixing, pH control, and ultimately, lactic acid production. Hence, the purpose of this research is to produce a new type of PCS that could enhance lactic acid production with no clumping and medium channelling problems.

The specific objectives of this study were to develop method in producing ring and disc PCS containing 50 % polypropylene and 50 % agricultural products; to select the best PCS blend by the fractional factorial design method, to develop methods in evaluating the biofilm population, the nutrients leaching property, the hydrophobicity, the porosity, and lactic acid absorption property of the PCS; to verify the performance of the best PCS blend in long-term fermentation; and to evaluate factors such as yeast extract concentration, starting glucose concentration, and medium recyling rate for optimizing lactic acid fermentation.

Dissertation organization

This dissertation follows an alternative format and is divided into three papers. Each paper contains Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgement, and References with the tables and figures included at the end . The papers are written to conform to the specifications of Applied and Environmental Microbiology, the journal to which the papers have been submitted. A general introduction, chapters including a literature review, and a general conclusion are also included. References cited in those chapters follow the general conclusion. All experiments, data collection, and data analysis were performed by the candidate.

Literature review

Lactic acid

Lactic acid or 2-hydroxypropionic acid is a naturally occurring organic acid that can be produced by fermentation or chemical synthesis. It is also a major metabolic intermediate in most living organisms from anaerobic prokaryotes to human beings. Lactic acid was first isolated from sour milk by the Swedish chemist Scheel in 1780 [53]. It is one of the smallest molecule that exists in two optically active configurations, the L(+)- and D(-)-isomer (Fig.1).

```
        COOH                          COOH
          |                             |
          |                             |
  H ———— C ———— OH          OH ———— C ———— H
          |                             |
          |                             |
        CH₃                           CH₃
```

D(-)-Lactic acid   **L(+)-Lactic acid**
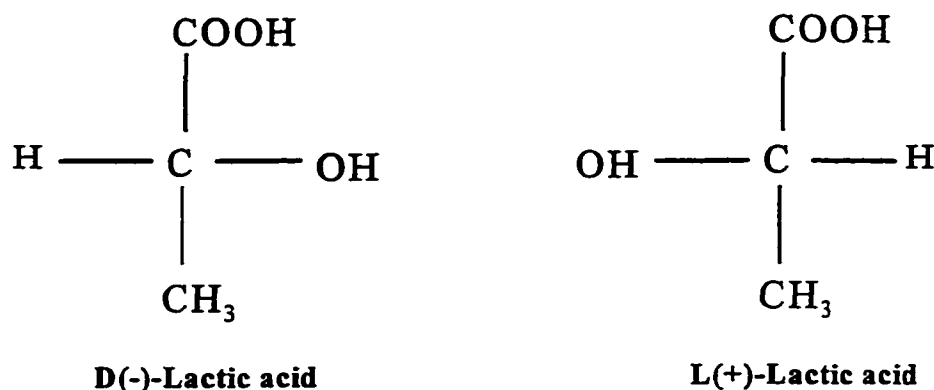
Fig. 1 Enantiomers of lactic acid [82].

Economic importance of lactic acid

The first successful uses of lactic acid in the leather and textile industries began about

1894 [33] and production levels were about 5 tons/yr [40]. Technical-grade lactic acid was

used as a textile printing developer, and as an acidulent for deliming hides and dying wool.

In 1942, about half of the 2.7×10^3 kg/yr produced in the United States was used by the

leather industry, and an emerging use in food products consumed about 20% [31]. In 1982,

world-wide production of lactic acid was $2.4 - 2.8 \times 10^4$ kg/yr. More than 50% of the lactic

acid produced was used in food as an acidulent and a preservative. The production of

stearoyl-2-lactylates consumed another 20%. The rest of the lactic acid was used by the

pharmaceutical industry and other industrial applications. In the 1990s, approximately 4 x

10^4 tons of lactic acid is produced annually. The major worldwide producers of lactic acid

are C.V. Chemie Combinatie Amsterdam, the Netherlands; Industria Quimica de Sintesis y

Fermentacoes, Brazil; Luis Ayuso S.A., Spain; Mushaimo Chemical Lab, Ltd., Japan; and

Sterling Chemicals Inc., United States [43, 22]. With the joining of Ecological Chemical

Products (EcoChem), Archer Daniels Midland Company, and Cargill into the manufacturer

list during the 1990's, United States has shifted from a lactic acid importer to a lactic-acid

self-sufficient nation with export potential [43].

Lactic acid and the food industry

The use of lactic acid in food and food-related applications accounts for

approximately 85% of the lactic acid demand in the United States. The nonvolatile and

odorless lactic acid is classified as GRAS (generally recognized as safe) food additive by the

FDA in the U.S.

Lactic acid is a very good preservative and pickling agent for sauerkraut, olives, and

pickled vegetables. It is also used as an acidulent, a flavoring agent, a pH buffering agent or

an inhibitor of bacterial spoilage in a wide variety of processed foods, such as candy, breads,

and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies,

mayonnaise, and processed eggs [82].

More than 50% of the lactic acid for food-related uses goes to the production of

emulsifying agents, especially for the bakery goods. The four important emulsifying agents

from lactic acid are calcium and sodium, stearoyl-2-lactylate, glyceryl lactostearate, and

glyceryl lactopalmitate [82]. Calcium stearoyl lactylates is a very good dough conditioner,

and sodium stearoyl-2-lactylate is both a conditioner and an emulsifier for yeast-leavened

bakery products. The glycerates and palmitates are used in cake mixes and other bakery

liquid shortenings. The manufacture of these emulsifiers requires heat-stable lactic acid; thus

only the synthetic or the heat-stable fermentation grades are used for this application [22].

An emerging new use for lactic acid or its salts is in the disinfection and packaging of carcasses. Lactic acid exerts both a bactericidal and a bacteriostatic effect that results in extended shelf life of meat. According to Snijders et al. [76], the immediate bactericidal effect of lactic acid decontamination on beef, veal, and pork carcasses reduced the aerobic plate count (APC) by 1.5 $\log_{10}$ per cm^2. Smulders and Woolthuis [75] reported a 62 % increased in APC reduction at 14 d postmortem, indicating some delayed bacteriostatic effects of lactic acid. Hot lactic acid spray immediately after dehiding or after evisceration were also proved to be effective in lowering the APC, *Salmonella* and *Listeria* population on beef carcasses [3]. Siragusa and Dickson [72, 73] had demonstrated a better reduction in *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli* O157:H7 by applying lactic acid contained in calcium alginate gel to lean-beef tissue surfaces. The addition of aqueous solutions of lactic acid and its salts during the processing of poultry and fish can also increase the shelf life and reduce the growth of anaerobic spoilage organisms such as *Clostridium botulinum* [22].

Applications of lactic acid in other industries

Lactic acid and ethyl lactate have long been used in pharmaceutical and cosmetic applications and formulations, particularly in topical ointments, lotions, and biodegradable polymers for medical application (such as surgical sutures, controlled-release drugs, and prostheses). The calcium salt is widely used for calcium-deficiency therapy and as an effective anti-caries agent. As humectants in cosmetic applications, the lactates are often superior to natural products and more effective than polyols. Ethyl lactate is the active

ingredient in many anti-acne preparations. In addition, lactic acid is also a terminating agent

for phenol-formaldehyde resins, an alkyl resin modifier, a solder flux, a lithographic, and an

ingredient in adhesive formulations, electroplating baths, and detergent builders [22, 82].

Future markets of lactic acid

In the market lactic acid exists in the form of three different concentrations (50%,

80% and 88% solutions) and grades (technical, food, and pharmaceutical). At present, the

prices for the 88% solution for food grade and technical grade is $1.15/lb and $1.12/lb,

respectively [7]. The global market of lactic acid has been estimated to be growing at about 3

- 5 % annually, and lactic acid has been called a "commodity chemical sleeping giant" due to

its large potential market in the production of biodegradable polylactide polymers for the

packaging industry [52].

There is an increased interest in degradable plastics because of environmental

concerns over plastics disposal. Unlike plastic beverage bottles, certain disposables, such as

contaminated food packaging, diapers, hospital wastes, and feminine hygiene products, are

not suitable for collecting and recycling. Conventional plastic materials are not easily

degraded in the environment because of their high molecular weight and hydrophobic

character. The polyesters from lactic acid and lactide were found to be degradable by

moisture. This allows the application of polylactide in producing plastics that do degrade in

the environment [43]. Because of the nonvolatile, nontoxic, and degradable properties, the

use of lactate esters as 'green' solvents is also considered to be one of the future applications

of lactic acid [22]. In addition low-molecular-weight polymers of L-lactic acid have recently

been demonstrated to possess the ability of stimulating plant growth in a variety of crops and fruits when applied at a low level. This encourages the production of controlled release or degradable mulch films for large-scale agricultural applications in the future.

According to the estimation of Battelle, SRI, Cargill (1993) announcement and Argonne, the U.S. volume and selling price projections for degradable plastics, 'green chemicals, and plant growth regulators from lactic acid could be approximately 2.5 - 3.4 million tons per year and $3.1 - 4.4 billions per year, respectively [22]. Currently, polymerization grade lactide monomers are supplied by CCA Biochem b.v., Holland, and Boehringer Ingelheim KG, Germany, at prices of $30 - 40 /lb. While lactide polymers are sold as specialty resins at $100 /lb by Medisorb Technologies International L.P., United States, CCA Biochem b.v., Holland, and Boehringer Ingelheim KG, Germany [43]. The high price of the lactide monomers and polymers is partly due to their batch production and purification costs. Hence, for the large-scale commercialization of lactic acid as a competitive raw material in the polymer industry, the development of advanced fermentation processes, and new technologies for extraction and purification are required for reducing the production costs of lactic acid.
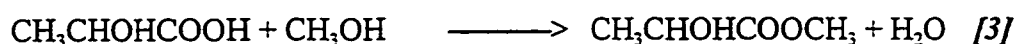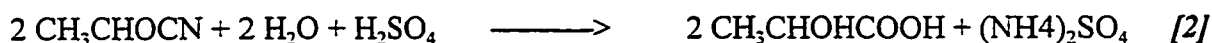
Production of lactic acid

Lactic acid can be produced chemically from coal, petroleum, and natural gas; and biologically through the bioconversion of carbohydrates, agricultural and industrial wastes, and plant biomass [82]. The chemical synthesis route produces only racemic lactic-acid mixture of L(+)- and D(-)-lactic acid; whereas pure L(+)- or D(-)-lactic acid solution can be

obtained through microbial fermentation. The configuration of lactic acid produced by the

bacteria depends upon the stereospecificity of the lactate dehydrogenase possessed by the

organisms [37].

Chemical synthesis

The commercial chemical method for producing lactic acid involves the conversion of

hydrogen cyanide and acetaldehyde to lactonitrile in the presence of catalyst (Equation *1*).

The crude lactonitrile is then purified and hydrolyzed to lactic acid by using either

concentrated hydrochloric or sulfuric acid (Equation *2*). The crude lactic acid is then

esterified with methanol to form methyl lactate (Equation *3*) which is then further distillate

and purified into different grades and concentrations of lactic acid (Equation *4*) [22, 82]

$$CH_3CHO + HCN \longrightarrow CH_3CHOCN \qquad [1]$$

$$2\ CH_3CHOCN + 2\ H_2O + H_2SO_4 \longrightarrow 2\ CH_3CHOHCOOH + (NH4)_2SO_4 \quad [2]$$

$$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O \quad [3]$$

$$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH \qquad [4]$$
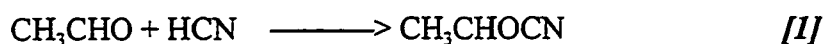
Carbohydrate fermentation

Most lactic acid bacteria are strictly fermentative and aerotolerant. Lactic acid

producing bacteria include species of the *Lactobacillus, Sporolactobacillus, Streptococcus,*

Leuconostoc, Pediococcus, and *Bifidobacterium.* They produced lactic acid through either

the homofermentative, the heterofermentative, or the bifidum pathway.

The heterofermentative pathway involves the formation of carbon dioxide, lactic acid, and ethanol from glucose. Initially, glucose through the oxidative pentose phosphate cycle gives rise to ribulose-5-phosphate which epimerizes to xylulose-5-phosphate. Xylulose-5-phosphate then cleaves into glyceraldehyde-3-phosphate and acetyl phosphate by the action of the enzyme, phosphoketolase. Finally, glyceraldehyde-3-phosphate and acetyl phosphate is converted to lactic acid and ethanol, respectively. The bifidum pathway involves the formation of lactic acid and acetic acid from the breakdown of one glucose molecule.

Homofermentative pathway (Fig. 2) yields 2 molecules of lactate per molecule of glucose. Glucose is degraded via the Embden-Meyerhof-Parnas pathway to pyruvate.
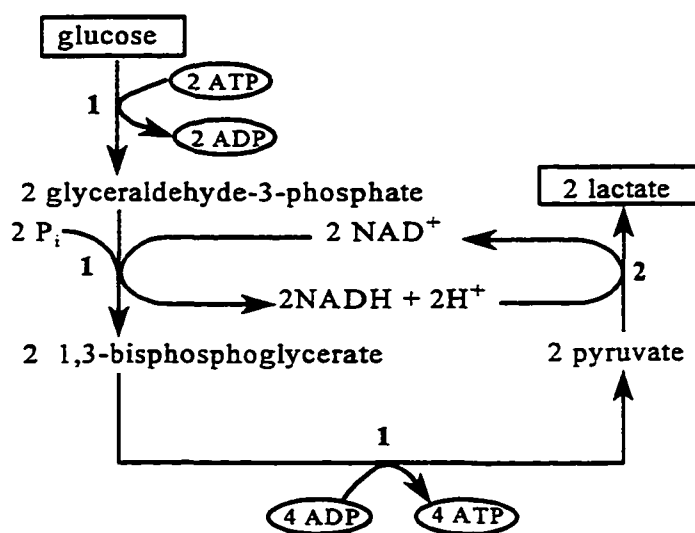
Fig. 2 Formation of lactate from glucose by the homofermentative pathway. 1, enzymes of the glycolysis pathway; 2, lactate dehydrogenase [37].

Pyruvate acts directly as H-acceptor and 2 ATPs are yielded per molecule of glucose.

Only the homofermentative organisms are of industrial importance because of their greater lactic acid yield and lower byproducts concentration in the fermentation process [43, 82]. Examples of homofermentative and heterofermentative organisms are listed in Table 1.

Table 1. Homo- and heterofermentative lactic acid bacteria [37].

Genera and species	Homofermentative	Heterofermentative	Lactic acid configuration
Lactobacillus			
L. delbrueckii	+	-	D (-)
L. lactis	+	-	D (-)
L. bulgaricus	+	-	D (-)
L. casei	+	-	L (+)
L. curvatus	+	-	DL
L. plantarum	+	-	DL
L. brevis	-	+	DL
L. fermentum	-	+	DL
Streptococcus			
S. faecalis	+	-	L (+)
S. cremoris	+	-	L (+)
S. lactis	+	-	L (+)
Pediococcus			
P. damnosus	+	-	DL
Leuconostoc			
L. mesenteroides	-	+	D (-)
L. dextranicum	-	+	D (-)
Bifidobacterium			
B. bifidum	-	+	L (+)

Fermentation of lactic acid

It has been demonstrated that lactic acid fermentation is in-between the type I (growth associated) and type II (non-growth associated) fermentation [21]. As illustrated in Fig. 3, the production of lactic acid is at first directly proportional to the medium cell density (i.e., growth associated). As the medium cell density curve plateaued, the lactic acid concentration still continued to increase (non-growth associated).
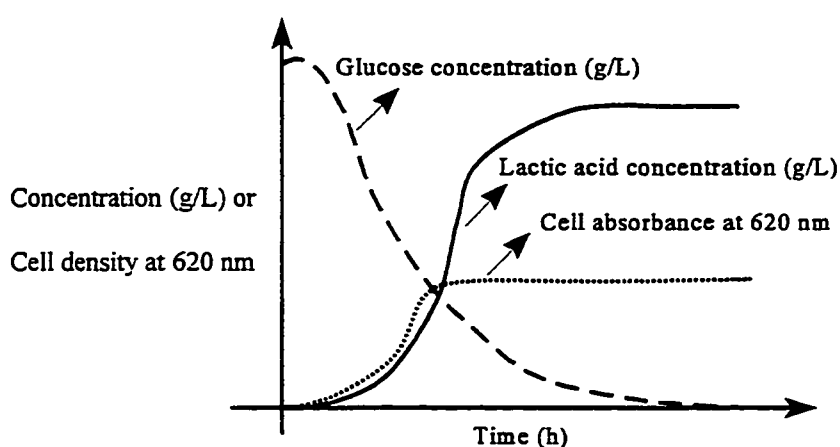
Fig. 3 Glucose consumption, lactic acid production, and cell density of *L. casei*.

The non-growth associated fermentation probably is a result of the 'uncoupling' effects of lactic acid inside the bacteria (e.g. *L. casei*) cytoplasm. At high medium lactic acid concentration, the pH of the medium will be closed to the pKa (3.85) of lactic acid. The undissociated form of lactic acid can diffuse freely through the cell membrane of the bacteria [6]. However, because the internal pH is higher than the external pH, acid dissociation will occur inside the cell (Fig. 4). As a result, the anion concentration of the cytoplasm will be higher than that of the surrounding external medium. This causes a net efflux of anions from

the cell. Hence, for every molecule of acid entering the cell, one proton is internalized [9].

Thus, there is a need to produce energy (ATP) via the homofermentative pathway for

maintaining the internal pH by pumping out protons as the lactic acid concentration inside

and outside the cell increased [37, 41, 67].
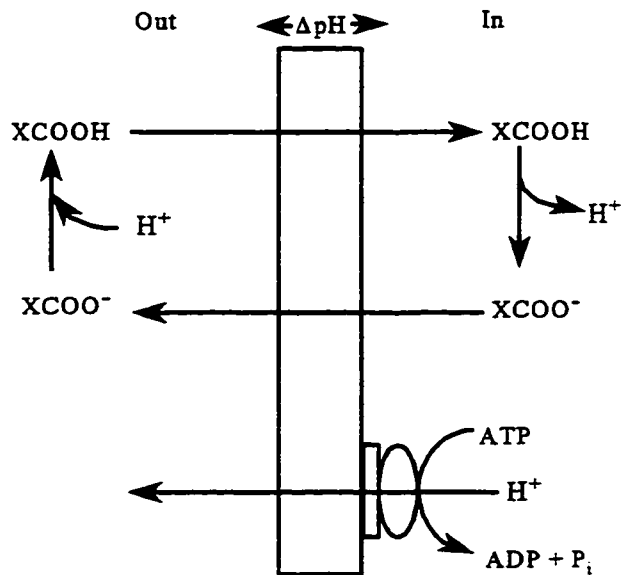
Fig. 4 Schematic representation of uncouplers action of organic acid and the dissipation of

protonmotive force [67].

Batch fermentation is generally employed in industrial-scale lactic acid production

[43, 82]. Continuous process with cell recycling [54, 39] and cell immobilization system [47,

77] have also been proposed; however, uncertainties in scaling up keep them from being used

without further testing. Cell immobilization is one of the current method commonly used to

increase the cell concentration in the bioreactor and to lactic acid production.

Immobilization

Immobilization of biocatalyst (e.g. microorganisms) into or onto a solid support

material is a common method in reducing bioreactors cell washout, increasing the biocatalyst

concentration, and optimizing biocatalyst contact with the substrate. C. D. Scott [68]

defined immobilization of microorganisms as any technique that limits the free migration of

cells and classified immobilization into two main types: 1) entrapment, where the organisms

are caught in the interstices of fibrous or porous materials or are physically restrained within

or by a solid or porous matrix such as a stabilized gel or a membrane; and 2) attachment,

where the microorganisms adhere to surface or other organisms by self-adhesion or chemical

bonding.

Entrapment of cells

Encapsulation or physical entrapment of organisms inside a polymeric matrix, is one

of the most widely used techniques for cellular immobilization. Suspended cells are added to

an aqueous solution of hydrocolloidal gels such as alginate or carrageenan to form biocatalyst

beads. The resulted suspension is then forced through a nozzle, an orifice, or by dispersing it

into a noninteracting liquid medium to form droplets. The droplets are subsequently

stabilized into biocatalyst beads with entrapped organisms by polymerization or other types

of cross-linking. Alginate droplets can be stabilized with divalent ions such as Ca^{2+}, and

carrageenan droplets are generally crosslinked with K^-. Both of these materials interact

reversibly with the cation and tend to disintegrate when it is removed [74].

Encapsulation and lactic acid fermentation

Different fermentor configurations, such as stirred tank, packed bed, and fluidized bed reactors, have been tested for encapsulation of cells. In a continuous fermentation, Stenroos et al. [77], immobilized *L. delbrueckii* in calcium alginate, which produced 12 g/L lactic acid at a rate of 0.2 g/L/h. Tuli et al. [79], immobilized *L. casei* in polyacrylamide gels and obtained a maximum lactic acid concentration of 31 g/L with a 0.64 g/L/h production rate. Because of the displacement of calcium ions in the alginate beads by the lactate ions, shrinkage and decreased strength of calcium alginate beads are often observed and reported during lactic acid fermentation [29, 66]. The difficulty in stirred tank reactors is the breakage of alginate beads during stirring. The major draw backs of packed bed column configuration are difficult pH control, plugging of column by leaked cells, and decalcification of calcium alginate beads. The fluidized bed reactor also faced the alginate beads cell-leakage problem [66]. In addition, mass transfer limitation is another important factor that inhibited the application of encapsulated cells in polymer beads and granules for lactic acid production [65].

Cells can be restrained by semipermeable membrane materials (hollow-fiber bioreactor) that isolate the organisms from the bulk liquid. Growth must be controlled to prevent an excessive build-up of biomass since it could cause pressure that would rupture the membrane. Membrane systems that involve electrodialysis fermentations center around a cell-recycling system are not commercialized due to their chronic membrane fouling problems high costs [68].

Porous materials (cotton mats, nylon mesh, cloth, metallic mesh, foam and sponge etc.) have voids that allow organisms to penetrate and grow into large colonies. Silva and Yang (1995) [71] adopted a fibrous-bed bioreactor for continuous production of lactic acid from unsupplemented acid whey. The fibrous bed was essentially stainless steel screen topped with cotton terry cloth and spiral-rolled to form a tubular bioreactor packing. Productivity of the fibrous-bed bioreactor was found to be 10-times higher than that of the batch fermentor with free cells. However, product inhibition and diffusion limitation still have to be overcome for improving cell efficiency and reactor productivity.

Attachment and biofilms

Biofilms are formed when microorganisms first attach to a submerged surface in an aquatic environment and then grow, multiply, and produce extracellular polymers while being attached. In flowing systems where nutrients are continually replenished, adsorption to substratum surfaces would enable bacteria to have a better chance of obtaining nutrients for growth [85]. This is now generally accepted as the main reason for microbial attachment.

A biofilm system generally consists of the attached micbrobial layer, the overlying gas and/or liquid layer, and the substratum to which the biofilm adheres. It can be divided into five compartments; the substratum, the base film, the surface film, the bulk liquid, and the gas [17]. The accumulation of the biofilm follows a fixed pattern which is arbitrarily divided into three consecutive phases: lag phase, biofilm production phase, and steady-state phase [36] (Fig. 5). All events in biofilm formation contribute to three sequential activities: a) colonization, b) maturation, and c) detachment or sloughing [57].
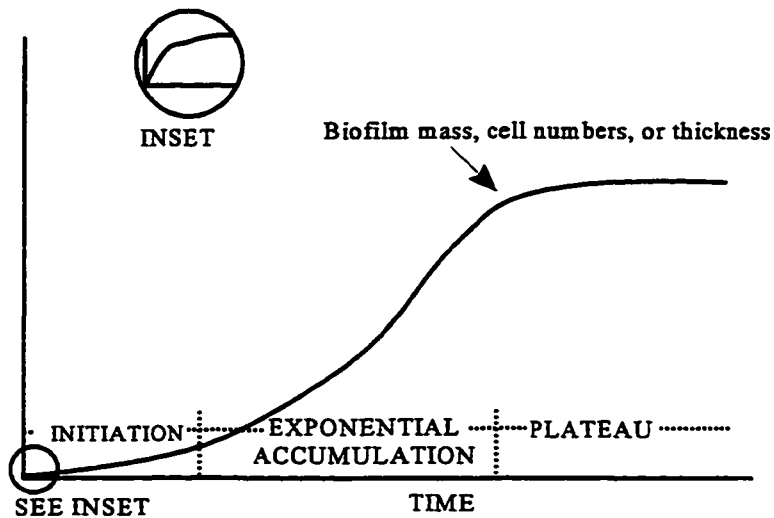
Fig. 5 The three phases of biofilm accumulation. The inset describes the initial

accumulation of the conditioning film, which is negligible in terms of thickness or

mass deposition [17].

Colonization

In natural environments like fresh water streams, ditches or inner surface of pipelines,

the formation process starts when organic molecules accumulate at the substratum, producing

a conditioned substratum. Dispersed microbial cells, mostly heterotrophic bacteria, in the

bulk water are transported to the conditioned substratum (Fig. 6).

In a quiescent environment, the predominant mechanism for bacterial transport may

be sedimentation or motility of the organism. In laminar flow, the primary transport

mechanism may be diffusion (Brownian motion), whereas in turbulent flow convective

mechanisms related to fluid motion dominate [16].
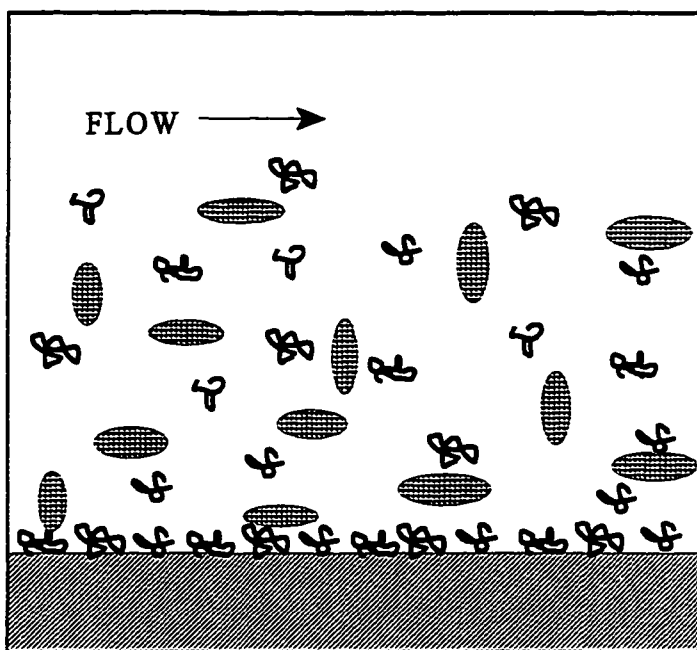
Fig. 6 Transport and adsorption of organic molecules on a clean substratum, forming a

conditioning film [17].

Initially, bacteria experience electrostatic attraction or repulsion to or from the

conditioned substratum. Then a fraction of the cells that reach the substratum adsorb to the

surface via extracellular polymers and surface appendages such as pili, fimbriae, flagella, and

prosthecae [18]. Since the attractive interactions between the substratum and the bacterium

at this stage are relatively few and weak, detachment of bacteria can be easily brought about

by fluid shear forces and electrical repulsion present among the surfaces around the bacteria.

Adhesion is therefore reversible [55].

Subsequently a fraction of the reversibly adsorbed cells remains immobilized and becomes irreversibly adsorbed. This irreversible adsorption is mostly due to multiple weak polymer-surface bonds that are formed between the substratum and the extracellular polymers secreted by each cell [20, 63] (Fig. 7).
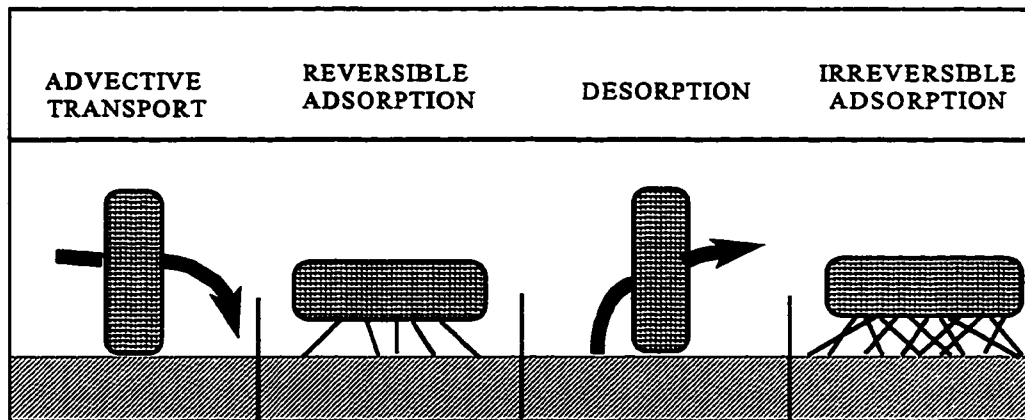
Fig. 7 Transport of microbial cells to the conditioned substratum, and adsorption, desorption, and irreversible adsorption of cells at the substratum [17].

Maturation

The irreversibly adsorbed cells grow at the expense of substrate and nutrients in the bulk water, increasing biofilm cell numbers and forming other metabolic products. Filamentous microorganisms, like fungi and *Actinomycetes*, and prosthecae bacteria develop within one or two weeks of active growth into the primary population of the biofilm.

Exopolymers are then secreted by the bacteria. The majority of the layer probably consists of polysaccharides with oligosaccharide repeating units [19, 46]. Cerning et al. [15] showed that *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Lactobacillus casei* ssp. *casei* produced exopolymer layers containing primarily galactose

and glucose with a trace amounts of mannose, rhamnose and pentoses. While Kojic et al.

[43] found that *Lactobacillus casei* CG11 produced exopolysaccharides containing 75%

glucose and 15% rhamnose and a trace amount of arabinose, mannose, and galactose.

Chemical forces like dispersion forces, electrostatic interactions and dipole moments

of the exopolymer layer accumulate and glue the bacteria together. The biofilm now takes on

the macroscopic appearance of a gel. The polysaccharides molecules of the exopolymer

layer are quite stiff and extended. Sometimes the exopolymer layer may be present in a

variety of forms. Leppard and Bakke demonstrated the presence of 5 nm electron-opaque

fibrils in biofilms of *Psuedomonas aeruginosa* [18].

The exopolymer layer slows down the diffusional transport of dissolved molecules

(nutrients and products) to and away from the biofilm surface. Hence, the formed gel,

together with the mycelia of the fungi and/or *Actinomycetes*, act as a penetration barrier to

toxic substances and a trap for debris and nutrients, and other organic materials from the bulk

phase [20].

Because of the limitation of transfer through the gel, the inner layers of the biofilm

may become deficient in oxygen. This new environment stimulates the development of an

anaerobic microbial population in the reduced zone. In the final phase of biofilm

development, protozoa may attach to the film. Large populations of ciliate or flagellate

protozoa are found in many natural mature biofilms. They feed on the bacterial population

and appear to be important participants in maintaining the microbial community in a stable

condition.

Detachment

Detachment can be categorized into two processes, shearing and sloughing. Shearing occurs when microbial cells on the surface of the biofilm are being torn away by the shear stress of the flowing fluid. This process may occur at the beginning or any other stages of the biofilm formation. Very often the cells detached by shearing can collide and reattach to the biofilm surface. This enables an exchange of the different kinds of microorganisms along the inner surface of the biofilm. The redistribution of microorganisms is very important in keeping a stable ecological condition in the biofilm.

Eventually the biofilm may experience a destructive release called sloughing, in which extensive portions of the biofilm are removed, leading to a nearly bare substratum. Such an event might be caused by excessive exopolymer or gas production in the anaerobic zone. Sloughing could result in pressure gradients and subsequent detachment of the film [16]. Following this kind of event, only a relatively thin exopolymer may remain on the substratum.

Economic importance of biofilms

Biofilm formation in medicine is detrimental. The adherence of bacteria to the tooth surface forms a permeable layer within which acidic fermentation products of the bacteria accumulate and demineralize the enamel layer of the tooth [42]. This leads to the potential development of dental caries, malodor and peridontal disease. Biofilm formation on pacemakers and implants may result in serious infestation or fatal consequences. Survival of the biofilms is probably due to the body's immune system failing to recognize and kill the bacteria once the cells are transformed into biofilms [44].

In industry, biofilm formation is the major cause of microbial fouling and material deterioration in pipelines, tubings and ship hulls [16]. High costs are incurred in developing and buying antimicrobial agents that are able to suppress biofilm formation effectively and in the removal of biofilms.

As an alternative to suspended cell culture in a fermentor, immobilization of cells has been used in conventional fermentation. Biofilm formation is a natural way of immobilizing microorganisms to an inert support. Reactors with biofilms have been used industrially mainly in wastewater treatment plants and the 'quick vinegar' production process.

In a wastewater treatment plant, the collected wastewater passes through a settling tank where debris and large particles are removed and is then transferred to one or more bioreactors for the removal of organic matter, nitrogenous wastes and heavy metals. Wastewater treatment bioreactors can be divided into two main types: attached biomass reactors and nonattached biomass reactors. The first type of reactors mainly involves biofilm formation in a fixed (packed) bed reactor whereas the second type of reactors comprises the applications of activated sludge, contact process and sludge blanket systems [14, 58]. Conventionally, stones have been used as substratum for the biofilm in the packed bed reactor. Presently plastic media which have the advantage of lightness, higher specific exchange surface area, and higher interstitial spaces are used instead [2].

A trickling filter is used in quick vinegar production [21]. This is a recycled batch-fermentation process which takes about 4 -5 days for completion. The packed bed reactor is in the form of a wooden reactor filled with beechwood shavings. The starting material (ethanol brew as a result of the yeast ermentation of sugar) is sprayed over the surface of the

beechwood shavings which are coated with an acetic- acid bacteria biofilm (*Acetobacter* and/or *Gluconobacter*). After trickling through the shavings, the partially converted solution is cooled and recirculated over the beechwood chips. Generally about 88 - 90% of the added alcohol is converted to acetic acid through this trickling generator process [21]. The thickness of the biofilm is controlled by low growth rates and high substrate conversion. Although this is a batch process, the biofilm formed on the beechwood chips is retained for further batch processing.

In the 1970's, metal leaching and animal tissue culture were developed as additional industrial fermentations using biofilms. The ability of bacteria and its exopolymer to bind heavy metal ions has been documented by Lion et al. [51]. This property leads to the application of biofilms in the cycling of trace metals in nature, metal leaching [21], detoxification of industrial wastes containing heavy metals [50], formation of polysaccharide gels or aggregates by inorganic cations [12], treatment of water polluted by hydrocarbons [4], culturing of viruses [5], and the production of the mammalian cell (e.g. hepatocytes, bone marrow cells, lymphocytes; or cell-derived products) [10, 78].

Biomass support particle systems

In fixed biofilms wastewater treatment systems, unpredictable sloughing of the biofilm generally occurs when the microbial film is allowed to become too thick. Microorganisms nearest the substratum are depleted of nutrients and oxygen. This starvation together with the accumulation of unwanted or even toxic products causes sloughing and in some cases the collapse of the whole film. To overcome the above problem, small particles

called biofilm support particles (BSP) on which biofilms could form can be used in a stirred tank or fluidized bed fermentor to give a system called the completely mixed biofilm fermentor [5]. The small, irregular size and varied geometrical shape of the BSP provides a large surface area for microbial film development. This increases the reactor biomass concentration which in turn raises the overall substrate conversion rates.

In a BSP system, microorganisms remain in the particle by adsorption to the surfaces and aggregation to each other, forming a biofilm matrix throughout the support particle. Abrasion results from physical contact among the moving particles which causes removal of excess surface growth and allows a relatively constant film thickness to be maintained. Upward flow of water or a water and gas mixture in the fermentor generates upward forces that are large enough to overcome the falling motion of the BSP. This results in an uniform pattern of particle movement thus allowing thorough mixing within the bioreactor.

BSP types, shapes, and applications

BSP for wastewater treatment can be constructed from a variety of materials such as rocks, gravels, sands, wooden chips, ceramic, poly(ester) foams, plastic materials (high density polypropylene, polyvinyl chloride, or high density polyethylene), stainless steel wire, fritted glass particles in a range of sizes and shapes (spheres, mats, cubes, donut-shape, and porous pads) [12]. Recently, Massol-Deya et al. [56] employed granular activated-carbon as BSP for treating petroleum-contaminated groundwaters. Cell-free channel structures for facilitating transport among deep inner layers to the surface of biofilms were observed.

Black et al. [8], used stainless steel BSP and three plastic foam types of BSP of varying porosity to study ethanol production by *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in a fluidized bed reactor and in a continuous flow reactor system. In all cases, yields of ethanol were better than in freely suspended cultures. Webb et al. [84], employed a system similar to that of CMMFF with the filamentous fungus *Trichoderma viride* QM 9123 and stainless steel BSP. This system showed a three-fold higher cellulase production than that obtained using freely suspended cells.

Kunduru and Pometto III [48, 49] used polypopylene-composite chips containing 25% (w/w) agricultural materials (ground soybean hulls with zein or soybean flour) as BSP for continuous ethanol fermentation. Biofilm reactors out-performed suspension-culture reactors, with 15 to 100-fold higher productivities and with higher percentage yields for *S. cerevisiae* and *Z. mobilis*.

BSP for lactic acid production

Lactic acid production using biofilm on inert supports have been extensively studied for their potential to improve the cell concentration, productivity, and the lactic concentration of the reactor. Goncalves et al. [35] made inert supports mainly from glass (Raschig rings of sintered glass, beads of sintered glass, and beads of porous glass) and compared *Lactobacillus delbrueckii* NRRL B445 attachment ability with irregular ceramic particles. Beads of sintered glass was found to be the best support, yielding the highest volumetric lactic acid productivity (2.5 g/L/h).

Guoqiang et al. [38] adsorbed *Lactobacillus casei* (DSM 20021) on foam glass particles pretreated with polyethyleneimine. In the stir-tank system, glucose was almost completely utilized (99.2%) with a lactic acid productivity of 5.2 g/L/h at a dilution rate of 0.18 /h. In the 14-days packed-bed experiment at 0.11 /h dilution rate, lactic acid yield and productivity was about 90% and 1.17 g/L/h, respectively. The use of these glass supports is hindered by their brittleness and sensitivity to attrition under stirred conditions.

Demirci et al. [24] had used pea gravels, aluminum oxide-ceramic spheres, and polypopylene-composite chips containing 25% (w/w) agricultural materials as BSP for lactic acid batch fermentation. Polypopylene-composite chips were shown to have the best biofilms formed among the three types of BSP.

Further application of polypropylene-composite chips containing 25% (w/w) agricultural materials for lactic acid fermentation had been studied. In short-term (7-days) studies, pure- and mixed-culture continuous fermentations produced 30 g/L/h and 35 g/L/h lactic acid, respectively [25]. In the long-term repeated-batch lactic acid fermentation, other than a higher lactic acid concentration (25% higher), the percentage yields, maximum productivity, glucose consumption rates, and growth rates of the polypropylene-composite chips bioreactor were similar to that of the polypropylene-alone supports bioreactor [26]. The main reason for the above results was due to supports clumping, which caused serious medium channeling and greatly reduced substrate-to-cell contact. Further studies for new shape and properties of BSP are required for overcoming the above problems.

Evaluation of bacterial attachment

Physical chemistry research of interfaces provides two theories for describing the adsorption of a particle to a surface. The first theory is the Derjaguin-Landau and Verwey-Overbeek (DLVO) theory of colloidal chemistry since a bacterial suspension may be described as a living colloidal system (liquid system containing large molecules or small particles) [59]. This theory describes the change in Gibbs energy as a function of the distance between two bodies. If steric effects are negligible, the total interaction Gibbs energy is obtained from the addition of the Van der Waals and the electrostatic interaction between the bacteria and its environment. The Van der Waals interaction is usually attractive, whereas the electrostatic interaction is usually repulsive, because in nature both bacteria and surfaces are predominantly negatively charged [81]. Attraction of bacteria to negatively charged interfaces depends upon interacting electrical double-layer-repulsion and Van der Waals attraction energies, the resultant energy being dependent upon the electrolyte valency and concentration in the liquid. Therefore, variations in the degree of adsorption have to be affected by the differences in the electrokinetic potentials of both the cells and the adsorbent particles [23]. The surface charge density of bacteria can be calculated from its zeta (ζ) - potential. ζ-Potential is generally measured by microelectrophoretic methods [30] using the equation of Smoluchowski:

$$\zeta = U\eta / \varepsilon \qquad [5]$$

where U is the electrophoretic mobility, η is the viscosity, and ε is the permissivity. The electrophoretic mobility of a particle depends upon the ratio of the conductivity of the particle to that of its environment. Hence, a modified Smoluchowski equation is used to

determine the ζ- potential of bacteria:

$$\zeta = U\eta \, (1 + \sigma_2/2\sigma_1) \, / \, \varepsilon \qquad\qquad [6]$$

where σ_1 and σ_2 are the conductivities of the particle and suspending medium, respectively [30]. Microelectrophoresis apparatus and techniques generally have the problems of failure to conform to the theorectical requirements, inadequate temperature control, and difficult to construct and manipulate. Gittens and James [34] modified the microelectrophoresis apparatus and incorporated a rectangular observation chamber, conforming to the theorectical requirements in which temperature can be accurately controlled.

The second theory describe bacterial adhesion as a result of thermodynamical values involving surface tensions among the interfaces, bacteria, suspended liquid, and solid substratum [45]. Surface tension is an indication of the forces of attraction that hold the molecules together in the liquid (or solid) state. Thus, liquid droplets tend to become spheres (the form of least surface area) because of the mutual cohesion of the molecules. Conversely, work must be expended to increase the surface area of a liquid, as in expanding a soap bubble. Hence surface tension can be regarded as a force (N/m) acting along the surface or as surface free energy (J/m^2), the two concepts being entirely equivalent. Whereas, older surface tension data will be given in the c.g.s. units of dyne/cm or erg/cm^2 [69]. According to Gibbs free energy of adhesion per interface unit area,

$$\Delta F_{adh} = \gamma_{SB} - \gamma_{SL} - \gamma_{BL} \qquad\qquad [7]$$

where ΔF_{adh} is the interfacial free energy of adhesion, γ_{SB} is the solid bacterium interfacial free energy, γ_{SL} is the solid-liquid interfacial free energy, and γ_{BL} is the bacterium-liquid interfacial free energy.

When electrical charge interactions are negligible, adhesion may be expected if:

$$\Delta F_{adh} \leq 0 \qquad\qquad [8]$$

whereas if:

$$\Delta F_{adh} > 0 \qquad\qquad [9]$$

adhesion is energetically unfavorable [1]. The solid as well as the liquid parameters that appear in equation [7] can be measured by employing contact angle measurements using the equation of Young

$$\gamma_{SV} - \gamma_{SL} = \gamma_{LV} \cos \theta \qquad\qquad [10]$$

where θ is the contact angle and the indices LV and SV mean liquid vapor and support vapor, respectively [61]. The Gibbs free energy of adhesion can be calculated by measurement of the contact angles for cell and support, and tables of converting the contact angles to surface free energy is published by Neumann et al. [60].

Interactions of the bacteria with the liquid droplets and problems connected with the drying of the bacterial layer have difficulty in the determination of surface free energies of bacterial surfaces by contact angle measurements. Busscher et al. [13] overcame these problems by placing the cellulose triacetate filter (pore diameter, 0.45 um; Gelman GA-6) with the bacteria lawn in a petri-dish containing a layer of 1%(wt/vol) agar containing 10% (vol/vol) glycerol until the filters were mounted onto a holder. The water contact angles, measured 2 s after the droplets were applied, are presented as a function of the drying time of the bacterial deposits. The contact angles were calculated from the measured parameters height (h) and length (l) of the image of the drop by the equation:

$$\tan \theta = 2h/l \qquad\qquad [11]$$

The value of θ considered as characteristic of the cells is deduced from the horizontal part of the respective curves. Bacteria can be separated roughly into three categories, hydrophobic (θ > 90°), moderately hydrophobic (θ = 50° to 60°), and hydrophilic (θ < 40°) [59].

Other than free surface energy (contact angle), hydrophobicity and cell surface charges of bacteria have also been used to predict bacterial attachment on solid surfaces. Relative hydrophobicity of bacterial cells has been characterized by bacterial adherence to hydrocarbons (BATH) [64]. However, considerable variation in values has been reported depending on the method of determination. Hydropbobic interaction chromatography (HIC) [59, 80] has also been employed in measuring the hydrophobicity of bacteria. Concerns about the filtration effect or nonspecific binding of the bacteria by the column gel have hindered its wide application. Electrostatic interaction chromatography (ESIC) has been used to measure the cell surface charge of bacterial cells [62]. The efficiency of the ESIC method is limited by the total surface area of the resin particles, and hydrophobic property of those bacteria with high charges are generally not detected by this method.

Studies by Absolom et al. [1] with five bacterial species had proved that the surface tension values obtained from bacterial adhesion are in good agreement with the values obtained from direct contact angle measurement on layers of bacteria. Dickson and Koohmaraie [28] in their study of bacterial attachment on meat surfaces also obtained a high correlation (r^2 = 0.8) for contact angle method and xylene BATH, and for contact angle method and HIC. Hence, contact angles are generally regarded as a good measurement of bacterial hydrophobicity and have a predictive value for adhesion.

Demirci et al. [24] estimated the degree of biofilm formation on lactic acid BSP by evaluating the extent of clumping of solid supports, weight gain and Gram stains of the supports. Siebel and Characklis [70], Wanda et al. [83], and Bryers and Banks [11] determined biofilm thickness microscopically and analyzed biofilm population indirectly by product, glucose, and dissolved oxygen concentration. Dickson and Koohmaraie [28], and J.S. Dickson [27] measured the population of loosely attached bacteria on meat surfaces by rinsing the BSP in phosphate buffer. They also obtained the relative physically attached bacterial population by enumerating the homogenized solution of the BSP. Fletcher and Loeb [32], Bryers and Banks [11] fixed the BSP with attached bacteria with Bouin fixative, stained them with ammonium oxalate crystal violet or acridine orange, and then counted the attached bacteria by bright-field or fluorescence microscopy. In experiments for evaluating BSP, it is generally recommended to include at least a method for estimating the tendency of bacterial attachment to the supports (e.g. contact angle method) and a method for enumerating the actual bacteria population on the supports.

INGREDIENTS SELECTION FOR PLASTIC COMPOSITE-SUPPORTS

USED IN L(+)-LACTIC ACID BIOFILM FERMENTATION BY

LACTOBACILLUS CASEI SUBSP. *RHAMNOSUS*

A paper submitted to Applied and Environmental Microbiology

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Abstract

Incorporation of oat hulls, soybean hulls, yeast extract, soybean flour, dried red blood cells, bovine albumen, and/or salts into plastic-composite supports was evaluated by two replications of a 2^{5-1} design. Plastic-composite supports containing 50% agricultural products and 50% polypropylene (w/w) were produced by twin-screw high-temperature extrusion. *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443) was incubated for 48 h at 37°C with uncontrolled pH in 50 bottles each with 5 g of discs and 20-mL minimal medium (2% glucose and mineral salts solution). Culture-fermentation medium lactic-acid concentration and cell density were analyzed by high-performance liquid chromatography (HPLC) and spectrophotometry, respectively. Stripping sand, scanning electron microscopy, and surface contact-angle method were used to study the plastic composite-supports biofilm population and relative hydrophobicity. Biofilm population was affected by the contact angle and relative hydrophobicity of supports ($r = 0.79$ to 0.82). Lactic acid production was by the

suspended cells (r = 0.96) and the biofilm on the PCS disc (r = 0.85). Soybean hulls, yeast

extract and salts gave less hydrophobic supports (P < 0.0001) and better cells attachment (P <

0.03). Yeast extract enhanced the growth of free and attached cells in minimal medium (P <

0.0001). Bovine albumen blended with soybean hulls, yeast extract, soybean flour, and salts

had the highest lactic acid concentration in the first (7.6 g/L) and twentieth (1.4 g/L)

simulated repeated-batch fermentation. Under all conditions, suspended cells and

polypropylene discs control gave negligible lactic-acid production and cell density. Plastic-

composite supports consistently outperformed the two controls in minimal-medium lactic-

acid biofilm-fermentation.

Introduction

Lactic acid is an organic hydroxy acid that exists in two optically active, L(+) and D(-

), enantiomers [16]. It is widely used by the food industry (acidulent, preservative, stearoyl-

2-lactylate synthesis) and nonfood industry (polylactic acid, green solvent, slow release

carrier) [5,12]. Polylactic-acid degradable plastic is a polyester of lactic acid with a projected

market of 300 million bushels of corn per year [5].

Lactic acid can be produced chemically from acetaldehyde and hydrogen cyanide or

via microbial fermentation, but only microbial sources can produce exclusively the L- or D-

isomers of lactic acid. Presently, lactic acid is produced by batch fermentation because it

exhibits both Type I (growth associates) and Type II (nongrowth associates) fermentation [3].

Lactic-acid production rates and concentration can be increased by strain development to

form high-production mutants [7] or by increasing cell density in the fermentor [8,9]. Cell

immobilization is a common way to increase cell density, although industrial application of cell immobilization with calcium alginate beads and polyacrylamide gels are few. This is mainly due to the high cost of immobilization, mass-transfer limitations, lack of stability of the biocatalysts, and changes in product pattern of reactions catalyzed by certain immobilized cells [12].

Biofilms are a natural form of cell immobilization that results from microbial attachment to solid supports in submerged environment [1]. This increases the cell density and enables the biofilm population to withstand stresses such as pH change and starvation. Attachment of cells on supports to form biofilm depends largely on the formation of extracellular polysaccharides and surface charge between the solid surface and the microorganisms [2]. Studies by Van Loosdrecht et al. [15] demonstrated that measurement of hydrophobicity of cell surfaces by contact-angle method gave more consistent results than methods such as the hexadecane test and partitioning of cells in the two-phase polyethylene glycol and dextran system.

Previous studies in our laboratory had successfully proved that biofilm fermentation with plastic-composite supports (PCS) chips containing 75% polypropylene (PP) and 25% agricultural material (w/w) benefitted lactic acid production. In pure- and mixed-culture continuous fermentation, 30 and 35 g/L/h productivity was achieved, respectively [8]. In addition, the PCS chips were shown to be effective in long-term (more than 2 months) repeated-batch lactic acid biofilm fermentation with both pure and mixed cultures [9]. However, medium channelling and clumping of cells among the PCS chips interfered with medium mixing, pH control, and ultimately, lactic acid production. To overcome these

problems, new PCS with a disc shape were used for this study. We demonstrated that lactic acid production was shown to have high correlation with the suspended cell density, biofilm population, and hydrophobicity of the new PCS discs.

Materials and methods

Plastic-composite supports

Plastic composite-supports (PCS) discs that contained 50% polypropylene [PP] (Quantum USI Division, Cincinnati, OH) and 50% agricultural materials (w/w) were produced by high-temperature extrusion in a Brabender PL2000 with twin-screw co-rotating extruder (Model CTSE-V, C.W. Brabender Instruments, Inc., South Hackensack, NJ). Incorporation of ground (20 mesh) oat hulls (OH) (Ralston Foods, Cedar Rapids, IA), ground (20 mesh) vacuum-dried (48 h at 110°C and 30 in Hg) soybean hulls (SH) (Cargill Soy Processing Plant, Iowa Falls, IA), defatted soybean flour (SF) (Archer Daniels Midland, Decature, IL), yeast extract (YE) (Ardamine Z, Champlain Industries Inc., Clifton, NJ), dried bovine albumin (BA) (American Protein Corp., Ames, IA), dried bovine red blood cells (RBC) (American Protein Corp., Ames, IA), and mineral salts (S) (0.2% sodium acetate, 0.12% $MgSO_4 \cdot 7H_2 0$ and 0.006% $MnSO_4 \cdot 7H_2O$) into PCS discs was evaluated.

Materials to be extruded were first mixed in a separate container on a weight base before being poured into the extruder hopper. The mixture was extruded (11 and 15 rpm for soybean-hulls and oat-hulls composite supports, respectively) as a continuous tube through a medium pipe die (3.2 mm ID and 12.7 mm OD) with barrel temperatures of 200, 220, and 200°C and a die temperature of 167°C. The barrel exhaust vent was plugged because the

release of moisture at the die was essential for producing a porus support. Composite tubing

was extruded onto a steel rod and air cooled slowly without fanning. Tubes with 10 to 11

mm OD were then cut into discs. Composition, physical properties, and treatments applied

for each PCS are listed in Table 1. Polypropylene discs were bored out of a polypropylene

sheet (3.5 mm thick) with cork borers 7 mm ID and 11 mm OD.

Hydrophobicity of L. casei and PCS discs

The relative hydrophobicity of each PCS-discs blend was determined by measuring

the contact angles with the sessile drop technique as described by J.F. James (Program Abstr.

5th Int. Pathog. Neisseria Conf., abstr. no. V119, 1986) [10]. A drop of deionized water (20

μL) was deposited on the cut surface of the disc (Fig. 1). The surface and the drop was

photographed within 1 s of application. The photographic slides were developed, and the

contact angle (θ) of each water droplet on the discs cut surface was measured.

The relative hydrophobicity of L. casei was determined by measuring the contact

angles of deionized water droplets deposited on a cell lawn within 1 s of application (Fig. 1)

[6]. The cell lawn was prepared by filtering 300 ml of L. casei culture (18 h in 8% Glu LAF

medium [8]) with a 0.45-μm cellulose triacetate filter (Millipore HAWP, Millipore

Corporation, Bedford, MA). The moisture content of different cells lawns were standardized

by placing the filters with cells in a petri dish containing glycerol-agar (1% [w/v] in water

containing 10% [v/v] glycerol) for 3 h [14].

Bacterial culture preparation

Lactobacillus casei subsp. *rhamnosus* (ATCC 11443) is a homofermenter of L(+)-lactic acid. Stock cultures were maintained in Lactobacillus MRS broth (Difco Laboratories, Detroit, MI) at 4°C with monthly transfers to fresh medium. Ten milliliters of an active *L. casei* culture (18 h in MRS broth at 37°C) was inoculated into 100 ml of lactic-acid fermentation (LAF) medium [2% glucose (Glu), 0.4% yeast extract (YE) (Ardamine Z, Champlain Industries Inc., Clifton, NJ), and salt solution (0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.1% sodium acetate, 0.06% $MgSO_4 \cdot 7H_2O$, and 0.003% $MnSO_4 \cdot 7H_2O$)] [8], which was then incubated for 18 h at 37°C. Centrifugation (16300 x *g*, 20 min) followed by a rinsing step with 100 ml of minimal medium (MM) (2% Glu, 0% YE, and salt solution) were used to remove LAF medium from the active cells. The rinsed active cells were then resuspended into 100 ml of sterilized MM and used in the simulated repeated-batch fermentation assay.

Batch fermentation (BF) studies

Batch fermentation without pH control was used to characterize each PCS blend performance. Suspended cells and PP discs were used as controls. PCS or PP discs (5 g) were sterilized dry (45 min at 121°C) in a 50-ml screw-cap cultured tube. The sterilized discs were aseptically transferred into a dilution bottle containing 20 ml of sterilized MM and soaked at 37°C in duplicates for 24 h. This process was used to wet each plastic disc and to remove all the rapidly leachable materials. The initial soaking solution was decanted aseptically, and each dilution bottle was refilled with 20 ml of sterilized MM. Then, each dilution bottle was inoculated with 0.2-ml active *L. casei* culture (18-h culture) and incubated in a 37°C water bath for 48 h.

The BF medium was aseptically decanted and evaluated for lactic acid produced,

glucose consumed, and suspended cell density (620 nm). L(+)-Lactic acid and D-glucose

concentrations were analyzed by a Waters high-performance liquid chromatograph (HPLC)

(Milford, MA) equipped with Waters model 401 refractive index detector and a Bio-Rad

Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA)

using 0.012 N H_2SO_4 as the mobile phase. Bacterial growth in LAF medium was followed

by measuring the absorbance at 620 nm with a Spectronic 20 spectrophotometer (Milton Roy

Co., Rochester, NY).

Viable biofilm population on the supports

A modified method of Dickson et al. [10] was used to enumerate the relative biofilm

population on the PCS. After the first simulated RBF in MM, five discs (both PCS and PP)

were aseptically removed from each RBF bottle and transferred into a dilution bottle

containing 100 ml sterile 0.1% peptone water. Each bottle was vigorously shaken to remove

nonbiofilm cells. The rinsed supports were then aseptically transferred into a screw-cap

culture tube with 9 ml sterile 0.1% peptone water and 5 g of sterile sand. The culture tube

was subsequently vortexed vigorously at 30-s intervals for a total of 1.5 min. The culture

tube medium was then serially diluted into sterile 0.1% peptone (10^3 to 10^5). Colony-

forming units (cfu) were determined for each tube by using Lactobacillus MRS agar spread

plates in duplicate. Finally, the five sand-stripped discs from each BF bottle were rinsed

(water), convection-oven-dried (70°C, 24 h), and weighed.

Scanning Electron Microscope

Two supports were retrieved aseptically from the dilution bottle and immediately

fixed with 4% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) overnight at 4°C. Fixed

samples were washed three times in the same buffer at room temperature and postfixed in 1%

osmium tetroxide with shaking for 1 h at 4°C. Postfixed samples were again washed three

times in the same buffer and then dehydrated through an ethanol graded series (50, 70, 75,

80, 85, 90, 95, 100, 100, and 100%). The dehydrated samples were then critical-point-dried

by a hexamethyldisilazine (HMDS) solution series [30-min intervals of 1:1 (v/v)

HMDS/100% ethanol, 100% HMDS, 100% HMDS, and 100% HMDS] at room temperature

with shaking. Samples were allowed to dry overnight inside a solvent hood in a crack-lid

petri dish containing HMDS-saturated filter paper at room temperature. SEM micrographs of

gold-coated critical-point-dried supports were taken with a JEOL JSM-35 scanning electron

microscope at 25 kV(JEOL, Japan).

Statistical analysis

Two replications of a 2^{5-1} design [4] were used to evaluate the effect of SH, OH, YE,

SF, RBC, BA, and S on the characteristics of PCS in all the tests performed. The five factors

evaluated in the first replication were hulls (OH or SH), YE, SF, RBC, and S. The five

factors studied in the second replication were hulls (OH or SH), YE, SF, BA, and S. In each

replication 16 treatments in duplicate were examined. The data from each test were analyzed

by Least Significant Difference (LSD) and Analysis of Variance (ANOVA) using Statistical

Analysis System Package (version 6.03) (SAG Institute, Inc., 1985).

Results

Relative hydrophobicity effects of supports and bacteria on biofilm formation

As the bacterial contact-angle curve (Fig. 2) plateaued temporarily at 1.5 - 2.5 h, the

contact angle, θ, of *L. casei* was determined to be 26.7°. Because θ was less than 40°, *L.*

casei was considered to be hydrophilic [14]. The contact angles range of the PCS and PP

discs was 88.3° to 112.0° and 93° to 99°, respectively, (Table 1), which indicated that all

supports possessed a hydrophobic surface. Statistical analysis of the contact angles showed

that SH, YE, and S ($P < 0.0001$) decreased the supports hydrophobicity, whereas OH, RBC,

and BA increased the hydrophobicity of the supports ($P < 0.005$). Van Loosdrecht et al. [15]

concluded that hydrophobic bacteria will adhere to hydrophobic surfaces more readily than

hydrophilic bacteria. Therefore, the hydrophilic *L. casei* will favor attachment to less

hydrophobic PCS with SH, YE, and salts than to the more hydrophobic PCS with OH, RBC

and BA. This observation was supported by viable attached-cells counts on the supports

(Fig. 3). All supports with SH ($P < 0.03$), YE ($P < 0.0001$), and S ($P < 0.007$) increased the

biofilm population. Indeed, the contact angle of supports with SH-PCS and OH-PCS had a

high correlation ($r = 0.79$ and 0.82, respectively) with the viable attached-cells on the

supports (biofilm population) (Fig. 3) with the exception of PCS containing just YE and

RBC together. In addition, the SH-PCS had a relatively higher cell-attachment range (5.7 to

23.0 x 10^8 cfu/g supports) than the OH-PCS (2.6 - 7.5 x 10^8 cfu/g supports). Furthermore,

the YE and S hydrophobicity reduction effects had a greater impact on the BA-PCS than the

RBC-PCS. For example, the contact angle of OHYEBAS-PCS and SHSFYEBAS-PCS was

much less than the contact angle of OHBA-PCS and SHSFBA-PCS by 14.3° and 6°,

respectively, whereas, the contact angle of OHYERBCS-PCS and SHSHYERBCS-PCS was

1.7° and 2.7° less than the OHRBC-PCS and SHSFRBC-PCS, respectively. Hence, BA-PCS

with SH, YE, and S had a less hydrophobic surface and might have a greater biofilm

population. This was supported by the SHSFYEBAS-PCS, which had the greatest viable

cells attachment, 2.3×10^9 cfu/g supports, among all the supports (Fig. 3). In all instances,

PP-discs had the fewest average viable-cells attached on its surfaces, 4.1×10^4 cfu/g supports,

although its average contact angle, 96.7°, was lesser than most of the PCS supports (Table 1).

This might be due to the lack of complex nutrients leaching and/or porosity of the PP discs.

Batch fermentation of lactic acid

Lactic acid concentration in the PCS simulated RBF was highly associated with the

MM cell density (r = 0.96) (Fig. 4), and discs viable attached-cell-counts (r = 0.85) (Fig. 5).

These results indicated that the lactic acid production in all instances was determined by both

the free suspended-cells concentration in the MM and the immobilized-cell (biofilm)

population on the supports. Negligible lactic acid concentration and cell absorbance was

observed in the suspended cells controls and the PP discs controls under all conditions.

Because the biofilm population of PCS was affected by its relative hydrophobicity,

lactic acid production was expected to be influenced also by the contact angle of the supports.

As indicated in Fig. 6, the lactic acid concentration and contact angle of SH-PCS and OH-

PCS had a 0.66 and 0.79 correlation coefficient (r), respectively. Statistical analysis of the

influence of PCS agricultural ingredients on the suspended cells density and lactic acid

production was comparable to their effects on contact angle and biofilm population. SAG

ANOVA output indicated that SH (P < 0.0009) and YE (P < 0.0001) increased cell

absorbance in the MM and SH (P < 0.02), YE (P < 0.0001), and SF (P < 0.02) enhanced

lactic acid production. The impact of SH, YE, and SF on the suspended cells density (620

nm) of BA-PCS was greater than that of the RBC-PCS. Consequently, SHYEBA-PCS and

SHSFYEBAS-PCS had 1.5 and 1.8 cell absorbance, respectively, whereas, SHYERBC-PCS

and SHSFYERBCS-PCS had 1.1 and 1.4 cell absorbance, respectively (Fig. 4). Similarly,

BA-PCS and RBC-PCS increased lactic acid production. Hence, SHYEBA-PCS and

SHSFYEBAS-PCS had 6.8 and 7.6 g/L lactic acid, respectively, whereas, SHYERBC-PCS

and SHSFYERBCS-PCS had 5.6 and 6.8 g/L lactic acid, respectively (Fig. 5).

Scanning electron microscopy (SEM)

SEM photographs illustrated that the hulls were well mixed and that agricultural

materials were spread among the PP matrix in the PCS (Fig. 7C). This produced a network

with grooves (g), ridges (r), and pits (Fig. 7C), whereas the PP discs had a relatively smooth

and flat surface (Fig. 7A). The agricultural material increased surface area and provided

sheltered regions from hydraulic shear forces for bacterial attachment on the PCS surfaces.

These observations matched with those obtained by Masol-Deya et al. [13] and also partly

explained why the less hydrophobic PP discs did not result in a higher cells attachment.

SEM micrographs also indicated that the YE-PCS had denser and larger cell clusters (Fig.

7D) than PP discs (Fig. 7B). Furthermore, extensive electron-opaque fibrillar networks (f)

were observed (Fig. 7D). This suggested the production of exopolysaccharides by *L.casei*

biofilm. The exopolysaccharide network as observed in SEM micrographs paralleled those

of Leppard and Bakke (1986), which also showed the presence of 5 nm electron-opaque

fibrils in biofilms of *Psuedomonas aeruginsoa* [1].

Discussion

The results strongly indicate that PCS, besides being able to provide surfaces and hydraulic shear-forces shelters for biofilm formation, were also able to supply complex nutrients for bacterial growth. The contact angle of *L. casei* was within the value range of *Thiobacillus* and *Bacillus* contact angles (26.8° and 32.6°, respectively) as reported by Van Loosdrecht et al. [15]. Hulls were necessary for increasing PCS surface area and providing sheltered regions from hydraulic shear forces by forming porous network with grooves and ridges. Of the two types of hulls evaluated, SH outperformed OH by being able to lower PCS hydrophobicity and by having better lactic acid production. This result was similar to that of Demirci et al. [8] with PCS chips (25% agricultural materials), which demonstrated that SH-PCS had a better yield (93.6%) and higher productivity (0.8 g/L/h) than OH-PCS (yield: 90.6%, productivity: 0.64 g/L/h) in continuous lactic acid fermentation with *L. casei*. Salts were another ingredient that could reduce the hydrophobicity of PCS and enhance attachment of cells on the supports.

Because of the MM used, negligible cell density and lactic acid production in the two controls (suspended cell culture and PP discs) confirmed *L. casei* fastiduous nature [11]. Moreover, this also demonstrated that PP discs in minimal medium could not supply the required complex nutrients to the bacteria for growth and lactic acid production. Among the minor agricultural ingredients added, YE was the most outstanding additive. YE, not only lowered the hydrophobicity of the PCS dramatically, but it also exerted a large impact on the increase of both the biofilm and the MM cell density, which in turn, greatly enhanced lactic-acid production. The favorable effects of YE were a result of its hydrophilic and solubility

property. This is an important factor because it indicates the possibility of lactic acid fermentation in minimal or reduced complex nutrients medium, which in turn, would lower the overall fermentation cost significantly. Among all the PCS evaluated, SHSFYEBAS-PCS had the greatest viable attached-cells counts, the highest lactic-acid concentration and suspended cell density in the BF and a relatively small contact-angle. All these results indicated that SHSFYEBAS-PCS possessed the greatest potential to be used as nutrient carrier and biofilm support for commercial production of lactic acid in reduced complex nutrient medium.

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Table 1.   Composition of plastic composite supports.

Supports	Ingredients of extrusion mixture % (w/w)[a]						S
	PP	HULLS	SF	YE	RBC	BA	
SH	50	50, SH	-	-	-	-	-
OHSF	50	40, OH	10	-	-	-	-
OHYE	50	40, OH	-	10	-	-	-
SHSFYE	50	40, SH	5	5	-	-	-
OHRBC	50	40, 0H	-	-	10	-	-
SHSFRBC	50	40, SH	5	-	5	-	-
SHYERBC	50	40, SH	-	5	5	-	-
OHSFYERBC	50	35, OH	5	5	5	-	-
OHS	50	50, OH	-	-	-	-	+
SHSFS	50	40, SH	10	-	-	-	+
SHYES	50	40, SH	-	10	-	-	+
OHSFYES	50	40, OH	5	5	-	-	+
SHRBCS	50	40, SH	-	-	10	-	+
OHSFRBCS	50	40, OH	5	-	5	-	+
OHYERBCS	50	40, OH	-	5	5	-	+
SHSFYERBCS	50	35, SH	5	5	5	-	+
OHBA	50	50, OH	-	-	-	10	-
SHSFBA	50	40, SH	5	-	-	5	-
SHYEBA	50	40, SH	-	5	-	5	-
OHSFYEBA	50	35, OH	5	5	-	5	-
SHBAS	50	40, SH	-	-	-	10	+
OHSFBAS	50	40, OH	5	-	-	5	+
OHYEBAS	50	40, OH	-	5	-	5	+
SHSFYEBAS	50	35, SH	5	5	-	5	+
PP (Control)	100	-	-	-	-	-	-

a   PP: polypropylene, OH: oat hulls, SH: soybean hulls, SF: soybean flour, YE: yeast extract, RBC: dried bovine red blood cells, BA: dried bovine albumen, S: salt.

Table 2.   Physical properties of plastic composite supports.

Supports [a]	Bulk volume cm³ [b]	Weight/disc mg/disc [c]	Supports contact angle (degree) [d]	Extrusion mixture water % [e]
SH	21	204	106	0.7
OHSF	22	159	99	2.4
OHYE	24	150	94	2.4
SHSFYE	20	182	102	0.6
OHRBC	24	161	102	2.6
SHSFRBC	19	176	104	1.0
SHYERBC	22	179	112	1.1
OHSFYERBC	24	150	103	2.8
OHS	23	185	101	2.8
SHSFS	19	197	104	0.8
SHYES	20	179	93	1.1
OHSFYES	21	182	95	3.0
SHRBCS	20	170	104	1.2
OHSFRBCS	24	164	92	2.1
OHYERBCS	25	170	100	3.2
SHSFYERBCS	20	174	101	1.2
OHBA	22	200	103	2.0
SHSFBA	20	197	99	0.8
SHYEBA	20	181	100	0.9
OHSFYEBA	25	164	93	3.3
SHBAS	20	154	101	1.0
OHSFBAS	23	182	92	2.3
OHYEBAS	24	176	88	2.4
SHSFYEBAS	20	168	93	1.0
PP (Control)	24	217	97	/

a   PP: polypropylene, OH: oat hulls, SH: soybean hulls, SF: soybean flour, YE: yeast extract, RBC:dried bovine red blood cells, BA: dried bovine albumen, S: salt.
b   Bulk volume of 5 g of PCS. Values were average of two replicates.
c   Each sample contained 5 g of supports. Values were average of two replicates.
d   Values were average of three replicates.
e   Percent moisture was obtained by convection drying method. Values were means of two replicates.

Figure legends list

Fig. 1. Shape of deposited water droplet on PCS/PP discs and *L.casei* cells lawn for contact

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Fig. 2. Relationship between contact angle of water droplet and drying time of *L. casei* cells

lawn.

Fig. 3. Relationship between contact angle and *L. casei* attachment on supports surface

during batch fermentation. LSD (P < 0.05) of contact angle and bacterial attachment

was 4.3° and 0.58 x 10^9 cfu/g support, respectively. The ● was not included in the

calculation of r.

Fig. 4. Relationship between *L. casei* suspended cell density of minimal medium and lactic

acid production during batch fermentation. LSD (P < 0.05) of cell density and lactic

acid concentration was 0.58 absorbance and 2 g/L, respectively.

Fig. 5. Relationship between biofilm population on supports and lactic acid production

during batch fermentation. LSD (P<0.05) of viable cells attached on supports and

lactic acid concentration was 0.57 x 109 cfu/g supports and 2 g/L, respectively.

Fig. 6. Relationship between *L. casei* contact angle and lactic acid production during batch

fermentation. The LSD (P<0.05) of contact angle and lactic acid concentration was

4.3° and 2 g/L, respectively. The ● was not included in the calculation of r.

Fig. 7. Scanning electron micrographs of the outer surface of the PCS and polypropylene

disc with *L. casei* biofilms in minimal medium. (A) Smooth outer surface of

polypropylene discs. Bar, 25 μm. (B) Enlargement of the box in (A) to show a small

cluster of *L. casei*. Bar, 5 μm. (C) Rough outer surface of OHYERBCS-PCS discs

with grooves [g] and ridges [r]. Bar, 180 µm. (D) Surface of OHYERBCS-PCS discs

enlarged to show large clusters of *L. casei* with fibrillar networks [f] formed from the

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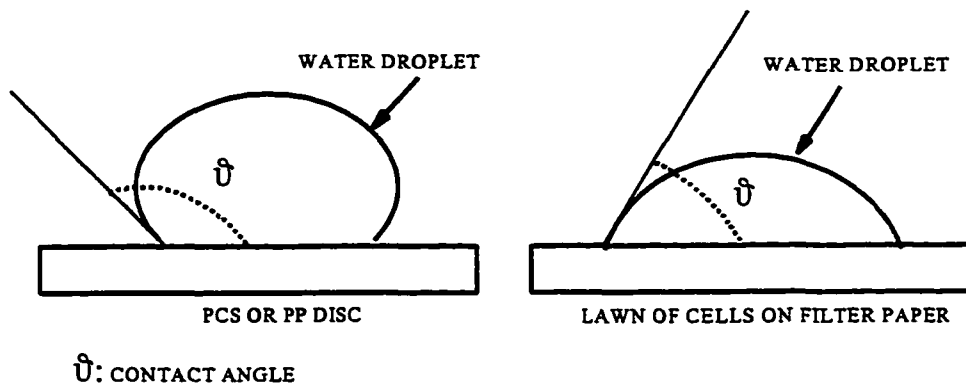
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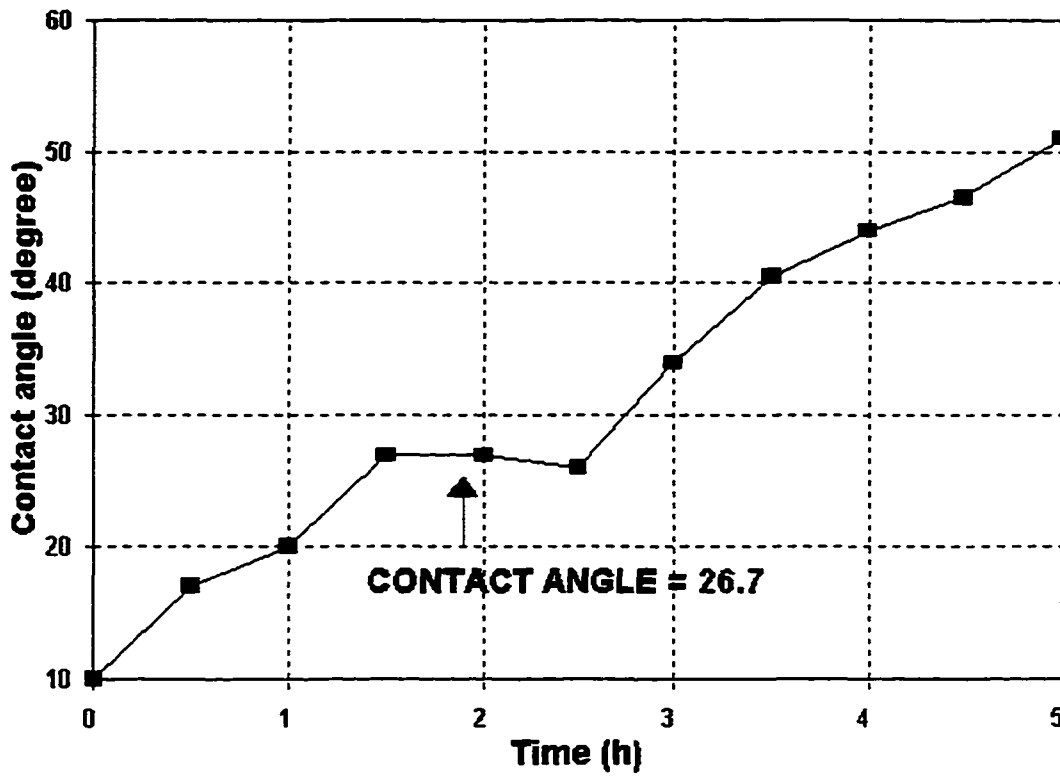
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NUTRIENTS LEACHING AND END PRODUCT ACCUMULATION IN PLASTIC COMPOSITE-SUPPORTS FOR L(+)-LACTIC ACID BIOFILM FERMENTATION

A paper submitted to Applied and Environmental Microbiology

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Abstract

Plastic composite-supports (PCS) (50% polypropylene(PP) and 50% agricultural products) produced by twin-screw high-temperature extrusion were soaked consecutively in 20-mL and then three 100-mL sterilized minimal medium at 37°C to simulate twenty 20-mL repeated-batch fermentation (RBF). Leached nutrients in minimal medium by the PCS and PP discs (control) were evaluated by Micro-Kjeldahl Method, by absorbance at 260, 275, and 280 nm, and by bioassays using *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443). Leached nitrogen in 20 mL initial soaking solution had a high correlation with the bioassay (cell density, r = 0.87) and with the absorbance at 260 nm (r = 0.95). PCS with only yeast extract as the minor agricultural ingredient had 51 to 60% initial leached nitrogen. PCS blended with bovine albumen, red blood cells, and soybean flour-leached nutrients gradually (20 - 30% initial leached nitrogen) and could still maintain 1 g/L lactic acid and cell density (absorbance at 620 nm:0.4-0.6) after the twentieth 20-mL SRB. PP discs under all circumstances gave negligible lactic acid production and cell density. PCS discs (20 mL bulk volume) were soaked in 30% lactic acid solution for 72 h at 45°C. Soaked PCS discs were

rinsed three times, then heat-treated (121°C, 15 min) in 15 mL deionized water. The amount

of lactic acid in the residual 30% lactic-acid soaking-solution and the PCS-disc-extracted

solution were determined by high-performance liquid chromatograph (HPLC). Lactic acid

accumulation in PCS was shown to be mainly due to absorption and had no correlation with

lactic acid production or biofilm formation.

Introduction

Polylactic-acid-degradable plastics are polyester thermoplastics with properties

similar to acrylics and polystyrene. Some of its applications are disposable thermoplastic

products (wraps, cups, insulators), refuse and retail bags, and agricultural mulch film, which

constitutes a potential market size of 600 to 800 million lb/yr [3,11]. To compete in the

commodity plastics market, the development of advanced and economic fermentation

processes that yield a relatively pure lactic acid solution is required. Homofermentative

Lactobacillus is the typical production organism. *Lactobacilli* are fastidious organisms, and

their growth requires a specific supply of complex nutrients such as vitamins, nucleotides,

amino acids and inorganic salts [10]. Hence, expensive complex nutrients such as yeast

extract, when added to the culture medium, significantly increase the overall fermentation

cost [11].

Biofilms are a natural form of cell immobilization by which microorganisms attach to

solid surfaces [1]. Immobilized cells are used to increase the overall cell density in the

bioreactor [5,6]. This laboratory recently confirmed lactic-acid biofilm fermentation by

using plastic composite-supports discs (50% polypropylene and 50% agricultural products

[w/w]) with *L. casei* subsp. *rhamnosus* in minimal medium. The results in that study

indicated that, along with being a biofilm support, the PCS was also a complex nutrients

carrier [9]. Hence, methods for evaluating the PCS total complex nutrients, the amount of

available complex-nutrients leached, and the operational time of PCS as a complex nutrient

carrier are essential for PCS blend selection.

Lactic acid is known to have antimicrobial activity [8] and end-product inhibition on

lactate dehydrogenase, the enzyme that catalyzes the formation of lactic acid from pyruvate

acid [15]. The PCS discs were shown to be porous and permeable to liquid [9]. This

indicates possible lactic-acid accumulation inside the PCS, which in turn, might impede

biofilm formation and/or lactic acid fermentation.

The study reported here demonstrated that accumulation of lactic acid inside the PCS

was mainly due to absorption and that it had no impact on lactic acid production nor biofilm

formation. In addition PCS containing soy hulls, soybean flour, yeast extract, and bovine

albumen behaved as a slow-release-complex nutrients carrier for lactic-acid biofilm

fermentation in minimal medium environment.

Materials and methods

Plastic composite-supports

Plastic composite-supports (PCS) discs, which contained 50% polypropylene [PP]

(Quantum USI Division, Cincinnati, OH) and 50% agricultural materials (w/w), were

produced by high-temperature extrusion in a Brabender with PL2000 twin-screw co-rotating

extruder (Model CTSE-V, C.W. Brabender Instruments, Inc., South Hackensack, NJ).

Incorporation of ground (20 mesh) oat hulls (OH) (Ralston Foods, Cedar Rapids, IA), ground

(20 mesh) vacuum-dried (48 h at 110°C and 30 inches Hg) soybean hulls (SH) (Cargill Soy

Processing Plant, Iowa Falls, IA), defatted soybean flour (SF) (Archer Daniels Midland,

Decature, IL), yeast extract (YE) (Ardamine Z, Champlain Industries Inc., Clifton, NJ), dried

bovine albumin (BA) (American Protein Corp., Ames, IA), dried bovine red blood cells

(RBC) (American Protein Corp., Ames, IA), and mineral salts (S) (0.2% sodium acetate,

0.12% $MgSO_4 \cdot 7H_2 0$ and 0.006% $MnSO_4 \cdot 7H_2O$) into PCS discs was evaluated. Materials to

be extruded were first mixed in a separate container on a weight basis before being poured

into the extruder hopper. The mixture was extruded (11 and 15 rpm for soybean hulls and oat

hulls composite supports, respectively) as a continuous tube through a medium pipe die (3.2

mm ID and 12.7 mm OD) with barrel temperatures of 200, 220, and 200°C and a die

temperature of 167°C. The barrel exhaust vent was plugged because the release of moisture

at the die was essential for producing a porus support. Composite tubing was extruded onto a

steel rod and air cooled slowly without fanning. Tubes with 10 to 11 mm OD were then cut

into discs. Composition and interstitial volume of each PCS are listed in Table 1.

Polypropylene discs were bored out of a polypropylene sheet (3.5 mm thick) with cork borers

7 mm ID and 11 mm OD.

Lactic acid accumulation assay

L(+)-Lactic acid absorption to PCS was evaluated according to the procedures

outlined in Fig. 1. Screw-cap culture-tubes (in replicates of three) with weighed supports

were sterilized dry (45 min at 121°C) alongside control culture tubes without supports, with

equivalent-weight of filter papers, unground oat hulls, unground soy hulls, or PP discs. Each

culture tube was filled with 20-mL sterilized deionized-water and incubated in a 45°C water bath for 72 h to hydrate the materials inside the culture tubes and to solubilize any readily leachable material from the PCS and hulls. Initial residual soaking-solution volume was measured, discarded, and aseptically replaced by 15 mL sterilized 30% L(+)-lactic-acid-solution (LAS) (30% lactic acid, 0.4% yeast extract, 2% glucose and mineral salts solution). The screw cap junctions were wrapped with parafilm (to prevent moisture lost due to evaporation) and the culture tubes were subsequently incubated in a covered water bath at 45°C for 2 weeks. After incubation, the 30% LAS in each culture tube was decanted, and the supports in each tube were rinsed three times in 15 mL deionized water. The 30% LAS and the rinsed solutions from each tube were collected in a 100-mL volumetric flask. The final flask volume was made up to 100 mL with deionized water. The lactic acid concentration of the 30% LAS after treatment was regarded as the final residual lactic acid not absorbed by the materials, whereas, the lactic acid concentration of the culture tube without supports/materials was treated as the initial 30% LAS lactic acid concentration. Treated and washed materials from each tube were subsequently transferred to a 100-mL dilution bottle containing 15 mL deionized water and were autoclaved (15 min at 121°C) in an effort to release the absorbed lactic acid. L(+)-Lactic acid concentration of the initial 30% LAS, final residual 30% LAS, and the hot-water treatment solution was determined by high-performance liquid chromatography (HPLC). The interstitial volume of each type of support was estimated by the following formula:

$$\text{Interstitial volume (mL)} = \frac{\text{lactic acid absorbed in the PCS (mg)}}{\text{Initial 30\% LAS lactic-acid concentration mg/mL}} \quad (1)$$

Total nitrogen in PCS

A modified standard AOAC micro-kjeldahl method (920.39C) was used to determine the total nitrogen in the unground soybean hulls, unground oat hulls, soybean flour, yeast extract, dried bovine albumen, dried red-blood cells, and PCS. Two grams of PCS (0.2 g for agricultural material) was weighed and digested by 10-mL concentrated sulfuric-acid digestion in the presence of Copper(II) selenite dihydrate (Aldrich Chemical Company, Inc., Milwaukee, WI) and potassium sulfate (catalysts). The liberated ammonia was distilled and collected by a receiving flask containing 10 mL 1% boric acid. The amount of ammonia in the distillate was determined by titration with 0.1 N HCl.

Leaching ability of PCS

Five grams of PCS or PP (control) discs were sterilized dry (45 min at 121°C) in a 20-mL screw-cap cultured tube in duplicate. Twenty milliliters of sterilized minimal medium (MM) (2% glucose and mineral salts solution) was aseptically added to the culture tubes, which were then incubated a 37°C water bath for 48 h. The initial soaking solution was aseptically transferred into sterilized screw-cap culture tubes.

Ten milliliters of the initial MM soaking-solution was retrieved from each culture tube, and the amount of nitrogen present in the solution was determined by the modified standard AOAC micro-kjeldahl method already described. The initial leached nitrogen % from the PCS was calculated by the formula:

Initial Leached Nitrogen (%) = $\dfrac{\text{Nitrogen in 20 mL initial soaking solution} \times 100\ \%}{\text{Total nitrogen in 5 g PCS}}$ (2)

Another sample was retrieved from the remaining initial soaking-solution for absorbance

measurement (with appropriate dilution) at 260, 275, and 280 nm by a Beckman Model DU 50 UV/VIS spectrophotometer (Beckman Instrument Inc., Irvine, CA) to evaluate leachable aromatic compounds.

Bacterial culture preparation

Lactobacillus casei subsp. rhamnosus (ATCC 11443), a homofermenter of L(+)-lactic acid, was obtained from the American Type Culture Collection (Rockville, MD). Stock cultures were maintained in Lactobacillus MRS broth (Difco Laboratories, Detroit, MI) at 4°C with monthly transfers to fresh medium. Ten milliliters of an active L. casei culture (18 h in MRS broth at 37°C) was inoculated into 100 mL of lactic acid fermentation (LAF) medium [2% glucose (Glu), 0.4% yeast extract (YE) (Ardamine Z, Champlain Industries Inc., Clifton, NJ), and salt solution (0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.1% sodium acetate, 0.06% $MgSO_4·7H_2O$, and 0.003% $MnSO_4·7H_2O$)] [4] which was then incubated for 18 h at 37°C. Centrifugation (16300 x g, 20 min) followed by a rinsing with 100 mL of MM were used to remove LAF medium from the active cells. The rinsed active cells were then resuspended into 100 mL of sterilized MM and used in the bioassay of the PCS soaking solution.

Bioassays of PCS leachate

Five grams of PCS or PP (control) discs were sterilized dry (45 min at 121°C) in a 20-mL screw-cap culture tube. The sterilized discs were aseptically transferred into a dilution bottle and soaked in 20 mL of sterilized MM at 37°C water bath in duplicate for 48 h. Ten milliliters of the MM soaking solution was retrieved from each dilution bottle, and the amount of nitrogen present in the solution was assayed by the modified standard AOAC

micro-kjeldahl method already described. Another 5 mL of the soaking solution was

aspetically transferred from the bottle into a sterilized 20-mL screw-cap culture tube for

bioassays. Each culture tube was inoculated with 0.05-mL washed and active *L. casei* culture

(18-h culture) and incubated in a 37°C water bath for 48 h. Lactic acid produced, glucose

consumed, and cell density (absorbance at 620 nm) of the bioassayed soaking solution were

determined by methods to be described. Another sample was retrieved from the remaining

soaking solution, which was then appropriately diluted for absorbance measurement at 260,

275, and 280 nm. The whole process was repeated two times by adding 100-mL sterilized

MM, which simulated five and ten 20-mL repeated-batch fermentations.

Simulated Repeated-batch fermentation (RBF) studies

Simulated RBF assay without pH control was developed to characterize the supports'

nutritional benefits. Suspended cells and PP discs were used as controls. PCS or PP discs (5

g) were sterilized dry (45 min at 121°C) in 20-mL screw-cap culture tubes. The sterilized

discs were aseptically transferred into a dilution bottle containing 20 mL sterilized MM and

incubated in a 37°C water bath for 24 h. This initial soaking solution was decanted

aseptically.

Each dilution bottle was then refilled with 20 mL of sterilized MM, inoculated with

0.2-mL active L. casei (18-h culture) then reincubated in a 37°C water bath for 48 h. The

first RBF medium was aseptically decanted, and 80 mL of fresh sterilized MM was refilled

into the dilution bottle, thus simulating an equivalent of five 20-ml RBF. The decanting and

refilling processes were repeated with three consecutive transfers in 100-mL sterilized MM,

which subsequently simulated ten, fifteen, and twenty 20-mL RBF. Decanted fermented

media from each simulated RBF were evaluated for lactic acid produced, glucose consumed, and cell density (620 nm).

Viable biofilm population on the supports

A modified method of Dickson et al. [7] was used to enumerate the relative biofilm population on the PCS. After the first simulated RBF in MM, five discs (both PCS and PP) were aseptically removed from each RBF bottle and transferred into a dilution bottle containing 100 mL sterilized 0.1% peptone-water. Each bottle was vigorously shaken to remove nonbiofilm cells. The rinsed supports were then aseptically transferred into a screw-cap culture tube with 9 mL sterilized 0.1% peptone water and 5 g of sterilized sand. The culture tube was subsequently vortexed vigorously at 30-s intervals for a total of 1.5 min. The culture tube medium was then serially diluted into sterilized 0.1% peptone (10^3 to 10^5). Colony-forming units (cfu) were determined for each tube by using Lactobacillus MRS agar spread plates in duplicates. Finally, the five sand-stripped discs from each RBF bottle were rinsed (water), convection oven dried (70°C, 24 h), and weighed. Preliminary data on some PCS blends showed that the biofilm population in minimal medium with no pH control was similar for 20 batches of repeated-batch fermentation. Hence, only the PCS biofilm population of the first RBF was evaluated in this study.

Bioassayed and fermented media analysis

L(+)-Lactic acid and D-glucose concentrations were analyzed by a Waters high-performance liquid chromatograph (HPLC) (Milford, MA) equipped with Waters model 401 refractive index detector and a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using 0.012 N H_2SO_4 as the mobile phase. Bacterial

growth in LAF medium was followed by measurement of the absorbance at 620 nm with a

Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, NY).

Statistical analysis

Two replications of a 2^{5-1} design [2] were used to evaluate the effect of SH, OH, YE,

SF, RBC, BA, and S on the characteristics of PCS in all the tests performed. The five factors

evaluated in the first replication were hulls (OH or SH), YE, SF RBC, and S. The five

factors studied in the second replication were hulls (OH or SH), YE, SF, BA, and S. In each

replication 16 treatments in duplicate (unless otherwise stated) were examined. The data

from each test were analyzed by Least Significant Difference (LSD) and Analysis of

Variance (ANOVA) using Statistical Analysis System Package (version 6.03) (SAS Institute,

Inc., 1985).

Results and discussion

Lactic acid accumulation and interstitial volume of PCS

If the accumulation of lactic acid was mainly due to absorption, then the lactic- acid

concentration inside the PCS and the lactic acid concentration in the medium will be the

same. Furthermore, the lactic acid accumulated inside the PCS (5 g) should be inversely

proportional to the amount of residual lactic acid in the outside medium, and their sum

should be equal to the initial amount of lactic acid in the 15 mL of 30% LAS. Results

showed that all the PCS behaved similarly to the filter paper controls, which resembled

absorption. The amount of lactic acid accumulated in the PCS, as indicated by the total lactic

acid being extracted from the PCS through high-temperature treatment, was inversely

correlated (r = - 0.95) with the amount of lactic acid left in the 30% LAS after 2 weeks'

soaking (Fig. 2). This strongly supported the assumption that there were no specific sites in

the PCS for the binding of lactic acid and that the accumulation of lactic acid in the supports

was mainly due to absorption, which could be easily retrieved by physical methods such as

high temperature treatment. This assumption was further confirmed in that the amount of

lactic acid accumulated in the PCS neither had any correlation with the biofilm population on

the supports nor with the lactic acid production of *L. casei* during batch fermentation (Fig. 3).

The lactic acid absorbed in the PCS served as a marker to determine the PCS

interstitial volume. The larger the interstitial volume, the more lactic acid was absorbed

inside the PCS (r = 0.77) and the lesser the amount of lactic acid remaining in the medium

outside the PCS (r = - 0.71) (Fig. 4). The interstitial volume of the PCS was calculated by

using equation [1] (Table 1). From SAS ANOVA, SH (P < 0.0005), RBC (P < 0.0001) and

BA (P < 0.0044) addition increased the interstitial volume of PCS, which in turn, suggested

that the liquid transport capacity of these PCS was greater.

PCS total nitrogen and leaching ability

The percentage protein of the agricultural materials resulted from the micro-kjeldahl

analysis were similar to the values obtained from literature [12, 17] and product-

specification data sheets (Table 2). SH had greater protein content than OH. Among the

minor agricultural ingredients, dried RBC and BA had more protein content than did YE and

SF. The PCS total nitrogen (mg/g) obtained from Micro-kjeldahl analysis matched the sum

of the agricultural ingredients nitrogen content (r = 0.96) (Fig. 5). Thus indicating that most

of the protein was retained in the PCS after the extrusion process.

Results of the initial soaking-solution Micro-kjeldahl analysis showed that supports with high total nitrogen did not correlate with high initial leached-nitrogen percentage. As shown in Fig. 6, the initial leached-nitrogen percentage of SHSFYEBAS-PCS was 23% when its total nitrogen was 114 mg nitrogen per g support, whereas OHYE-PCS had 63% initial leached nitrogen when its total nitrogen was 49 mg nitrogen per g support. These results indicated that complex nutrients in PCS with higher initial leached-nitrogen-percentages depleted quickly (within 1 to 5 RBF). This, in turn, led to the reduction of L. casei growth in long-term RBF, whereas PCS with lower initial leached-nitrogen percentage, released its complex nutrients gradually and thus sustained a longer operational life. This was supported by the simulated RBF analysis, which showed that, although OHYE-PCS and OHSFYES-PCS both had similar total nitrogen content (49 and 42 mg/g support, respectively), they performed quite differently at the 20th RBF (Fig. 7). As indicated by the negligible lactic acid concentration in the minimal medium, OHYE-PCS with a 63% initial leached nitrogen could no longer provide nutrients for the growth of L. casei at the 20th simulated RBF, whereas OHSFYE-PCS having a 42% initial leached nitrogen still supported bacterial growth and obtained a final 1.18 g/L lactic acid concentration in the minimal medium. Indeed, SHSFYEBAS-PCS, which had the highest lactic acid concentration (1.44 g/L) by the end of the 20th simulated RBF, possessed only an initial 23% leached nitrogen. SAS ANOVA supported this observation and indicated that SH, SF, RBC, and BA slowed down the released of nitrogenous compounds from the PCS, whereas OH and YE both hastened the leaching of nitrogenous compounds from the supports (P < 0.0001).

The leaching ability of PCS was evaluated also by measuring the soaking solution absorbance at 260, 275, and 280 nm. The selection of these wavelengths was based on the compounds expected to be leached out from the PCS. The 260 nm corresponds to the λ_{max} of L-phenylalanine [13], riboflavin [16], and some lignin-derived aromatic compounds such as vanillic acid [14]. The 275 nm corresponds to λ_{max} of L-tyrosine [13], cobalamin [16], syringic acid, guaiacol, and catechol [14]. The 280 nm correpsonds to λ_{max} of L-tryptophan, folacin [16], vanillin, and veratraldehyde [14]. Hence, the absorbance at these wavelengths could indicate the concentration or presence of nutrients such as aromatic amino acids (nitrogenous compounds) and water-soluble vitamins or of the released of lignin-derived aromatic fragments from the hulls, which might inhibit the growth of microorganisms. SAS ANOVA output demonstrated that PCS with SH had greater absorbance than supports with OH at all three wavelengths ($P < 0.0001$). Among all the minor agricultural ingredients, PCS with YE had greater absorbance than supports without YE ($P < 0.0001$).

Bioassays of the PCS soaking solution showed that the cell density and lactic acid concentration of the initial soaking solution had a correlation of 0.80 to 0.86 and 0.70 to 0.76, respectively, with the soaking solution absorbance at 260, 275, and 280 nm (Table 3 and Fig. 7). This indicated that sufficient complex nutrients were being released from the PCS to support the growth of *L. casei* in minimal medium. For PCS with hulls alone as the agricultural ingredients (OH-PCS and SH-PCS), negligible lactic-acid-concentration was detected, although an increase in cell density were observed. In contrast, as shown by SAS ANOVA, YE significantly increased the cell density of the soaking solution in the bioassays analysis ($P < 0.0001$). This strongly suggested that hulls alone partly met the complex

nutrients requirements of *L. casei* and the presence of minor complex ingredients such as YE was essential for the PCS to exhibit its nutrients carrier role. Indeed, the correlation coefficient between the initial soaking-solution lactic acid concentration and its absorbance at 260 nm was improved from 0.76 to 0.84 (Fig. 8) when OH-PCS and SH-PCS values were removed from the data set. In addition, a high correlation of the initial soaking-solution leached-nitrogen concentration (analyzed by Micro-kjeldahl) with its cell density (r = 0.87) (Fig. 9) absorbance at 260, 275, and 280 nm (r ≥ 0.90) (Table 3) was demonstrated. This further proved that the nutrients in the form of nitrogenous compounds was a component of the PCS leachate, whereas the amount of lignin-derived aromatic compounds in the leachate was relatively small and posed no harmful effect on the growth of this fastidious bacteria. Bioassays of PP discs soaking solution demonstrated no leached nitrogenous-compounds for the bacteria to grow in the minimal medium environment.

Among the three wavelengths (260, 275, and 280 nm) used, 260 nm exhibited the highest correlation with the soaking solution leached-nitrogen Micro-kjeldahl values, the initial leached-nitrogen percentage, and the cell density of the initial soaking solution (Table 3). This strongly suggested that 260 nm would be the optimum wavelength to be used for evaluating the amount of nitrogenous compounds leached from the PCS.

Accumulation of lactic acid in PCS due to absorption greatly removed the worry of end product accumulation in the supports. SH outperformed OH by having greater protein content and possessing the ability to increase interstitial volume of PCS. As indicated by the leached nitrogen and 260 nm absorbance of soaking solution (Fig. 10), YE was the most outstanding minor complex ingredient for supplying nitrogenous compound in the PCS.

However, because of the fast leaching rate of YE from the PCS, other minor agricultural ingredients such as RBC, BA, and SF were essential to ensure a gradual release of nitrogenous compounds from the PCS. The results of this study showed that there is high potential for SHYE-PCS with SF, BA, and/or RBC to perform as slow-releasing nutrient-carriers. Finally, SHSFYEBAS-PCS was demonstrated to have the highest lactic-acid concentration and cell density in 1st and 20th RBF, the greatest viable attached cells counts, a relatively small contact angle [9], and a gradual complex nutrients releasing rate. Hence, SHSFYEBAS-PCS is recommended for long- term biofilm lactic-acid fermentation studies in minimal medium.

Acknowledgements

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Table 1.   Composition and physical properties of soaking materials in lactic acid

absorption assay

Supports	Ingredients of Plastic composite-supports % (w/w)[a]						S	Interstitial volume
	PP	Hulls	SF	YE	RBC	BA		(mL) / g support[b]
SH	50	50, SH	-	-	-	-	-	0.27
OHSF	50	40, OH	10	-	-	-	-	0.30
OHYE	50	40, OH	-	10	-	-	-	0.24
SHSFYE	50	40, SH	5	5	-	-	-	0.30
OHRBC	50	40, 0H	-	-	10	-	-	0.32
SHSFRBC	50	40, SH	5	-	5	-	-	0.32
SHYERBC	50	40, SH	-	5	5	-	-	0.33
OHSFYERBC	50	35, OH	5	5	5	-	-	0.30
OHS	50	50, OH	-	-	-	-	÷	0.25
SHSFS	50	40, SH	10	-	-	-	÷	0.29
SHYES	50	40, SH	-	10	-	-	÷	0.32
OHSFYES	50	40, OH	5	5	-	-	÷	0.30
SHRBCS	50	40, SH	-	-	10	-	÷	0.36
OHSFRBCS	50	40, OH	5	-	5	-	÷	0.31
OHYERBCS	50	40, OH	-	5	5	-	÷	0.31
SHSFYERBCS	50	35, SH	5	5	5	-	÷	0.36
OHBA	50	50, OH	-	-	-	10	-	0.32
SHSFBA	50	40, SH	5	-	-	5	-	0.30
SHYEBA	50	40, SH	-	5	-	5	-	0.31
OHSFYEBA	50	35, OH	5	5	-	5	-	0.28
SHBAS	50	40, SH	-	-	-	10	÷	0.30
OHSFBAS	50	40, OH	5	-	-	5	÷	0.30
OHYEBAS	50	40, OH	-	5	-	5	÷	0.33
SHSFYEBAS	50	35, SH	5	5	-	5	÷	0.30

Oat hulls	-	100, OH	-	-	-	-	-	-
Soy hulls	-	100, SH	-	-	-	-	-	-
Filter paper	-	Cellulose	-	-	-	-	-	-
PP	100	-	-	-	-	-	-	0

a   PP: polypropylene, OH: oat hulls, SH: soybean hulls, SF: soybean flour, YE: yeast extract, RBC: dried bovine red blood cells, BA: dried bovine albumen, S: Salt.

b   Values were average of three replicates.

Table 2. Percentage of protein (dry-weight basis) of agricultural ingredients determined by Micro-kjeldahl.

Protein %[a]	Soybean hull	Oat hull	Soybean flour	Yeast extract	Dried red blood cells	Dried bovine albumen
Micro-kjeldahl Method	9.8	3.4	52.1	68.8	86.8	84.7
Published value	8.8[b]	1.6 - 5.7[c]	54.0[b]	68.0[d]	90.0[e]	80.0[e]

a % Protein = N x 6.25

b Adapted from table presented by Pearson [12].

c Adapted from table presented by Youngs and Brown [17].

d Adapted from product specifications of Champlain Industries Inc. (Clifton, NJ).

e Adapted from product specifications of American Protein Corporation (Manning, IA).

Table 3.   Correlation of initial soaking solution absorbance at 260, 275, and 280 nm

with initial soaking-solution leached nitrogen, initial nitrogen-leached

percentage, and initial soaking-solution cell density.

Initial soaking solution wavelength	Correlation coefficient (r)		
	Leached nitrogen in 20 mL initial soaking solution [a]	Initial nitrogen leached percentage [b]	Cell density of initial soaking solution at 620 nm [c]
260 nm	0.95	0.82	0.86
275 nm	0.92	0.80	0.82
280 nm	0.90	0.76	0.81

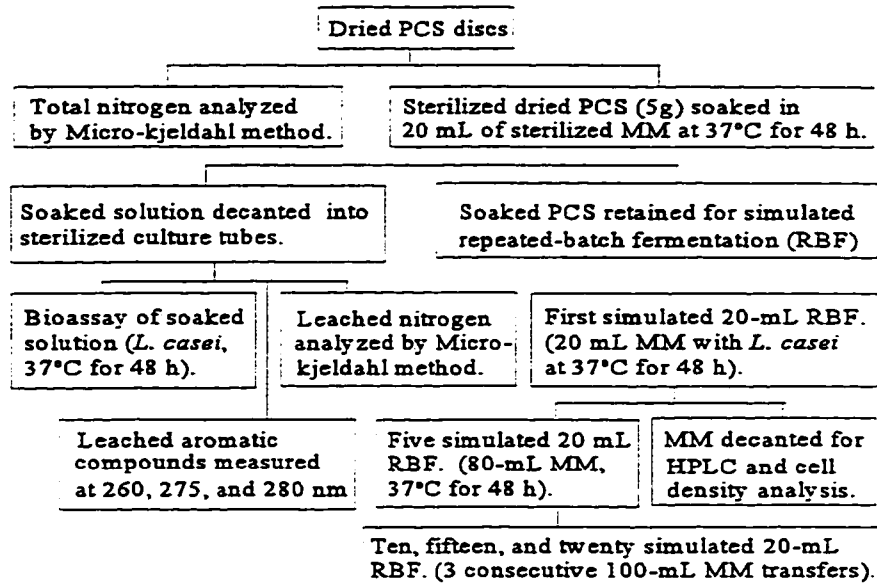a   Nitrogen concentration determined by Micro-kjeldahl analysis.

b   Initial leached nitrogen percentage = $\dfrac{\text{Nitrogen in initial soaking solution (mg)}}{\text{Total nitrogen in 5 g PCS (mg)}} \times 100\%$

c   Cells absorbance of soaking solution (100 mL) at 620 nm.

Figure legends list

Fig. 1. Schematic diagram of nutrient-leaching and lactic-acid accumulation. PCS:plastic composite supports, MM:minimal medium, PP:polypropylene, LAS:lactic-acid solution.

Fig. 2. Relationship between residual lactic acid (g) of 20-mL initial soaking solution and absorbed lactic acid (g) in plastic composite supports.

Fig.3. Effects of absorbed lactic acid (g) in plastic composite supports on first RBF lactic acid production and biofilm population of *L. casei*.

Fig. 4. Relationship of interstitial volume (mL/g support) with plastic composite-support absorbed lactic acid and residual lactic acid in 20 mL initial soaking solution.

Fig. 5. Relationship between estimated and Micro-kjeldahl total nitrogen value (mg/g support) of plastic composite supports.

Fig. 6. Relationship between initial leached-nitrogen percentage and total nitrogen of plastic composite supports as determined by Micro-kjeldahl.

Fig. 7. Relationship between initial leached-nitrogen percentage and *L. casei* 20th simulated RBF lactic acid concentration of plastic composite supports.

Fig. 8. Relationship between 100-mL soaking-solution absorbance at 260 nm and its bioassay cell-density (620 nm) *of L. casei* and lactic acid concentration.

Fig. 9. Relationship between the amount of leached nitrogen in 20 mL initial- soaking solution and its bioassay cell density (620 nm) *of L. casei*.

Fig.10 Relationship between the leached nitrogen (mg) present in 20-mL soaking solution and its absorbance at 260 nm.

LEACHED NUTRIENTS ANALYSIS

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                    ┌─────────────────┐
                    │ Dried PCS discs │
                    └─────────────────┘
          ┌───────────────────────┴──────────────────────────┐
┌──────────────────────────┐   ┌──────────────────────────────────────┐
│ Total nitrogen analyzed   │   │ Sterilized dried PCS (5g) soaked in    │
│ by Micro-kjeldahl method. │   │ 20 mL of sterilized MM at 37°C for 48 h.│
└──────────────────────────┘   └──────────────────────────────────────┘
        ┌────────────────────┴───────────────────────┐
┌───────────────────────────┐   ┌──────────────────────────────┐
│ Soaked solution decanted into│  │ Soaked PCS retained for simulated│
│ sterilized culture tubes.   │   │ repeated-batch fermentation (RBF)│
└───────────────────────────┘   └──────────────────────────────┘
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| Bioassay of soaked solution (*L. casei*, 37°C for 48 h). | Leached nitrogen analyzed by Micro-kjeldahl method. | First simulated 20-mL RBF. (20 mL MM with *L. casei* at 37°C for 48 h). |

| Leached aromatic compounds measured at 260, 275, and 280 nm | Five simulated 20 mL RBF. (80-mL MM, 37°C for 48 h). | MM decanted for HPLC and cell density analysis. |

Ten, fifteen, and twenty simulated 20-mL
RBF. (3 consecutive 100-mL MM transfers).

LACTIC-ACID ACCUMULATION ANALYSIS

Empty culture tube and Culture-tube with
5 g hulls, filter paper, PP, or dried PCS.

Materials in the culture-tube were sterilized
dry at 121°C for 45 min.

Sterilized materials were soaked with 20 mL
sterilized deionized water for 72 h at 45°C
and then decanted.

| Hydrated materials were resuspended in 15 mL sterilized 30% LAS for 2 weeks at 45°C. | The decanted soaked solution volume was measured. |

| Lactic acid concentration of the 30% LAS after suspension was determined by HPLC. | Materials in the culture tube were transferred to a 100-mL dilution bottle containing 15 mL deionized water and then autoclaved. |

Lactic acid concentration of hot-water
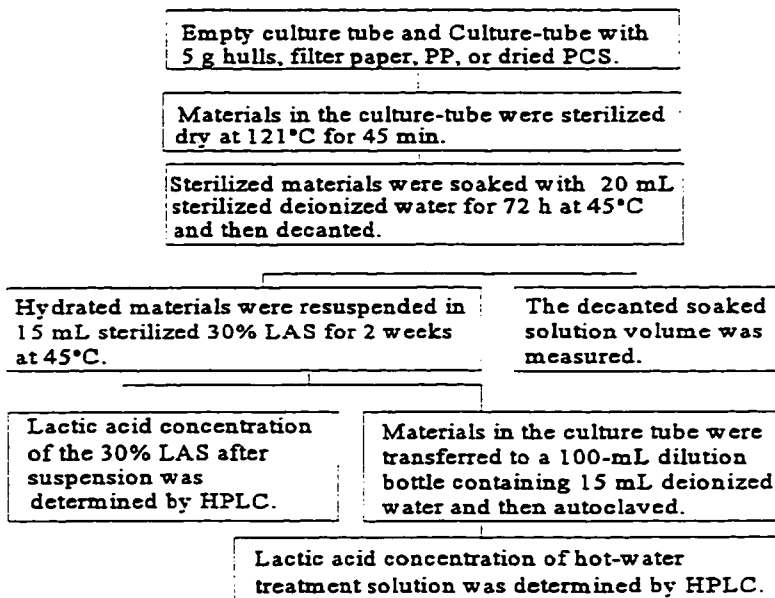treatment solution was determined by HPLC.

Fig. 1. Schematic diagram of nutrient-leaching analysis and lacti-acid accumulation analysis. PCS: plastic-composite support, MM: minimal medium, PP: polypropylene disc, LAS: lactic-acid solution.

Fig. 2.   Relationship between residual lactic acid (g) of 20-mL initial soaking-solution and absorbed lactic acid (g) in plastic composite-supports.
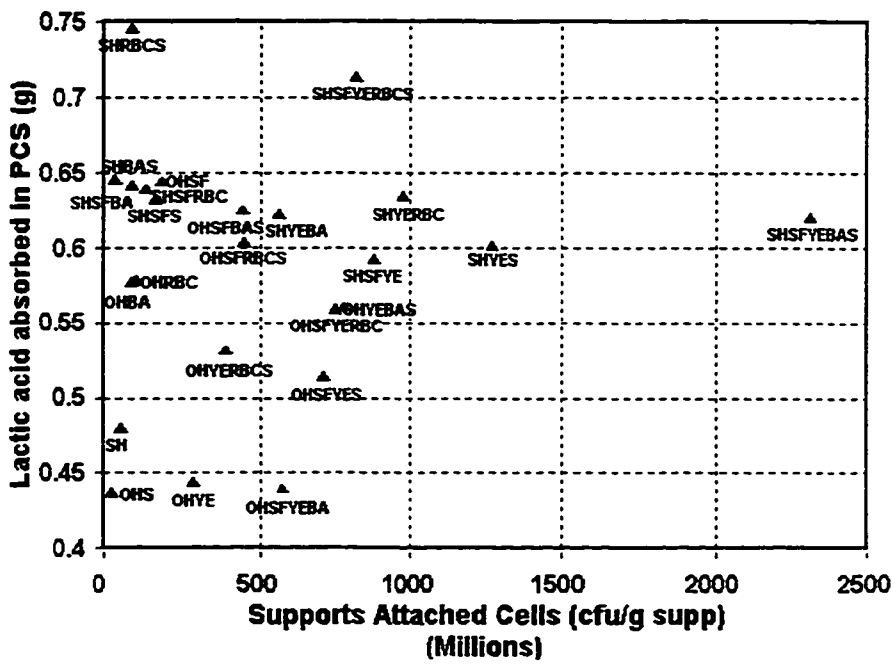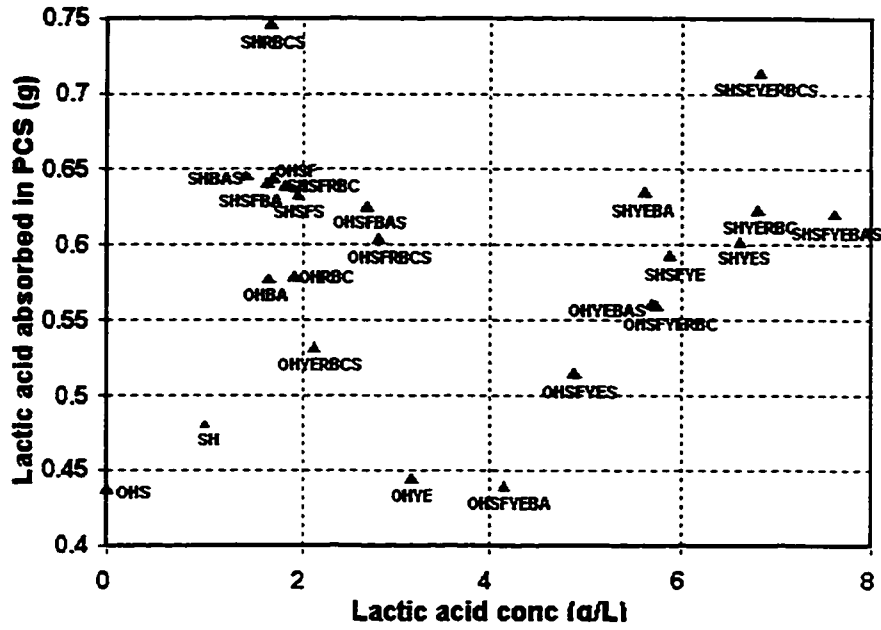
Fig. 3. Effects of absorbed lactic acid (g) in plastic composite-supports on 1st RBF lactic acid production and biofilm population of *L. casei*.
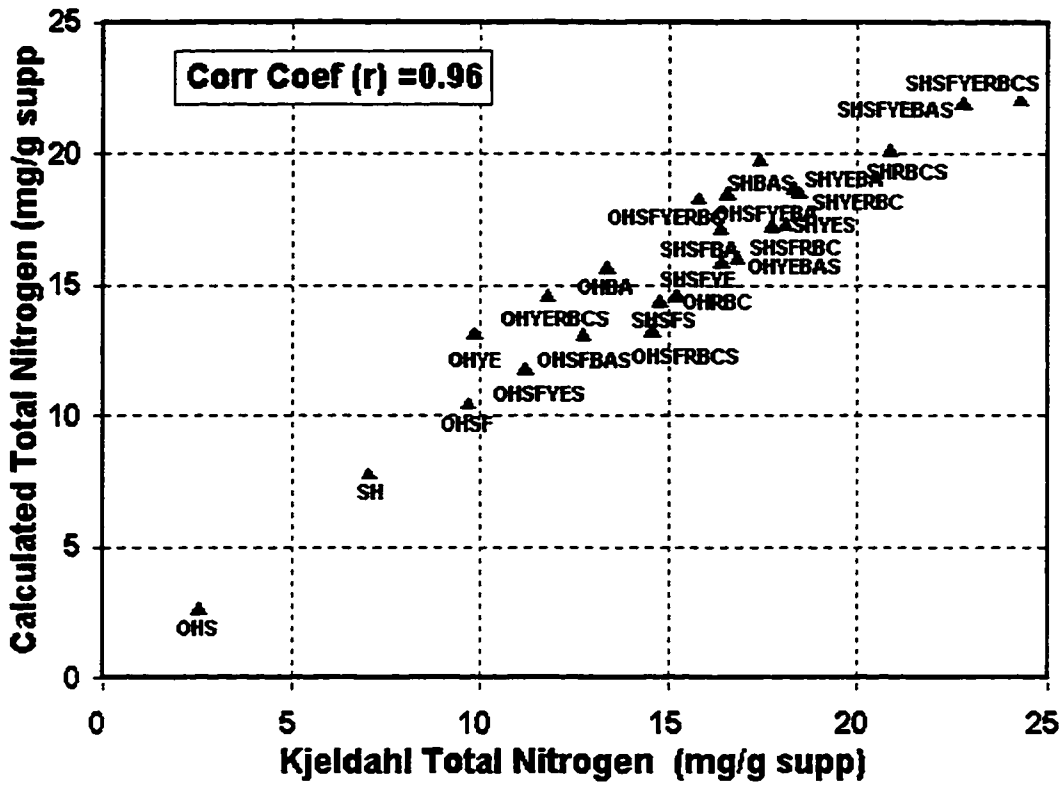
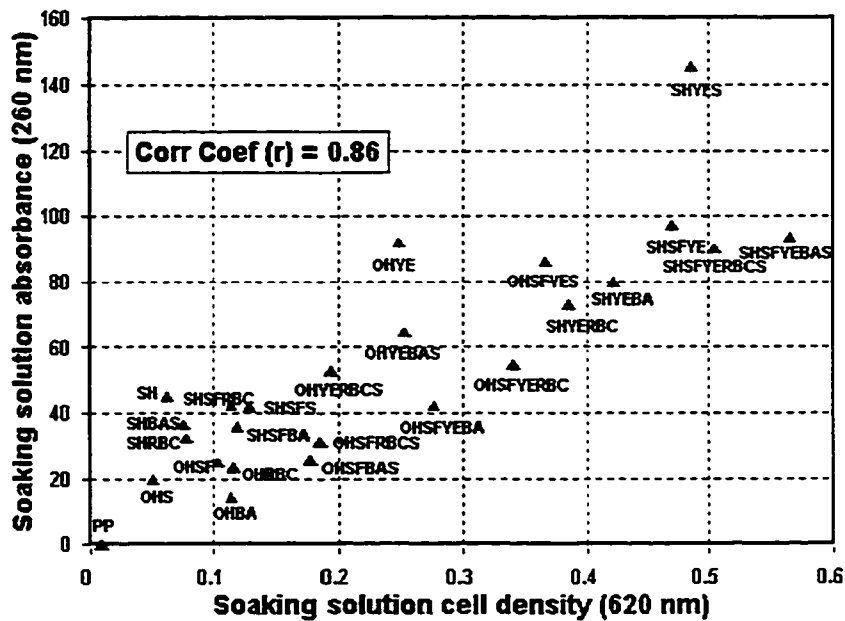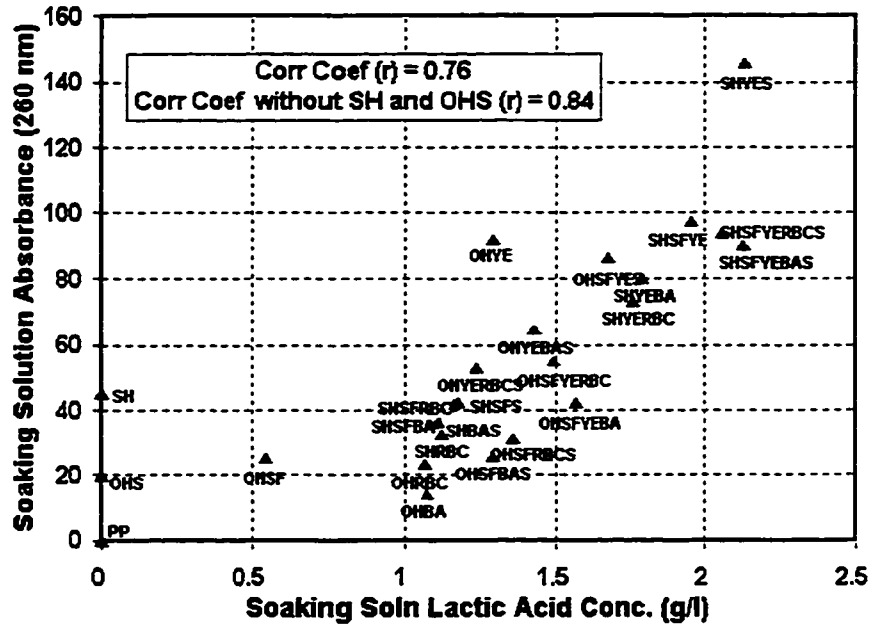Fig. 4. Relationship of interstitial volume (ml/g support) with plastic composite-support absorbed lactic acid and residual lactic acid in 20 mL initial soaking-solution.

Fig. 5. Relatioship between estimated and Micro-kjeldahl total-nitrogen value (mg/g support) of plastic composite-supports.

Fig. 6. Relationship between initial leached-nitrogen percentage and total nitrogen of plastic composite-supports as determined by Micro-kjeldahl.

Fig. 7. Relationship between initial leached-nitrogen percentage and *L. casei* 20th simulated RBF lactic-acid-concentration of plastic composite-supports.

Fig. 8. Relationship between 100-mL soaking-solution absorbance at 260 nm and its bioassay cell-density (620 nm) of *L. casei* and lactic acid concentration.

Fig. 9. Relationship between the amount of leached nitrogen in 20-mL initial-soaking solution and its bioassay cell density (620 nm) of *L. casei*.

Fig. 10.   Relationship between the leached-nitrogen (mg) present in 20-mL soaking solution and its absorbance at 260 nm.

OPTIMIZATION OF L(+)-LACTIC ACID PRODUCTION BY RING/DISC PLASTIC COMPOSITE-SUPPORTS THROUGH REPEATED-BATCH BIOFILM FERMENTATION

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Abstract

Plastic composite-supports (PCS) (35% soybean hulls, 5% yeast extract (YE), 5% soybean flour, 5% dried bovine albumen, and 50% polypropylene) rings and discs, produced by twin-screw high-temperature extrusion. They were used to stimulate biofilm formation, to supply nutrients for *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443), and to reduce medium channeling. Four customized reactors, three with 72.7 g rings and 72.3 g discs and one with suspended cells (control), were operated at 600 mL, 37°C and controlled pH 5 for 66 days. Effects of yeast extract concentration (0.2, 0.4, and 0.8%), starting glucose concentration (SG) (4, 6, 8, 10, 12, and 16%), and medium recycling rate (0.75, 1.5, 3, 6 cycles/h) were studied. L(+)-Lactic acid production and glucose consumption were determined by high-performance liquid chromatography (HPLC). Biofilm formation was evaluated by the stripping-sand method, scanning electron microscopy, and measurement of freeze-dried medium exopolysaccharides. The viable cells count on the PCS surface in 0.2, 0.4, and 0.8% YE Lactic-Acid-Fermentation medium (LAF) (8% glucose) was 7.1×10^9, 8.5

x 10^9, and 2.4 x 10^{10} cfu/g PCS, respectively. PCS-bioreactors in 0.4 and 0.8% YE LAF

medium shortened the lag time by 3-fold (control:11 h, PCS:3.5 h) and 6-fold (control:9 h,

PCS:1.5 h), respectively. PCS-bioreactors, at all YE concentration, increased LA

productivity by 40-70 %. PCS-bioreactors' total fermentation time with 0.2, 0.4, and 0.8%

YE LAF medium were 1.4, 2.1, and 2.6 times faster than that of the control, respectively.

PCS-bioreactors had its fastest productivity (4.26 g/L/h) at 10% SG, whereas the control

(2.78 g/L/h) was at 8%. PCS biofilm lactic-acid fermentation can drastically improve

fermentation rate under reduced complex nutrient addition.

Introduction

A lactic acid molecule has two optical active isomers, D(-) and L(+) forms [12].

Optically-pure lactic acid solution is important for the production of polylactide because the

physical properties of the polylactide are dependent on the stereochemistry of the individual

lactic acid molecule [10]. Microbial fermentation is the only source for producing optically-

pure lactic acid isomers. *Lactobacillus casei* subsp. *rhamnosus* is a homofermenter that

produces solely L(+)-lactic acid [7]. Cell immobilization is a common way to increase cell

density in fermentation. However, immobilizations of cells through calcium alginate beads

and polyacrylamide gels are not widely employed in industry because of the high cost of

immobilization, mass-transfer limitations, lack of stability of the biocatalysts, and changes in

product pattern of reactions catalyzed by certain immobilized cells [3].

Biofilms are a natural form of cell immobilization [1]. Demirci and Pometto [4]

demonstrated that lactic acid fermentation was enhanced by biofilm fermentation with plastic

composite-supports (PCS) chips containing 75% polypropylene (PP) and 25% agricultural

material (w/w). Ho et al. [8, 9] evaluated 24 PCS disc blends containing 50% PP and 50%

agricultural materials for L(+)-lactic acid biofilm fermentation in minimal medium with no

pH control. Each PCS blend was evaluated for biofilm development, slow release of

nutrients, surface contact angle, and hydrophobic compatibility with *L. casei*, porosity, and

lactic acid absorption. The PCS disc that consistently demonstrated the highest performance

contained 50% PP, 35% soybean hulls, 5% soybean flour, 5% yeast extract, 5% dried bovine

albumen, and salts (SHSFYEBAS-PCS). Hence, the goal of this study was to compare the

performance of the bioreactors with and without SHSFYEBAS-PCS in long-term biofilm

repeated-batch fermentation at controlled pH (5) and temperature (37°C).

Factors optimized were medium yeast extract concentraton, starting glucose

concentration, and medium flow rate in the bioreactor recycling loop. In this study, we

demonstrated that, under optimized conditions, PCS-bioreactors significantly shortened the

lag phase and total fermentation time. *L. casei* maximum productivity was also improved

through biofilm formation and complex-nutrients leaching of PCS.

Materials and methods

Plastic composite-supports

The selected plastic composite-supports (PCS) discs and rings, which contained 50%

polypropylene (PP) (Quantum USI Division, Cincinnati, OH), 35% ground (20 mesh)

vacuum dried (48 h at 110°C and 30 inch Hg) soybean hulls (SH) (Cargill Soy Processing

Plant, Iowa Falls, IA), 5% defatted soybean flour (SF) (Archer Daniels Midland, Decature,

IL), 5% yeast extract (YE) (Ardamine Z, Champlain Industries Inc., Clifton, NJ), 5% dried

bovine albumin (BA) (American Protein Corp., Ames, IA) and mineral salts (S) (0.2%

sodium acetate, 0.12% $MgSO_4 \cdot 7H_2O$, and 0.006% $MnSO_4 \cdot 7H_2O$) (w/w), were produced by

high-temperature extrusion in a Brabender with PL2000 twin-screw co-rotating extruder

(Model CTSE-V, C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by

Ho et al. [8, 9]. The PCS rings (1 cm ID, 1.5 cm OD) and discs (0.3 cm ID, 1.1 cm OD) were

extruded through a 12.7-mm OD large pipe die with 9.5-mm and 3.2-mm ID, respectively.

Bacterial culture preparation

Lactobacillus casei subsp. rhamnosus (ATCC 11443), a homofermenter of L(+)-lactic

acid, was obtained from the American Type Culture Collection (Rockville, MD). Stock

cultures were maintained in Lactobacillus MRS broth (Difco Laboratories, Detroit, MI) at

4°C with monthly transfers to fresh medium. All fermentation inocula were prepared by

transferring 10 mL of an active L. casei culture (18 h in MRS broth at 37°C) into 100 ml of

lactic acid fermentation (LAF) medium (2% glucose [Glu], 0.4% yeast extract [YE]

[Ardamine Z, Champlain Industries Inc., Clifton, NJ] and mineral salt solution [0.05%

KH_2PO_4, 0.05% K_2HPO_4, 0.1% sodium acetate, 0.06% $MgSO_4 \cdot 7H_2O$, and 0.003%

$MnSO_4 \cdot 7H_2O$]) [5] followed by an 18-h incubation at 37°C.

Media Preparation

All the dry ingredients of mineral-salt stock solution (0.05% KH_2PO_4, 0.05%

K_2HPO_4, 0.1% sodium acetate, 0.06% $MgSO_4 \cdot 7H_2O$, and 0.003% $MnSO_4 \cdot 7H_2O$) were mixed

with 80 L of deionized water in a B-Braun 100-D fermentor (New Brunswick Scientific,

Allentown, PA) and sterilized with continuous agitation for 20 min at 121°C and 15 psi. The

pH of the mineral-salts stock solution was adjusted to pH 5 by 4 N NaOH. The 80 L of

sterilized mineral-salt stock solution was then aseptically distributed into four separate

sterilized (2 h at 121°C) 20-L carboys. The 70% glucose (Cerelose dextrose 2001 CPC

Brand, International Ingredient Corporation, St.Louis, MO), and the 10% yeast extract

(Ardamine Z, Champlain Industries Inc., Clifton, NJ) stock solutions for each bioreactor were

autoclaved (30 min at 121°C) separately in a 3-L carboy. Thiry-two liters of 5 N NH_4OH was

filtered sterilized with a 142 mm cellulosic triton free 0.45-µm filter (MSI, Westboro, MA)

and aseptically distributed into four sterilized (1 h at 121°C) 10-L carboys. All stock solution

delivery carboys contained an air vent capped with a 0.45-µm air filter and a medium

delivery line with a liquid break to prevent reservoir contamination.

Repeated-batch fermentation system

Four sets of customized bioreactors (Fig. 1) were built for the long-termed repeated-

batch lactic-acid biofilm fermentation. As illustrated in Figure 1, each set of customized

bioreactor was composed of a modified Nalgene magnetic culture vessel (MCV) (part no. 71-

2605-0001) (Nalge Nunc International, Milwaukee, WI), an alkaline reservoir, and a media

formulation-mixing reservoir. The right arm of the MCV was equipped with a medium feed

line, a filter-sterilized (0.45 µm) carbon dioxide feed line, and a medium draining and

sampling port. The left arm of the MCV was covered by a screw cap, which acted as the

SHSFYEBAS-PCS sampling port. The central opening of the MCV contained an

innoculation port, a medium recycling line, an alkaline feed line, a condenser, and a pH

probe. The alkaline feed line, the medium recycling line and the pH probe were further

connected to a PCS-free pH-controlled basket inside the MVC. The pH-controlled

customized basket was made from a 60-mL syringe with 100 bored holes (5-mm ID). A magnetic stirrer was placed at the bottom of the pH-controlled basket to ensure that the recycling medium was well mixed with the added alkaline for pH control. This allowed the immediate detection of alkaline addition by the pH probe and prevented the overshooting of the alkaline into the bioreactor. The system pH was constantly controlled at pH 5 ± 0.05. A hole was drilled on each side of the MVC (2 mm above the base) for connecting a 1/4-inch barbed bulkhead fitting (Nalge Nunc International, Milwaukee, WI). The side of the 1/4-inch barbed bulkhead fitting facing the interior of the MVC was fitted with a section of the silicone tubing that stretched across the bottom of the MVC. The other side of the 1/4-inch barbed bulkhead fitting was linked to a pump, which was connected to the medium recycling line.

The design of the medium recycling loop was to ensure the homogeneous mixing of the medium throughout the fermentation process. The refillable alkaline reservoir (a 50-mL burette) was connected to the 5 N NH_4OH stock solution and to a pump for controlling the alkali addition. The alkaline reservoir showed the quantity and rate of alkaline consumption of each bioreactor and thus served as a quick indicator for fermentation performance. The aseptic media-mixing reservoir (a 500-mL burette) was connected to various stock solutions (the mineral salts, the 10% yeast extract, and the 70% glucose). The LAF medium formulation flexibility was achieved by the media- mixing reservoir, which permitted aseptic blending of different amount of glucose, yeast extact and mineral-salt stock solution. The space between the pH-controlled basket and the MCV was filled with 145.0 g of SHSFYEBAS-PCS (406 discs, 72.7 g and 377 rings, 72.3 g). The SHSFYEBAS-PCS were

sterilized dry (45 min at 121°C) in a 2-L fleaker beaker before transfer into the MVC. After

the addition of the SHSFYEBAS-PCS into the MVC, 300 mL of deionized water was added,

and the customized bioreactor was autoclaved (20 min at 121°C). The sterilized customized

bioreactor was then aseptically connected to the 5 N NH_4OH, glucose, yeast extract, and

mineral-salts stock solution carboys. The soaking water in the bioreactor was subsequently

drained through the medium draining port, and the fermentation system was ready to be filled

with a specific medium blend for the repeated-batch fermentation.

Repeated-batch fermentation

The ratio of the culture-medium working volume (600 mL) to the SHSFYEBAS-PCS

bulk volume (560 mL) was kept at 1.07 throughout the study. The bioreactors were

maintained at 37°C and controlled at pH 5. The entire 66 days' repeated-batch fermentation

was divided into three phases. The first phase was to evaluate the effect of LAF-medium YE

concentration (2, 4, and 8%) on the performance of the L. casei. At this phase, the recyling

flow rate and the starting glucose concentration of each bioreactor was 60 mL/min (6

working volumes/h) and 8%, respectively. The LAF-YE concentrations of the three PCS-

bioreactors were kept constant at 2, 4, and 8%, respectively, whereas the YE concentration of

the control bioreactor (bioreactor with no PCS) varied randomly so that a total of two batch

fermentations of 2, 4, and 8% YE LAF-medium were run during this phase.

The second phase was to determine the effect of medium recycling flow rate on the

maximum productivity of each bioreactor under different LAF medium conditions. In this

phase, an 8% starting glucose concentration was also used. The maximum productivity for

each repeated-batch fermentation was determined by measuring the slope of at least three

points on the steepest region of the lactic acid production curve. The YE concentration of the three PCS bioreactors and the control bioreactor was kept at 2, 4, 8, and 8 %, respectively. The medium-recyling flow rates evaluated were 60, 30, 15, and 7.5 mL/min, which was equivalent to 6, 3, 1.5, and 0.75 working volumes/h, respectively.

The third phase was to analyze the effect of the starting glucose concentration on the bacterial performance. The YE concentration of each bioreactor was the same as that of the second phase. The medium recyling flow rate was kept at 60 mL/min. The six starting glucose-concentrations evaluated were 4, 6, 8, 10, 12, and 16%. In addition, the control customized bioreactor performance (60 mL/min flow rate, 8% starting glucose and 0.8% YE concentration) was compared with a standard bench-top continuous-stir tank fermentor (2-L B-Braun Biostat M B-Braun, [New Brunswick Scientific, Allentown, PA]) (8% starting glucose and 1% YE concentration) at 37°C and controlled pH 5.

Biofilms formation analysis

Five PCS discs and rings (approximately 1 g) were aseptically retrieved from each bioreactor, and their biofilm population was determined by the stripping-sand method [8]. The sample from each bioreactor was serially diluted, and colony-forming units (cfu) of the 10^7 to 10^9 dilutions were determined by using Lactobacillus MRS agar spread plates in duplicate. Finally, the five sand-stripped discs and rings from each RBF reactor were rinsed with water, convection-oven-dried (70°C, 24 h), then reweighed.

In addition, the PCS biofilm population from each bioreactor was also evaluated by scanning electron microscopy (SEM). Broken and whole PCS discs and rings were prepared following the procedures described by Ho et al. [8]. SEM micrographs of gold-coated

critical-point-dried supports were taken with a JEOL JSM-35 scanning electron microscope

(JEOL, Japan) at 25 kV. Biofilm formation was further analyzed indirectly by measuring the

amount of exopolysaccharides (total complex carbohydrates) present in the fermentation

medium of the four bioreactors by comparing the reducing sugar concentration as determined

by Somogyi Nelson and Phenol Sulfuric assays [13]. If a polysaccharide was present, then

the Phenol Sulfuric assay would hydrolyze the polymer to monosaccharide as indicated by a

high concentration of reducing sugars, whereas Somogyi Nelson assay measured only the

free reducing sugars in freeze-dried medium. The PCS total weight loss in each bioreactor

was determined by measuring the difference between the PCS initial weight and its

convection-dried (60°C overnight) weight after the 66 days' repeated-batch fermention.

Fermented media analysis

L(+)-Lactic acid and D-glucose concentrations were analyzed by a Waters high-

performance liquid chromatograph (HPLC) (Milford, MA) equipped with Waters model 401

refractive index detector and a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad

Chemical Division, Richmond, CA) using 0.012 N H_2SO_4 as the mobile phase. Suspended

cells growth in the LAF medium was followed by measuring the absorbance at 620 nm with

a Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, NY).

Results and discussion

Effects of PCS on repeated-batch fermentation

As shown in Fig.2, the fermentation in the 0.8%-YE PCS bioreactor and the 0.8%-YE

control bioreactor both followed the in-between Type I (growth associated) and Type II

fermentation (nongrowth associated) pattern [2]. The production of lactic acid was at first directly proportional to the medium cell density (i.e., growth associated). However, as the medium cell density curve plateaued, the lactic acid concentration still continued to increase (nongrowth associated). The nongrowth- associated fermentation probably was a result of the *L. casei* need to produced energy (ATP) for maintaining its internal pH by pumping out protons as the lactic acid concentration inside and outside the cell increased [7, 11].

The final suspended cell absorbance at 620 nm of *L. casei* in the 0.2, 0.4, and 0.8%-YE PCS bioreactor was 7.2, 10, and 13.5 absorbance units, respectively (Fig.3). Similarly, the suspended cell absorbance at 620 nm of the control bioreactor was 5, 12.5, and 16 absorbance units when the medium YE concentration was 0.2, 0.4, and 0.8%, respectively (Fig. 3). This indicated that the medium YE concentration had a great impact on *L. casei* growth. Although the glucose consumption and L(+)-lactic acid production rate of the 0.8%-YE PCS bioreactor (maximum productivity: 3.6 g/L/h) were much faster than the 0.8%-YE control (maximum productivity: 2.5 g/L/h), the medium cell density of the 0.8%-YE PCS bioreactor (final cell absorbance at 620 nm: 13.5) was significantly lower than that of the 0.8%-YE control (final cell absorbance at 620 nm: 16) (Fig. 2). This observation was also obtained for both the 0.4%-YE PCS and the control bioreactors (Fig. 3). This was an expected result, because the medium cell density does not represent the total cell mass of the PCS bioreactors, which consisted of suspended cells in the LAF medium and immobilized cells in the biofilms. However, in the 0.2%-YE LAF medium environment, the suspended cell density in the PCS bioreactor was higher than that of the control. This confirmed the slow release of the complex nutrient from the PCS to the suspended cell population. As the

YE concentration in the control bioreactor medium was reduced from 0.4 to 0.2%, the amount of complex nutrients was not enough for the normal performance of suspended *L. casei*, causing its cell density to drop abruptly by 2.5-fold (12.5 to 5, respectively). In contrast, the suspended *L. casei* of the PCS bioreactor received complex nutrients from the leachate of the PCS discs and rings and exhibited only a 1.4-fold decrease (10 to 7.2) in its final cell density for the 0.4 to 0.2% YE medium.

Figure 4 illustrated that the PCS bioreactors outperformed the control bioreactor under all YE medium concentrations. PCS bioreactors in 0.4 and 0.8%-YE LAF medium shortened the lag time by 3-fold (control:11 h, PCS:3.5 h) and 6-fold (control:9 h, PCS:1.5 h), respectively. The PCS bioreactors, at all YE concentrations, increased lactic acid maximum productivity by 40-70%. Moreover, the total fermentation time of the 0.2, 0.4, and 0.8%-YE PCS bioreactors were 1.4, 2.1, and 2.6 times faster than that of the control, respectively. Furthermore, the yield of the 0.2 and 0.8%-YE PCS bioreactors were 24 and 6% higher than the yield of the control, respectively. Indeed, as indicated in Fig.5, the performance of the 0.8%-YE control bioreactor was only equivalent to the performance of the 0.4%-YE PCS bioreactor. This demonstrated the high potential of using a reduced complex nutrient medium in commercial batch fermentation with SHSFYEBAS-PCS discs and rings.

Besides the complex nutrients-leaching benefit, the biofilm population on the surface of the PCS discs and rings also benefitted overall bioreactor performance. The biofilm population on the outer surface of the PCS in the 0.2, 0.4, and 0.8%-YE PCS bioreactors was 7.1×10^9, 8.5×10^9, and 2.4×10^{10} cfu/g of supports, respectively. This sequential pattern of

the cfu/g of support with a corresponding increase in YE concentration parallels the previous

cell density discussion. This was further demonstrated by the SEM micrographs (Fig. 6),

where the biofilm on the interior surface of the SHSFYEBAS-PCS in the 0.8%-YE PCS

bioreactor (Fig. 6C and D) appeared significantly denser than the PCS in the 0.2%-YE PCS

bioreactors (Fig. 6E and F). The biofilm cells of *L. casei* on the outer surface of the PCS

were mostly present in streptobacillus form rather than its usual nonfilamentous bacillus form

(Fig. 6A and B). This might be a result of the hydraulic stress experienced by the biofilm

cells [1]. Typical fibrillar extracellular materials (f) derived from exopolysaccharides (EPS)

of biofilms, as observed by Leppard and Bakke (1986) [1] and Ho et al. (1996) [8], were

observed with SHSFYEBAS-PCS in the 0.2%-YE PCS bioreactor (Fig. 6F). The relative

EPS present in the culture medium of the 0.2, 0.4, and 0.8%-YE PCS bioreactor, were 11, 12

and 13 mg/g of freeze-dried medium, respectively. The high correlation between the EPS

value and the biofilm population of each bioreactor (r = 0.97) (Fig. 7) demonstrated that the

EPS in the medium was also a good indicator of the biofilm population on the supports.

The weight loss of the PCS after 66 days of lactic-acid repeated-batch biofilm

fermentation in the 0.2, 0.4, and 0.8%-YE PCS bioreactors was 16.0, 15.5, and 13.8

g/bioreactor, respectively. The weight losses due to the agricultural product leaching (3.2

g/bioreactor) were similar in the three PCS bioreactors. Hence, the factor contributing to the

smaller weight loss by the PCS bioreactors at different YE medium probably was due to the

biofilm population present in the PCS discs and rings. This was supported by the high

correlation between weight loss and biofilm population of the PCS bioreactors (r = -0.99)

(Fig. 7).

The lactic-acid production curve of the 0.8%-YE control bioreactor and the 1%-YE

B-Braun Biostat-M fermentor were similar (Fig. 5). This demonstrated that our customized

bioreactor had equivalent pH control and medium mixing ability of a standard benchtop

continuous-stir tank fermentor. In addition, it also indicated that $L.$ $casei$ lactic acid

fermentation could be operated at a lower medium YE concentration (0.8% instead of 1%).

Effects of medium recycling rate and starting glucose concentration

The recycling rate of the customized bioreactors affected the pH-controlling ability of

the bioreactors. A high positive correlation (r = 0.95 to 0.99) between the maximum

productivity of the bioreactors and the medium recycling rate was observed (Fig. 8). The

diffusion of substrates, products, and complex nutrients in and out of the PCS was highly

depended on the movement, the mixing, and the recycling rate of the medium. Therefore, the

slopes of the correlation curve for the PCS bioreactors were steeper than that of the control.

The effect of starting glucose concentration (SGC) on the maximum lactic acid

productivity of $L.casei$ was shown in Fig. 9. The 0.8%-YE PCS bioreactor, the 0.4%-YE

PCS bioreactor, and the 0.8%-YE control bioreactor all demonstrated an optimum starting

glucose concentration peak in their curve patterns of 100, 60, and 80 g/L glucose,

respectively. The 0.8%-YE PCS bioreactor had its highest maximum productivity (4.3 g/L/h)

at 100 g/L SGC, whereas the 0.8%-YE control (2.8 g/L/h) Was At 80 g/L SGC. This again

illustrated the high stress-tolerant property of the biofilms. The 0.4%-YE PCS bioreactor had

its fastest maximum productivity (3.0 g/L/h) at 60 g/L SGC. This demonstrated that the

reduction in medium complex nutrients also lowered the ability of $L.$ $casei$ in withstanding

the inhibitory effect resulted from high SGC. This was supported by the decreasing trend

demonstrated by the maximum productivity of the 0.2%-YE PCS bioreactors (2, 4, 6, and 8% SGC corresponded to 1.8, 1.7, 1.5, and 1.1 g/L/h, respectively). Substrate inhibition by high starting glucose concentration was also reported by Goncalves et al. (1991) [6].

From the results presented, it was clear that, with the complex-nutrients-leaching and biofilms-formation properties, the SHSFYEBAS-PCS discs and rings shortened the lactic acid fermentation lag phase time and total fermentation time, increased suspended and immobilized cells population, enhanced maximum productivity, and improved *L. casei* stress-tolerance ability to high starting glucose concentration and low medium yeast-extract concentrations. The shorter batch-fermentation time resulting from PCS biofilm fermentation will lower the overall production cost of lactic acid. The reduced complex-nutrients concentration of the culture medium will enable the use of inexpensive complex nutrients (i.e. corn-steep liquor, soybean flour, etc.) in lactic acid batch fermentation. In addition, the lower complex-nutrients concentration of the medium will also enhance the downstream recovery of lactic acid. Hence, employing the SHSFYEBAS-PCS in commercial repeated-batch biofilm lactic acid fermentation deserves strong consideration.

Acknowledgements

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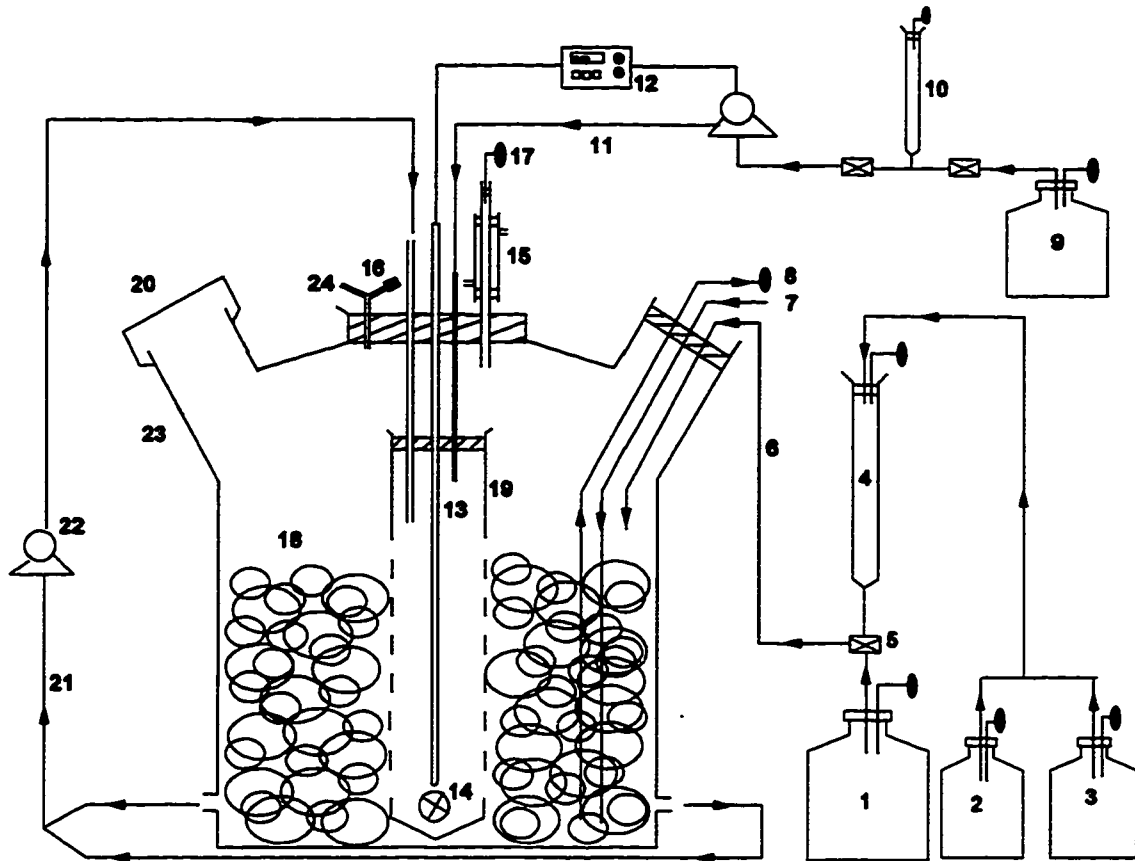
Figure legends list

Fig. 1. Schematic diagram of the repeated-batch fermentation system.

Fig. 2. Lactic acid production, glucose consumption, and *L. casei* growth curve of PCS and

suspended cells control bioreactor in 0.8% yeast-extract lactic acid fermentation

medium.

Fig. 3. Comparison between the *L. casei* cell density of the PCS bioreactors with the

suspended cells control bioreactor under different yeast extract concentrations.

Fig. 4. Comparison between the lactic acid production by *L. casei* of the PCS bioreactors

with the suspended cell control bioreactor under different yeast extract concentrations.

Fig. 5. Comparison between the lactic acid production of *L. casei* in PCS bioreactors,

suspended cells control bioreactor, and 1-L B-Braun stir-tank fermentor at different

yeast-extract concentrations.

Fig. 6. Scanning electron micrographs of *L. casei* on the exterior or interior surfaces of

SHSFYEBAS-PCS in different yeast-extract concentration media. (A) *L. casei*

biofilms formed on the exterior surface of SHSFYEBAS-PCS in 0.8%-YE medium.

Bar, 20 μm. Box in (A) was enlarged in (B) to show the long-rod filamentous

morphology of *L. casei*. Bar, 3 μm. (C) *L. casei* biofilms formed on the interior

surface of SHSFYEBAS-PCS in 0.8%-YE medium. Bar, 20 μm. Box in (C) was

enlarged in (D) to show the short-rod filamentous morphology of *L. casei*. Bar, 3 μm.

(E) *L. casei* biofilms formed on the interior surface of SHSFYEBAS-PCS in 0.2%-

YE medium. Bar, 20 μm. Box in (E) was enlarged in (F) to show the fibrillar

network (f) formed from the exopolysaccharide of *L. casei* biofilms. Bar, 3 μm.

Fig. 7. Relationship of the relative *L. casei* biofilm population with the PCS weight loss after

fermentation and the amount of exopolysaccharides present in the freeze-dried culture

medium of 0.2, 0.4, and 0.8%-YE PCS bioreactor.

Fig. 8. Relationship between the maximum lactic acid productivity by *L. casei* and working-

volume (600 mL) recycling rate of the PCS bioreactors and the suspended cells

control bioreactor.

Fig. 9. Effect of starting glucose concentration on the lactic acid maximum productivity by

L. casei of the PCS bioreactors and the suspended cell control bioreactor.

Keys:

1.	Mineral salt solution	13.	pH probe
2.	70% glucose solution	14.	Magnetic stirrer
3.	10% yeast extract solution	15.	Condenser
4.	Media mixing reservoir	16.	Innoculation port
5.	Stopcock	17.	0.45-um sterilized filter
6.	Medium feed line	18.	Plastic composite-supports
7.	CO_2 feed line	19.	pH-controlled basket
8.	Sampling and medium draining port	20.	PCS sampling port
9.	5 N NH_4OH stock solution	21.	Medium recycling line
10.	Alkaline reservoir	22.	Peristaltic pump
11.	Alkaline feed line	23.	Nalgene magnetic culture vessel
12.	pH meter	24.	Extra port

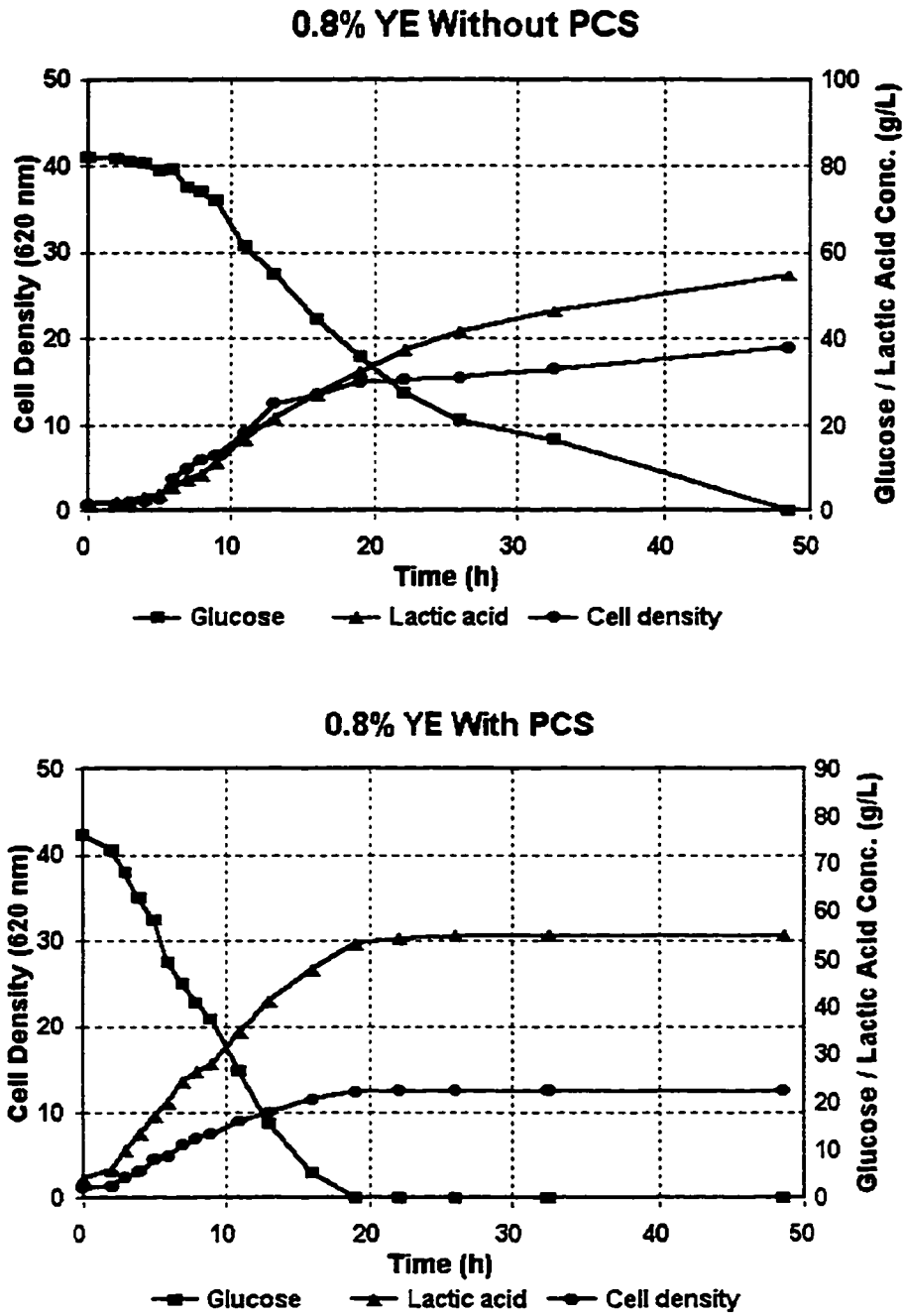Fig. 1. Schematic diagram of the repeated-batch fermentation system.

Fig. 2. Lactic acid production, glucose consumption, and *L. casei* growth curve of PCS- and suspended cells control bioreactor in 0.8% yeast-extract lactic-acid fermentation medium.
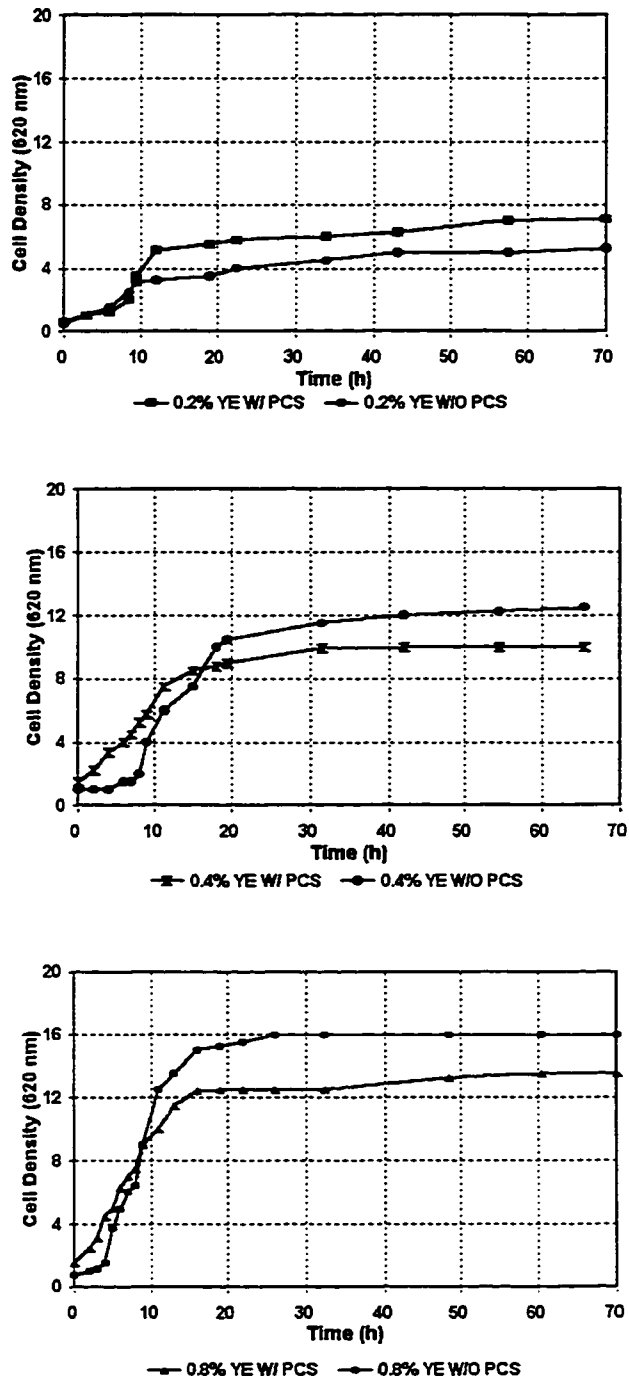
Fig. 3. Comparison between the *L. casei* cell density of the PCS bioreactors with the suspended cells control bioreactor under different yeast extract concentrations.
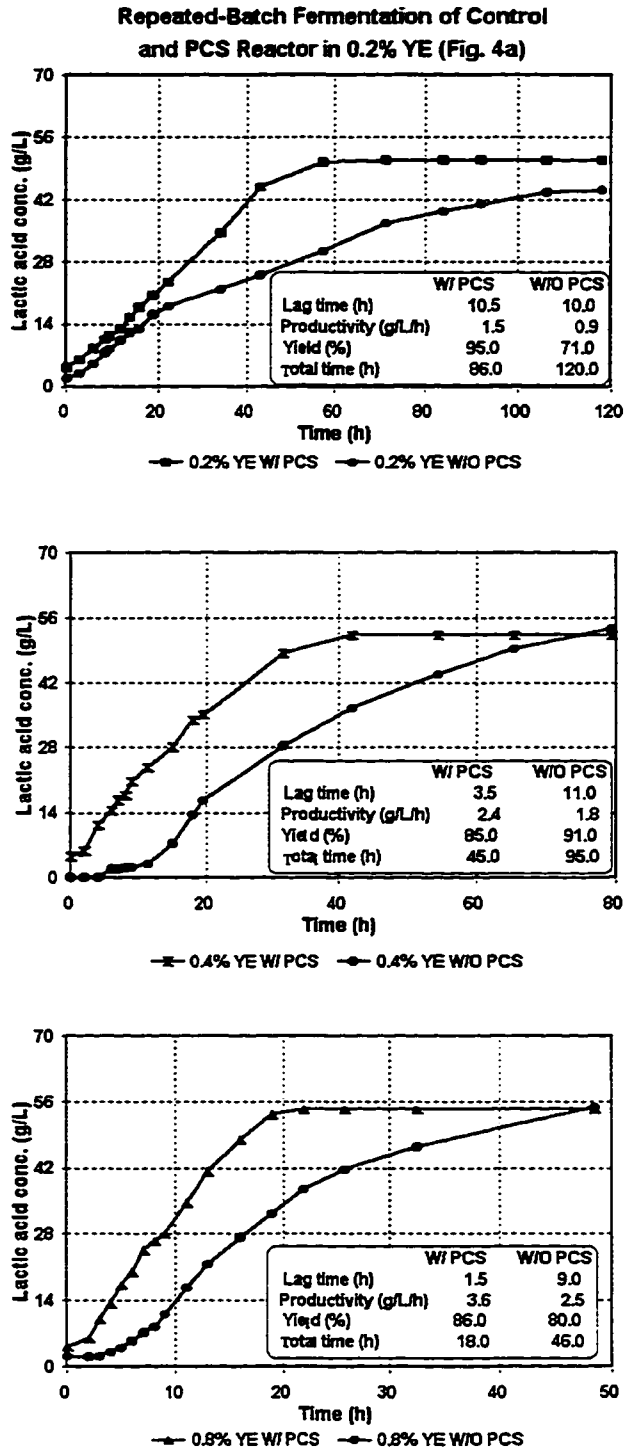
111

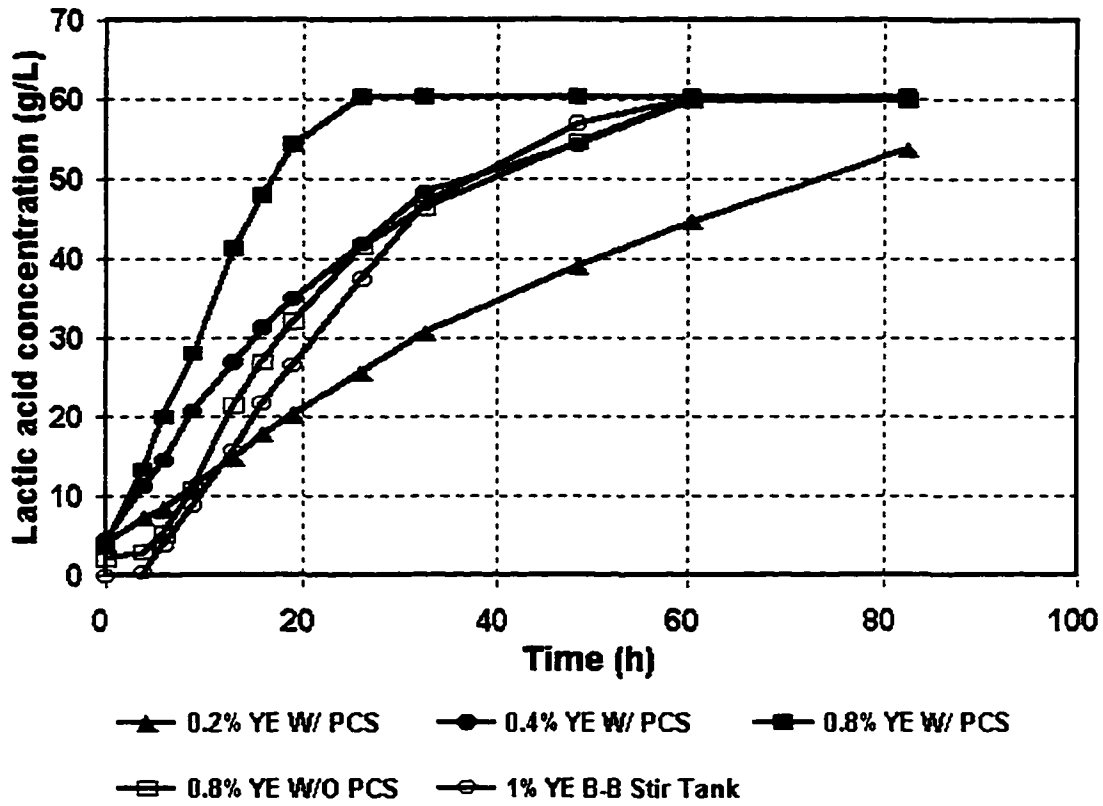Repeated-Batch Fermentation of Control and PCS Reactor in 0.2% YE (Fig. 4a)

	W/ PCS	W/O PCS
Lag time (h)	10.5	10.0
Productivity (g/L/h)	1.5	0.9
Yield (%)	95.0	71.0
Total time (h)	86.0	120.0

—■— 0.2% YE W/ PCS   —●— 0.2% YE W/O PCS

	W/ PCS	W/O PCS
Lag time (h)	3.5	11.0
Productivity (g/L/h)	2.4	1.8
Yield (%)	85.0	91.0
Total time (h)	45.0	95.0

—✖— 0.4% YE W/ PCS   —●— 0.4% YE W/O PCS

	W/ PCS	W/O PCS
Lag time (h)	1.5	9.0
Productivity (g/L/h)	3.6	2.5
Yield (%)	86.0	80.0
Total time (h)	18.0	46.0

—▲— 0.8% YE W/ PCS   —●— 0.8% YE W/O PCS

Fig. 4. Comparison between the lactic acid production by *L. casei* of the PCS- bioreactors with the suspended cell control bioreactor under different yeast extract concentrations.
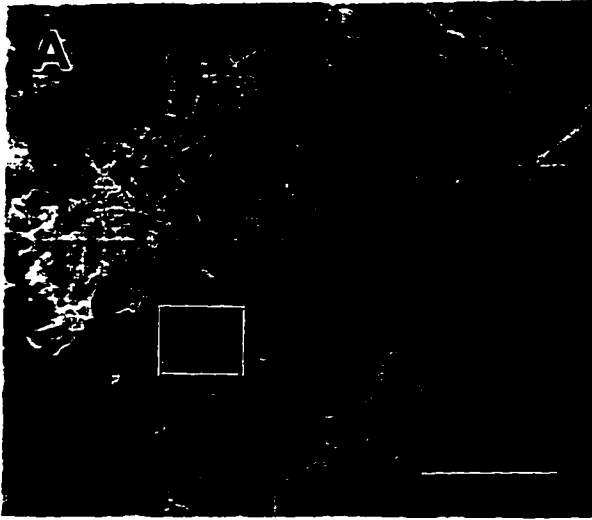
Fig. 5. Comparison between the lactic acid production of *L. casei* in PCS bioreactors, suspended cells control bioreactor, and 1-Liter B-Braun stir- tank-fermentor at different yeast-extract concentration.

Fig. 6. Scanning electron micrographs of *L. casei* on the exterior or interior surfaces of
SHSFYEBAS-PCS in different yeast-extract concentration media. (A) *L. casei*
biofilms formed on the exterior surface of SHSFYEBAS-PCS in 0.8%-YE medium.
Bar, 20 μm. Box in (A) was enlarged in (B) to show the long-rod filamentous
morphology of *L. casei*. Bar, 3 μm. (C) *L. casei* biofilms formed on the interior
surface of SHSFYEBAS-PCS in 0.8%-YE medium. Bar, 20 μm. Box in (C) was
enlarged in (D) to show the short-rod filamentous morphology of *L. casei*. Bar, 3 μm.
(E) *L. casei* biofilms formed on the interior surface of SHSFYEBAS-PCS in 0.2%-
YE medium. Bar, 20 μm. Box in (E) was enlarged in (F) to show the fibrillar
network (f) formed from the exopolysaccharide of *L. casei* biofilms. Bar, 3 μm.
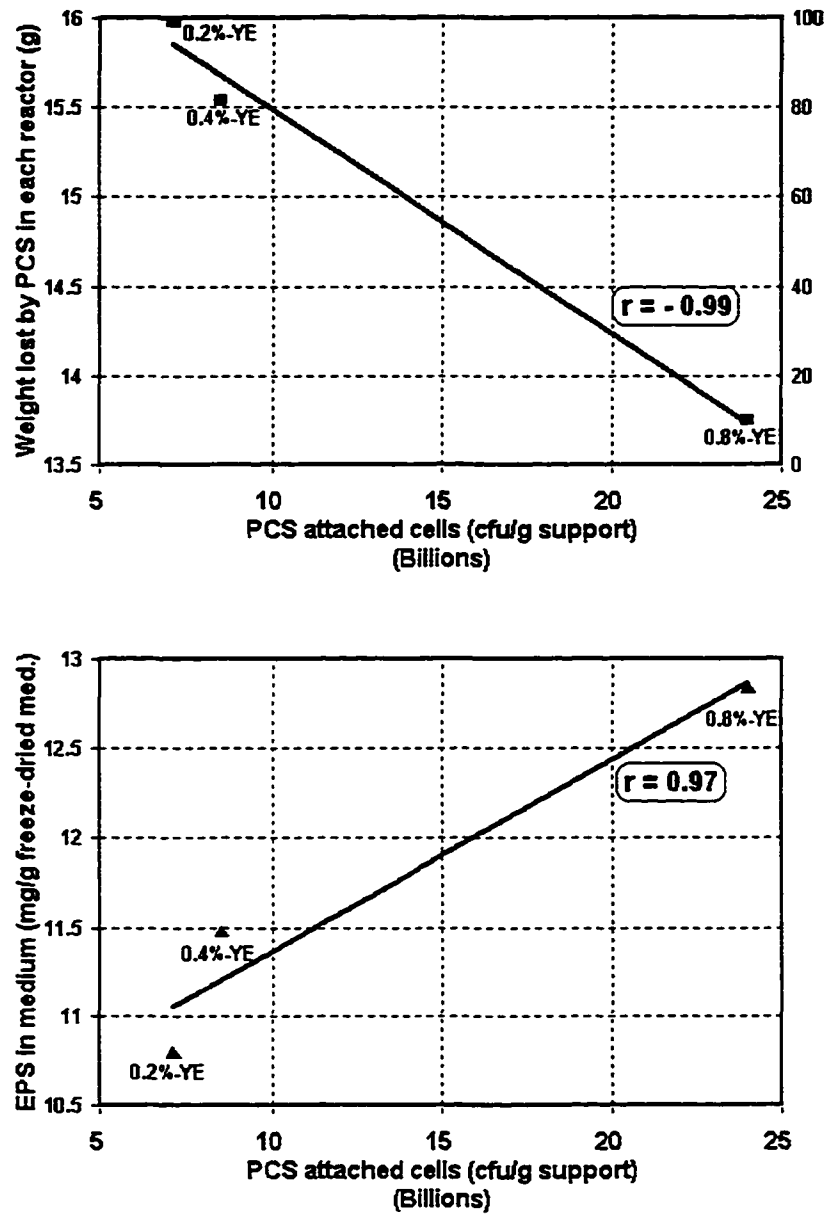
Fig.7. Relationship of the relative *L. casei* biofilm-population with the PCS weight loss after fermentation and the amount of exopolysaccharides present in the freeze-dried culture medium of 0.2, 0.4, and 0.8%-YE PCS bioreactor.
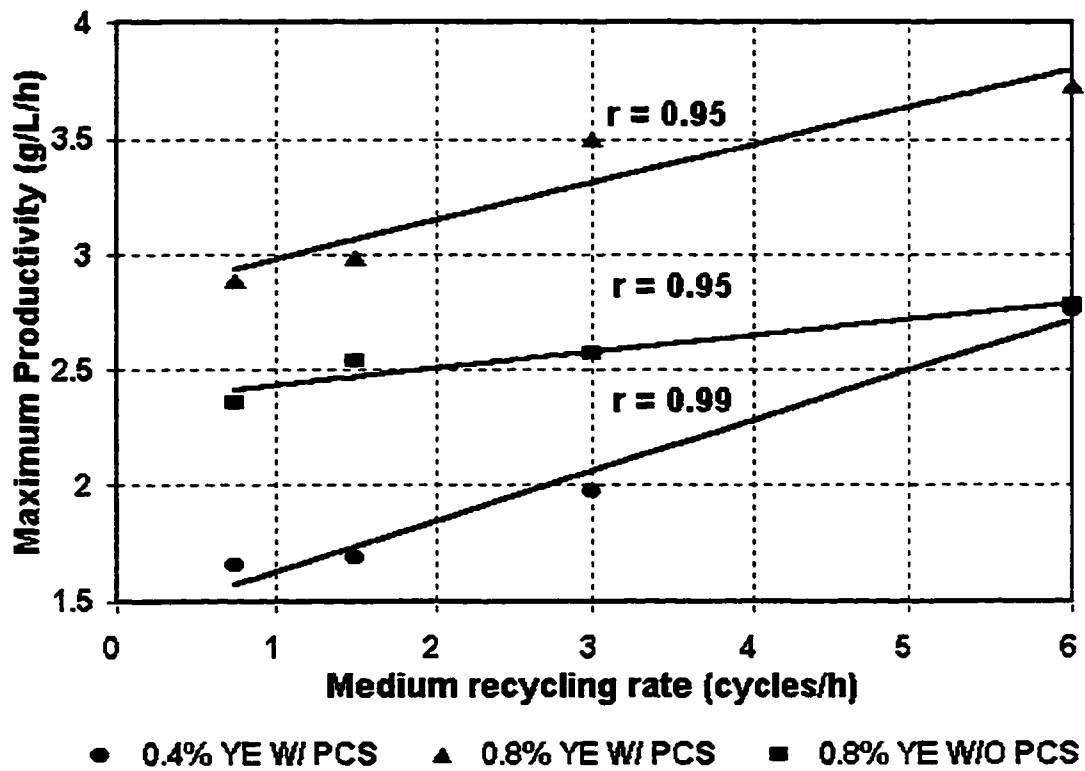
Fig.8. Relationship between the maximum lactic-acid productivity by *L. casei* and working volume (600mL) recycling rate of the PCS bioreactors and the suspended cells control bioreactor.
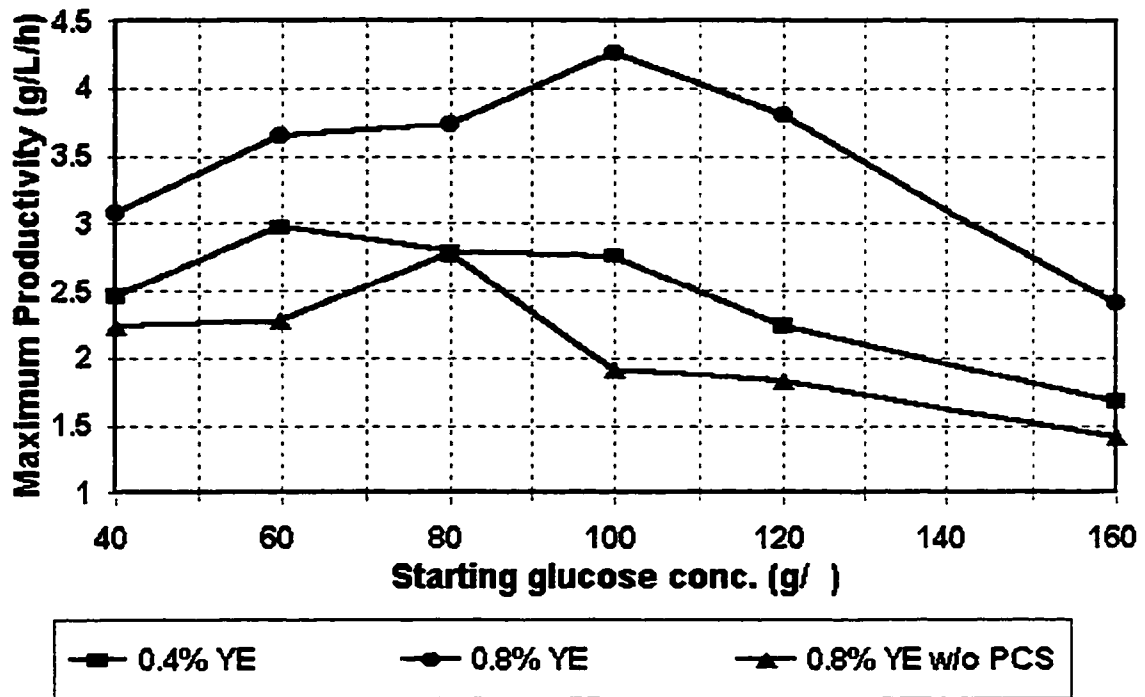
Fig.9. Effect of starting glucose concentration on the lactic-acid maximum productivity by
L. casei of the PCS bioreactors and the suspended cell control bioreactor.

GENERAL CONCLUSIONS

General discussion

High-temperature extrusion by the twin-screw co-rotating extruder (Model CTSE-V, C.W. Brabender Instruments, Inc., South Hackensack, NJ) was able to produce ring and disc plastic composite-supports (PCS) containing a maximum of 50 % agricultural materials. Two replications of a 2^{5-1} design had efficiently evaluated the effects of soybean hulls, oat hulls, soybean flour, yeast extract, dried red blood cells, dried bovine albumen, and salts on the properties of PCS.

Results showed that soybean hulls, yeast extract, and salts increased the biofilm population by reducing the hydrophobicity of the PCS. This was supported by the hydrophilic nature of the *L. casei* as its contact angle (27°) was determined to be less than 40°. As indicated by the leached nitrogen and 260 nm absorbance of soaking solution, yeast extract was the most outstanding minor complex ingredient for supplying nitrogenous compound in the PCS. However, because of the fast leaching rate of yeast extract from the PCS, other minor agricultural ingredients such as dried bovine albumen and soybean flour were essential to ensure a gradual release of nitrogenous compounds form the PCS. In addition, the verification of absorption being the main mechanism of lactic acid accmulation in the PCS had greatly removed the worry of end product accumulation in the supports. Among the twenty-four blends of PCS evaluated, SHSFYEBAS-PCS containing 50 %

polypropylene, 35 % soybean hulls, 5 % soybean flour, 5 % yeast extract, 5 % bovine albumen, and salts was demonstrated to have the highest lactic-acid concentration and cell density in the 1st and 20th simulated repeated-batch fermentation, the greatest viable attached cell counts, a relatively small contact angle, and a gradual complex nutrients releasing rate. Hence, SHSFYEBAS-PCS was selected for long-term repeated-batch fermenation study.

The results of polypropylene-alone supports being unable to support any bacterial growth and bifilm formation in all batch-fermentation circumstances demonstrated that the addition of agricultural products to the PCS was essential. In addition, the results of the batch fermentation of the 24 PCS-blends demonstrated that both the medium suspended cells and the PCS attached cells contributed to the production of lactic acid.

The long-term repeated-batch fermentation study showed that the operational life of the PCS was at least 66 days. The complex-nutrients-leaching and biofilms-formation properties of SHSFYEBAS-PCS discs and rings had shortened the lactic acid fermentation lag phase tiem and total fermentation time, increased suspended and immobilized cellls population, enhanced maximimum productivity, and improved *L. casei* stress-tolerance ability to high starting glucose concentration and low medium yeat-extract concentrations. These benefits greatly optimized the lactic acid fermenation process because the lower complex-nutrients concentration not only suggest the possibility of using inexpensive complex nutrients such as corn-steep liquor, but also implied a less complex medium for the downstream recovery process of lactic acid.

Recommendations for future research

The results obtained in this research demonstrated the optimization and improvement of lactic acid fermentation by the complex-nutrient release and biofilms formation properties of the plastic composite-supports (PCS). Kunduru and Pometto [48, 49] also showed the enhancement of ethanol production by the PCS. Hence, the study of using PCS in other industrial organic-compounds (acetic acid, butanol, polyols, and succinic acid) fermentation is essential for the confirmation of the PCS applications and benefits to a variety of potential commodity chemicals..

All current lactic acid facilities are batch fermentation and a swith to continuous fermentation could provide some economic savings. Potentially biofilm-lactic-acid continuous fermentation with PCS is possible. Thus, the optimized conditions and informations obtained from the repeated-batch fermentation are very useful for developing a continuous fermentation process involving the PCS. In addition, increasing the lactic-acid repeated-batch fermentation to pilot-plant scale is also essential for verifying the practicability of applying the PCS in industrial lactic-acid fermentation.

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