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Demirci, Ali, Ph.D.

Iowa State University, 1992

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Enhanced lactic acid production by strain development, and by a novel biofilm reactor

by

Ali Demirci

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Food Science and Human Nutrition
Major: Food Science and Technology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa

1992

To my parents; Latife & M. Ali
and
my wife and son; Ann & B. Kerem

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GENERAL INTRODUCTION

Lactic acid is a fascinating chemical. It has unwittingly been used for preserving food products since prehistoric times. Its chemical structure is simple (CH_3-CHOH-COOH), yet it is the smallest natural compound exhibiting two optically active forms.

Lactic acid can be produced from various agricultural products and from industrial waste streams by microbial fermentation. In homo-lactic acid fermentations two lactic acid molecules are produced per glucose molecule so acid production is very efficient.

In 1985, the production of lactic acid world-wide was 24 to 28 x 10^6 kg (60). Lactic acid production *via* fermentation presently provides only 50% of the world supply (46) with a current price of $1.03/pound. To qualify as an inexpensive chemical feedstock, lactic acid needs to sell at about $0.25/pound. Lactic acid is used to produce polylactic acid which is a plastic with good tensile strength, thermoplasticity, fabricability, and biodegrability 42). The current market price of polylactic acid is $500/pound. However, because of increased public demand for degradable polymers and because the current market for polyethylene is 9 billion pounds per year (17), Cargil Co., and Dupont Inc., have announced the future commercial production of polylactic acid by 1994 at a projected price of $1.00/pound (40). Polylactics are currently used for resorbable sutures, prosthetic devices, and slow-release carriers of herbicides and pesticides. Lactic acid can also

be used as a chemical feedstock for the chemical and biological production of other organic acids, such as acetic, propionic, acrylic acids, propylene glycol, ethanol, and acetaldehyde.

This dissertation addresses attempts to decrease the production costs of lactic acid by increasing lactic acid concentrations in the fermentation medium. To achieve this goal, chemical mutagenesis techniques and a novel biofilm reactor using a pure or mixed-culture were employed.

Explanation of Dissertation Format

This dissertation contains three manuscripts which will be submitted to scholarly journals. The American Society for Microbiology format was used throughout this dissertation. The first manuscript deals with enhancement of lactic acid production by chemical mutagenesis in *Lactobacillus delbrueckii*. The second manuscript deals with the development of a biofilm reactor for lactic acid production by selecting the best inert supports, biofilm formers and lactic acid bacteria. The third manuscript describes a comparison of pure- and mixed-culture biofilm reactors utilizing polypropylene composite supports for the continuous production of lactic acid at different flow rates. References cited in General Introduction, Literature Review, and Summary and Conclusion are listed following Summary and Conclusion section.

LITERATURE REVIEW

History of Lactic Acid

Lactic acid was discovered in 1780 by the Swedish chemist, Scheele (23). Other scientists, however, stated that Scheele's lactic acid was not a pure compound. Berzelius repeated Scheele's experiment in 1808 and confirmed that lactic acid was a pure acid. He also described for the first time some properties of lactic acid salts. A French chemist, Braconnot, rediscovered lactic acid in 1813; he believed that it was different from Scheels's acid. However, the German chemist, Vogel, proved the identity of Scheele's and Braconnot's acid. The final proof for the existence of lactic acid came from Gay-Lussac and others in 1833.

Chemistry

Lactic acid is 2-hydroxypropionic acid ($C_3H_6O_3$) (23). Initially, lactic acids produced by microbial fermentation and by animal muscle were considered identical. However, Liebig, in 1847, showed that there were differences in the crystals and solubilities of the salts of lactic acids produced by animals and bacteria. In 1869, Wislicenus discovered the D and L forms of lactic acid, making it the simplest hydroxy acid having an asymmetric carbon atom. It generally exists in a racemic mixture of the two optically active forms. In lactic acid there is one asymmetric carbon that carries four different groups: -COOH, -H, -OH, and -CH_3. These groups are ordered around this asymmetric carbon to give two

different arrangements that are mirror images of each other. Each arrangement is called an enantiomer of lactic acid. These two enantiomers of lactic acid can be distinguished by the letters L (levorotatory), and D (dextrorotatory). A racemic mixture consists of equal amount of these D and L forms. On the other hand, the optical activity (ability to rotate polarized light) of the two enantiomers is opposite of each other. This optical activity is defined as (-), and (+). Therefore, lactic acid isomers are designated as D(-)-lactic acid or L(+)-lactic acid.

Physical Properties

Most physical properties of the pure isomers lactic acid do not differ significantly from the properties of the racemic mixture (23). One important exception is the melting points, which are higher for pure isomers than the racemic mixture. The melting point of each isomer is 52.8-54°C, whereas for the racemic mixtures it is 16.8-33°C depending on the composition. Lactic acid is considered to be non-volatile (Table 1). Densities of aqueous solutions of lactic acid vary almost linearly with concentration and temperature (Table 2). The viscosity of lactic acid (85%) is 28.5 and 3.4 cp at 25 and 80°C, respectively. Lactic acid is soluble in water, but it has various solubilities in mixtures of different percentages of water and alcohols, phenols, ethers, aldehydes, ketones, and esters.

Table 1. Boiling points of anhydrous lactic acid at different pressures (23)

Pressure (mm Hg)	Boiling Point (°C)
0.1	78
0.3	80
0.5	82
1.0	98
12.0	119
15.0	122
20.0	140

Table 2. Lactic acid (LA) densities (g/ml) at different temperatures (23)

	Temperature (°C)						
% LA	20	30	40	50	60	70	80
25	1.058	1.053	1.047	1.052	1.035	1.03	1.023
50	1.120	1.113	1.106	1.099	1.090	1.084	1.075
75	1.178	1.169	1.160	1.152	1.143	1.136	1.125

Applications

Food applications

Lactic acid can be used in wide variety of foods (23). It is used in the food industry mainly as an acidulant and preservative. Lactic acid and its common salts are relatively nontoxic, and are deemed "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (F.D.A.). As an acidulant and preservative, lactic acid is used in combination with other acids to yield a mild and subtle acid taste in pickles, relishes, and salad dressings. It was demonstrated that 0.2% (w/v) lactic acid suppressed growth of the mesentericus group of bacteria, and 0.3% (w/v) completely suppressed growth of coliform bacteria. Calcium lactate is used in fruit preservation, to prevent the loss of apple slice firmness during processing. Calcium lactate, also, inhibits discoloration of fruit and some vegetables.

Lactic acid also is used in the food industry as a flavor enhancer and a buffer. It is added to beverages, including soft drinks, mineral waters and carbonated fruit juices, to improve flavor. It does not mask the flavor of natural fruits. Lactic acid is also used in beer, wine and other beverages to improve flavor and to adjust the pH of hard alkaline water.

By exogenous addition or by microbial fermentation, lactic acid is an essential part in the preparation of a great variety of dairy products. The production of processed cheese can be greatly simplified if a sufficient amount of lactic acid is

added to the freshly drained cheese curd to lower the pH to 4.8- 5.2 so that the curd can be processed without further curing.

Lactic acid has been used in the curing of meats to improve the taste. Lactic acid is also added to canned fish to retard bacterial spoilage and enhance the flavor.

In bread making, lactic acid is increasingly used as an acidifying agent to prevent spoilage by rope-forming organisms (60). Also, some lactic acid derivatives like stearyl-2-lactylic acid are used as an emulsifiers for shortening.

Non-food applications

Lactic acid and its derivatives have potential industrial uses (23). Lactic acid is used in tanning for deliming hides. It has also been recommended for use in soldering fluxes. Other uses of lactic acid are found in lithographic developers and in various types of ink. Also, variety of uses have been found in the textile industry. Polyesters of lactic acid yield a plastic material that possesses good tensile strength, thermoplasticity, fabricability, and biodegradability (41) that is currently being used to manufacture resorbable sutures, prosthetic devices, and slow-release carriers of herbicides and pesticides. Lactic acid can also be used as a chemical feedstock for the chemical and biological production of the other organic acids such as propionic, acrylic, and acetic acid, propylene glycol, ethanol, and acetaldehyde (41).

Production of Lactic Acid

Synthetic lactic acid production

Lactic acid is produced synthetically from a number of compounds by various chemical reactions (23). These chemical reactions are classified as:

1. Hydrolysis of derivatives of lactic acid,
2. Hydrolysis of 2-substituted propionic acids,
3. Decarboxylation of certain derivatives of 2-methylmalonic acid,
4. Reduction,
5. Oxidation,
6. Rearrangement and disproportion.

It has been shown that synthesis of lactic acid is possible by hydrolysis of its derivatives. For example, calcium lactate is converted into 2-chloropropionyl chloride with phosphorus pentachloride, then hydrolysis occurs to produce lactic acid.

$$(CH_3\text{-}CHOH\text{-}COO)_2Ca \xrightarrow{PCl_5} 2CH_3\text{-}CHCl\text{-}COCl + Ca^{++}$$

$$CH_3\text{-}CHCl\text{-}COCl \xrightarrow{C_2H_5OH} CH_3\text{-}CHCl\text{-}COOC_2H_5 \xrightarrow{OH^-} CH_3\text{-}CHOH\text{-}COOH + HCl + C_2H_5\text{-}OH$$

But this kind of synthesis has no practical value, because these derivatives are produced from lactic acid except lactonitrile. Lactonitrile is obtained by reaction of acetaldehyde and hydrogen cyanide, then it is hydrolyzed to lactic acid and ammonia.

$$CH_3\text{-}CHO + HCN \text{ -----> } CH_3\text{-}CHOH\text{-}CN$$

$$CH_3\text{-}CHOH\text{-}CN + 2H_2O \text{ -----> } CH_3\text{-}CHOH\text{-}COONH_4$$

$$CH_3\text{-}CHOH\text{-}COONH_4 \text{ -----> } CH_3\text{-}CHOH\text{-}COOH + NH_3$$

Decarboxylation of certain derivatives of 2-methylmalonic acid, such as 2-hydroxy-2-methylmalonic acid is used to produce synthetic lactic acid. Reduction of pyruvate to lactic acid is a well-known reaction. On the other hand, propylene glycol can be oxidized slowly to lactic acid by air in the presence of a spongy platinum catalyst. Some three-carbon atom compounds such as acetone, can be transformed into lactic acid.

Biological lactic acid production

Important genera of lactic acid bacteria (Table 3) are classified as homo- or hetero-lactic acid bacteria depending on the presence or absence of aldolase reaction in the metabolism, respectively. Homo-lactic acid bacteria such as some species of *Lactobacillus* degrade hexoses *via* glycolysis to produce lactic acid (Figure 1). Hetero-lactic bacteria, such as *Leuconostoc* species, do not have the enzyme aldolase. Therefore, they utilize an alternative pathway for hexose breakdown (Figure 2).

Table 3. Important genera of lactic acid bacteria (56)

Genus	Morphology	Type of Fermentation
Streptococcus	Cocci in pairs or chains	Homo-fermentative
Pediococcus	Cocci in pairs and tetrads	Homo-fermentative
Leuconostoc	Cocci in pairs and chain	Hetero-fermentative
Lactobacillus	Long and short rods	Both homo- and hetero-fermentative species

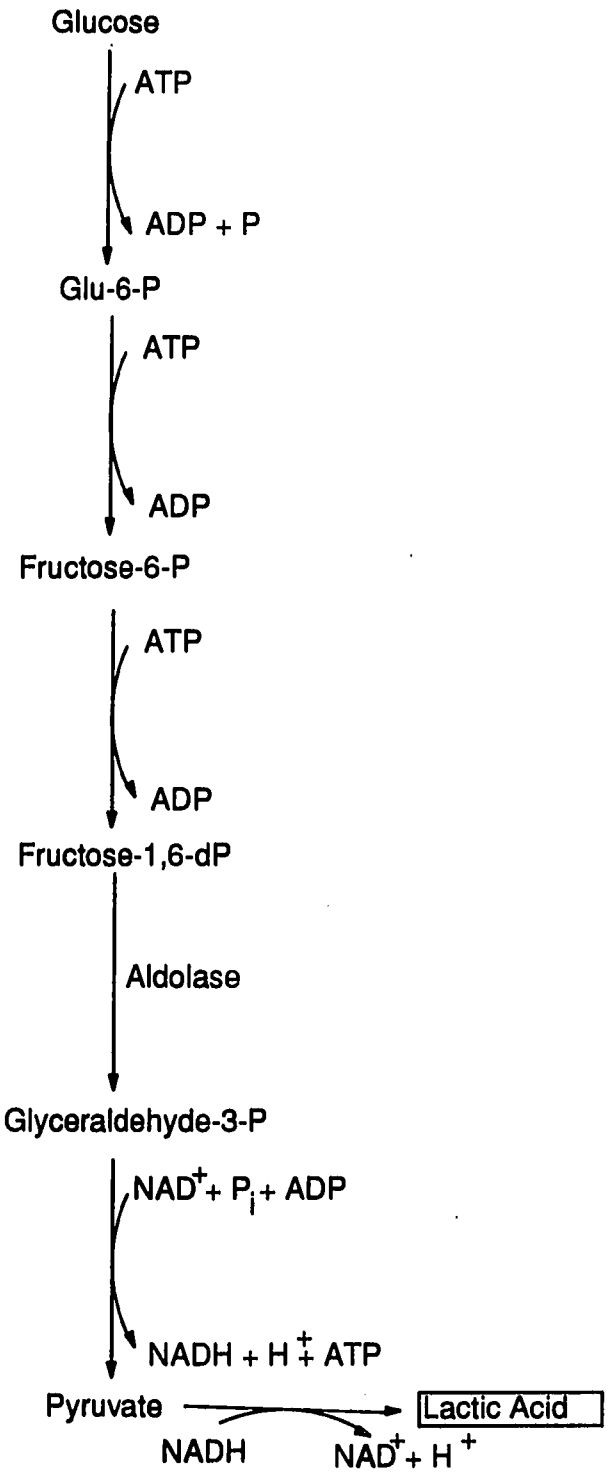


Figure 1. Homo-fermentative pathway of glucose to lactic acid

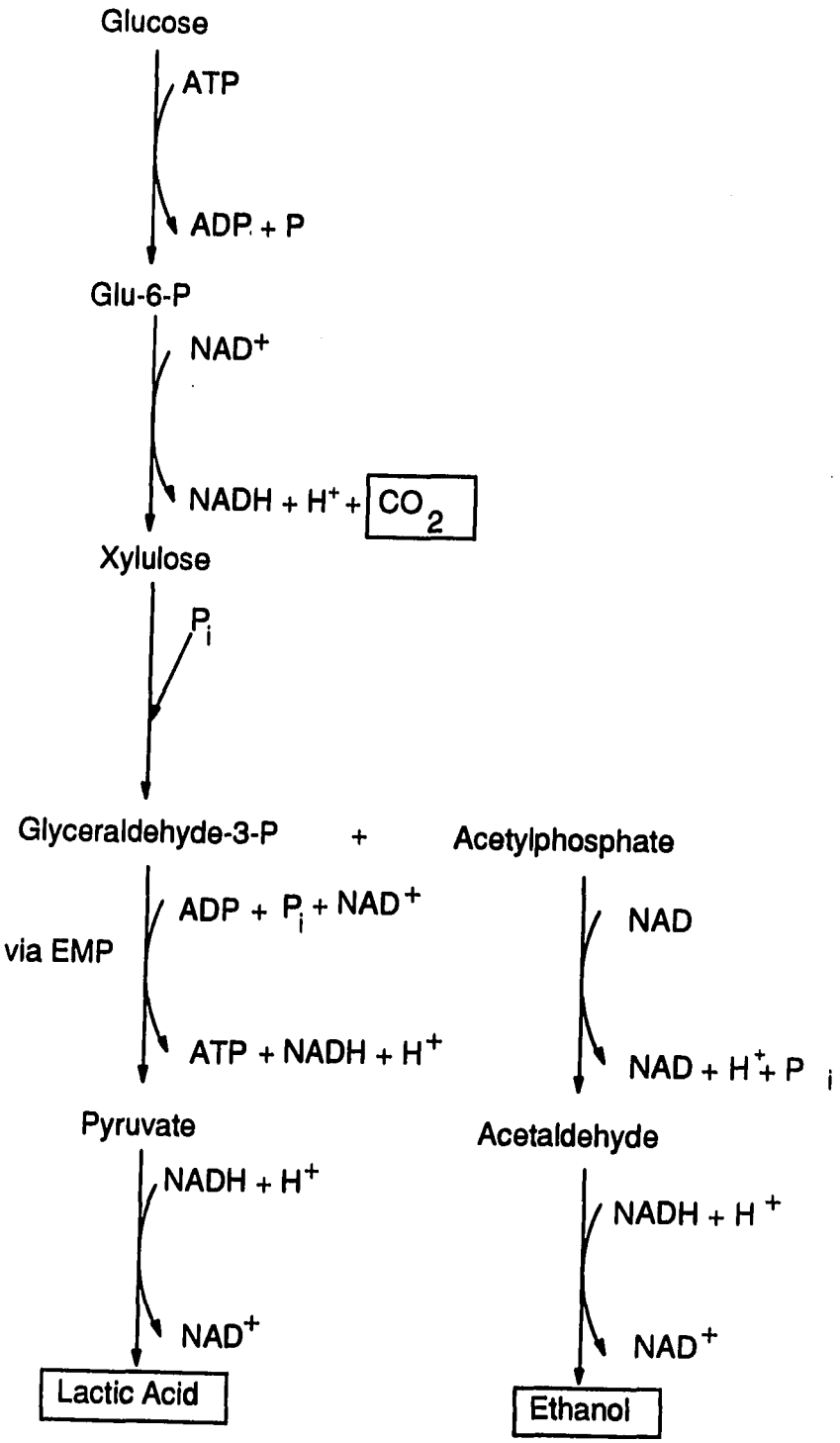


Figure 2. Hetero-fermentative pathway of glucose to lactic acid

Limitation

Although lactic acid is used in the food and non-food industries in large quantities, the production of lactic acid is still limited. Synthetic lactic acid is produced mainly from petroleum-based compounds. However, petroleum reserves are being depleted and synthetic lactic acid is expensive. Therefore, more efficient lactic acid production from a renewable sources, such as sugars or starch, *via* microbial fermentation will be needed to meet a growing demand for lactic acid. However, the production of lactic acid via fermentation provides only 50% of the world supply (46). Product inhibition and recovery are the two major limitations to conventional fermentation processes. Product feedback inhibition results from undissociated lactic acid which can diffuse through the cell membrane (44). Intracellular pH is decreased by increased levels of intracellular lactic acid. For example, *L. delbrueckii* is inhibited by 6% undissociated lactic acid whereas the calcium salt of lactic acid permits a tolerance to 12 to 18% lactate (14). Furthermore, some reactors have been designed to continuously remove lactic acid from the fermentation broth. However, these new fermentors are expensive to operate because of high capital and operating costs. Other approaches need to be developed. This is the subject of this dissertation.

Strain Development

The primary goals of strain development are to improve characteristics by mutating and selecting superior strains (16). These characteristics, in general, include increased yields of desired metabolites, removal of unwanted metabolites, improved utilization of inexpensive carbon and nitrogen sources, and/or alteration of cellular morphology which eases the separation of organism from the product. The urgent need for a superior antibiotic-producing strain of *Penicillium* during World War II led to perhaps one of the first efforts to develop a superior strain by mutagenic techniques. Industrial fungal production of penicillin initially yielded less than 100 units/ml; today yields of penicillin are about 85,000 units/ml (1).

Types of mutagenesis

Mutants are produced by errors in base pairing, covalent modification of bases, and the deletion or insertion of bases (16). Mutant isolation is a tedious and complex process. Isolation of a desired mutant depends on the procedure developed, and on luck. Formation of a stable mutant often is a race between the mutagenic agent activity and the repair mechanisms of the cell. Mutagenic agents cause some permutational lesions of DNA that can be one or more of the following:

1) Pyrimidine dimers, in which two adjacent pyrimidines on a DNA strand are coupled by covalent bonds which cause the DNA strand to lose ability to pair.

2) Chemical changes of a single base, such as alkylation and deamination which cause changes in the pairing properties of the DNA.

3) Crosslinks between the complimentary DNA strands, which prevent their separation in replication.

4) Intercalation of mutagenic agents into the DNA which causes frameshift mutations.

5) Single-strand breaks.

6) Double-strand breaks.

These structural changes can lead to mutations directly by causing pairing errors in replication or indirectly by error-prone repair (18). Mutagenesis is generally induced by using either ultraviolet light or mutagenic agents.

Ultraviolet light

Except for strongly pigmented fungal spores, cells are transparent. Shortwave ultraviolet (UV) light (200 to 300 nm) is used as a mutagen. UV radiation produces a variety of photo-products in DNA, among which thymine dimers have been identified as a major cause of lethal mutagenesis (6). These lesions in most cases are repaired by one of the three repair systems: photoreactivation, excision repair, and postreplication repair (16). These repair systems are error proof.

Mutagenic agents

There are many compounds which can produce desirable mutants. The best mutagen and set of conditions are not identical for all organisms. In general,

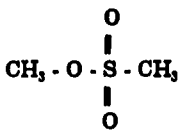
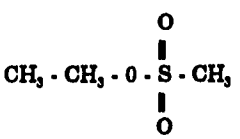
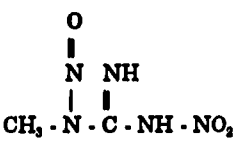
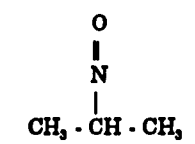
chemical mutagens are classified into two groups: (a) those that cause loss or addition of one or two bases in DNA during replication or repair (frameshift-mutagenesis), and (b) those that cause a chemical change in one or more of the nucleic acid bases (26).

Chemical mutagents

Nitrous acid is one of the chemical mutagens (29). Nitrous acid primarily converts amino groups to keto groups by oxidative deamination. For example, substitution of NH_2 on cytosine with OH by nitrous acid converts cytosine into uracil.

Alkylating agents are commonly used for mutagenesis (26) (Table 4). Alkylating agents modify DNA bases and they are more efficient mutagens than nitrous acid. They result in a variety of cellular constituents being subject to attack, and in the variety of reaction products being generated. The most commonly used alkylating agents are ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), and N-methyl-N-nitro-N-nitrosoguanidine (NG). These compounds are monofunctional alkylators, which means that only one alkyl group is donated. A number of alkylating agents are polyfunctional; these can induce both intrastand and interstrand DNA cross links along with additions of alkyl groups.

Table 4. Common alkylating agents (26)

	Methyl methanesulfonate
	Ethyl methanesulfonate
	N-methyl-N-nitro- N-nitroso guanidine
	Dimethylnitrosamine

Chemistry of alkylating agents

In nucleic acids, almost all susceptible positions of purine and pyrimidine ring systems are alkylated, depending on the agent and conditions. These positions are the N-1, N-3, and N-7 ring nitrogen of adenine and guanine, and the N-3 ring nitrogen of thymine and cytosine. However, the major product under most conditions is 7-alkyl-guanine (37, 52). Alkylation of the N-7 position of guanine introduces a positive charge to the imidazole ring of the purine nucleus (36). Singer (52) pointed out that the N-7 atoms of guanine are sterically available in the Watson-Crick structure for DNA, being situated in the "wide groove" of the double helix. Auerbach (4) noted that methylating agents are

usually more toxic than ethylating agents. Two mechanisms of ethylation-induced mutation have been proposed. One is based on downward shift of the pK for acidic dissociation of the N-1 hydrogen when guanine is alkylated at N-7 (35). Pairing of the ionized 7-ethylguanine with thymine would produce GC to AT transitions. The other mechanism is based on the increased rate of hydrolysis of glycoside linkage when deoxyguanylic acid is alkylated at N-7 (34). A mechanism of ethylation-induced mutation based on 3-ethyladenine has also been proposed (33). In DNA, this base probably exists in the imino form, and especially when protonated at N-1, it could be expected to pair with cytosine and thus produce an AT to GC transition.

Cell killing is apparently due to hydrolysis of ethylated purines rather than a direct result of alkylation. This is indicated by the enhanced sensitivity to post-treatment killing. Loveless (42) measured killing, alkylation, and post-treatment hydrolysis using radioactive alkylating agents and found that cell death correlates with the rate of hydrolysis. Using three different monofunctional alkylating agents, he discovered that slower hydrolysis rates caused a greater extent of alkylation at a specific percentage survival level.

Factors influencing mutagenesis

The mutagenesis rate can be influenced by many factors. These include the type of mutagen, dose of mutagen, physiological state of culture, and presence of repair inhibitors.

Type of mutagen. In every mutagenesis protocol, the kind of mutagenic agent is very important in terms of chemical stability of mutagenesis as well as the handling of mutagenic agent. The ethyl derivative is more stable than the methyl derivative, a finding paralleled by the greater alkali stability of 7-ethylguanosine compared to 7-methylguanosine. Therefore, Singer (52) generalized that ethylguanosines were more stable than methyl guanosines. Also, the rate of ethylation is much slower than that of methylation for all classes of reagents except alkyl iodides. This slow ethylation with EMS leads to some side reactions at neutrality with the N-6 of adenosine and N-4 of cytosine which have not been found to be alkylated by methylating agents (52). Similarly, the O-6 of guanine is ethylated but not methylated by EMS and MMS, respectively. Therefore, EMS is preferred for reasons of safety, stability, and mutagenicity for industrial microorganisms. N-Methyl-N-nitro-N-nitroso guanidine (NG) is one of the most effective mutagens. However, Bridges (8) cited dangers associated with the use of NG:

1) The mutagenesis produces tightly linked multiple lesions which may result in mutagenesis in more than one gene,

2) It is a highly carcinogenic compound, and

3) It may explode if heated to high temperatures.

Concentration of mutagen. The concentration of mutagen and time exposed to that dose play very important role in mutagenesis. At different concentrations, different products can be produced which may or may not be

mutagenic. Also, high concentrations can be lethal. On the other hand, low concentrations in some microorganisms induce error-free repair systems that confer increased resistance to the mutagenic and lethal actions. In terms of dose response, ethylating agents have unusual properties as mutagens. Their uniqueness could be attributed to their low toxicity which permits the use of much higher doses than any other alkylating agents, especially MMS (43).

Physiological state of culture. Repair mechanisms usually operate during replication (i.e. logarithmic growth) if there is a DNA lesion. Therefore, the physical state of a microorganism is very important in development of a stable mutant. Charles et al. (13), and Matijasevic and Zeiger (45) suggested that mutagenesis should be performed during the early stationary phase which is a period when DNA repair is slow.

Presence of repair inhibitors. Some compounds inhibit the enzymes used for repair of DNA in the cell by occupying active sites on the enzymes. Caffeine, acriflavine, and 8-methoxypsoralene are some examples of these compounds (30). Sideropoulos and Shankel (51) reported that caffeine prevents excision of thymine dimers, presumably by binding to the excising enzyme. This binding results in an impairment of DNA repair, which results in an increase in mutant numbers. Caffeine also is an inhibitor of the photoenzmatic repair of UV-induced lesions in *E. coli* DNA. Harm (22) demonstrated that caffeine inhibits photoenzymetic repair by competing with the photoactivating enzyme for binding sites at or close to the UV-induced pyrimidine dimers in DNA. Caffeine also slows

down error-prone post-replication repair mechanisms after chemical mutagenesis (57). Shetty et al. (49) successfully used caffeine in their mutagenesis protocol to develop a mutant of *Bacillus polymyxa* that produced L-phenylalanine from starch.

Reactor Designs for Lactic Acid Fermentation

As previously discussed, the chief limitations in lactic acid fermentations are low production levels and the separation/recovery of lactic acid from the fermentation broth. To overcome these limitations, several different fermentation techniques have been investigated.

Batch type fermenter

This is the oldest type of bioreactor. After seeding a liquid medium with a living cell inoculum, the reactor is sparged with gas (CO_2 or N_2) then is incubated until all the substrate is converted to product. The reactor is equipped with a pH controller, foam controller, agitation and gas sparging devices (Figure 3). Such equipment is still commonly used in the industry. However, it is generally agreed that microbial growth in batch culture may be limited either by availability of nutrients or by the accumulation of toxic materials. Therefore, removal of toxic metabolites from the broth is required for increased cell population, production rate, and final product level.

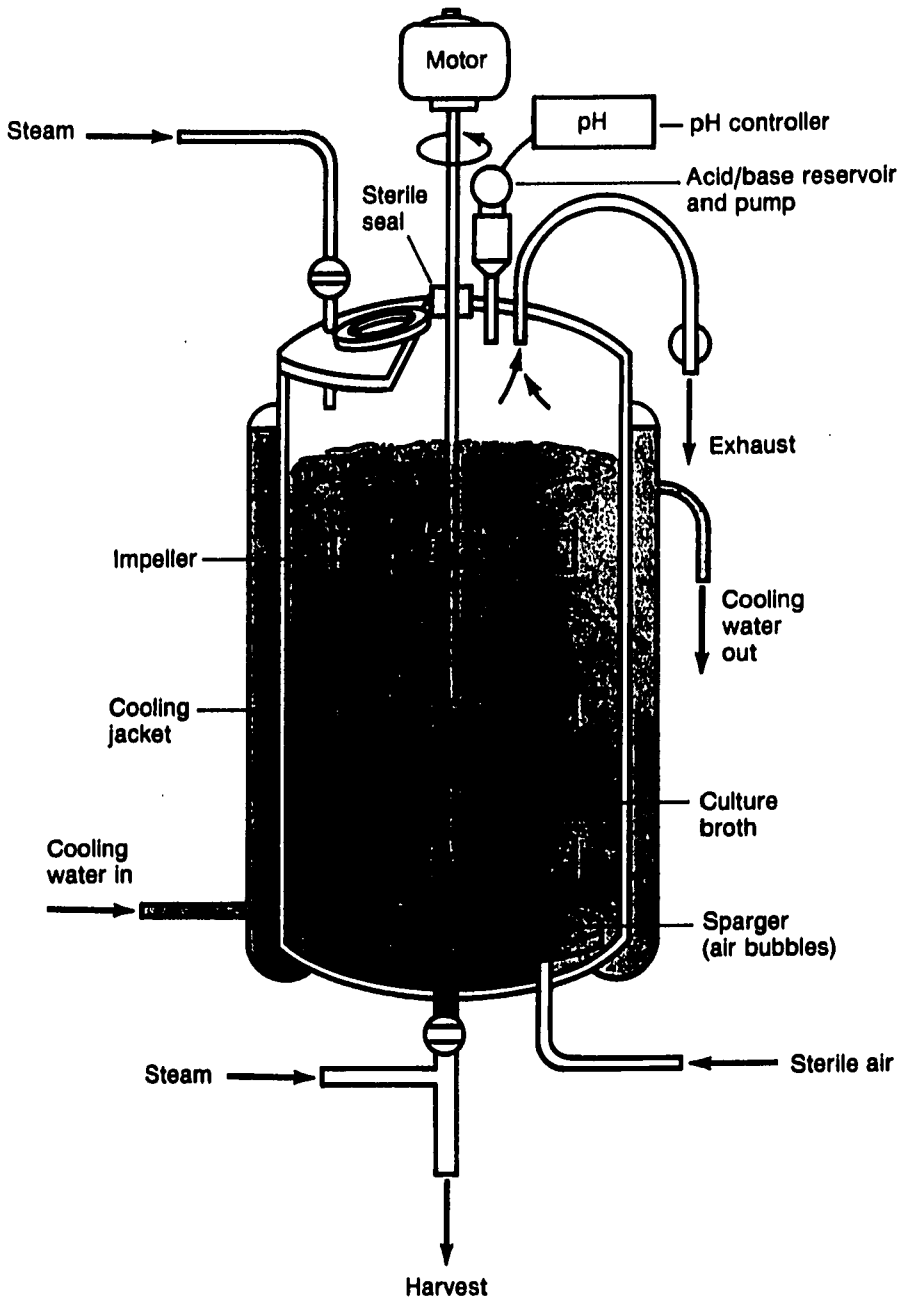


Figure 3. Schematic diagram of batch type fermenter (16)

Continuous stirred tank reactor (CSTR)

This reactor was developed to eliminate nutrient limitation to the microorganisms. Fresh medium is pumped-in at a certain flow rate, and an equal amount of medium is removed (Figure 4). The reactor has temperature and pH controllers. The medium in the reactor is agitated by means of an impeller, rising gas bubbles, or both. Higher productivity (g/l/h) can be achieved compared to a batch reactor, but cell wash-out at high dilution rates is a problem. Furthermore, toxic metabolites such as lactic acid, can cause microbial product inhibition. Therefore, in order to improve productivity, lactic acid should be continuously removed from the fermenter, and/or bacterial strains need to be developed that are tolerant to higher levels of acid so that higher cell densities can be maintained in the reactor.

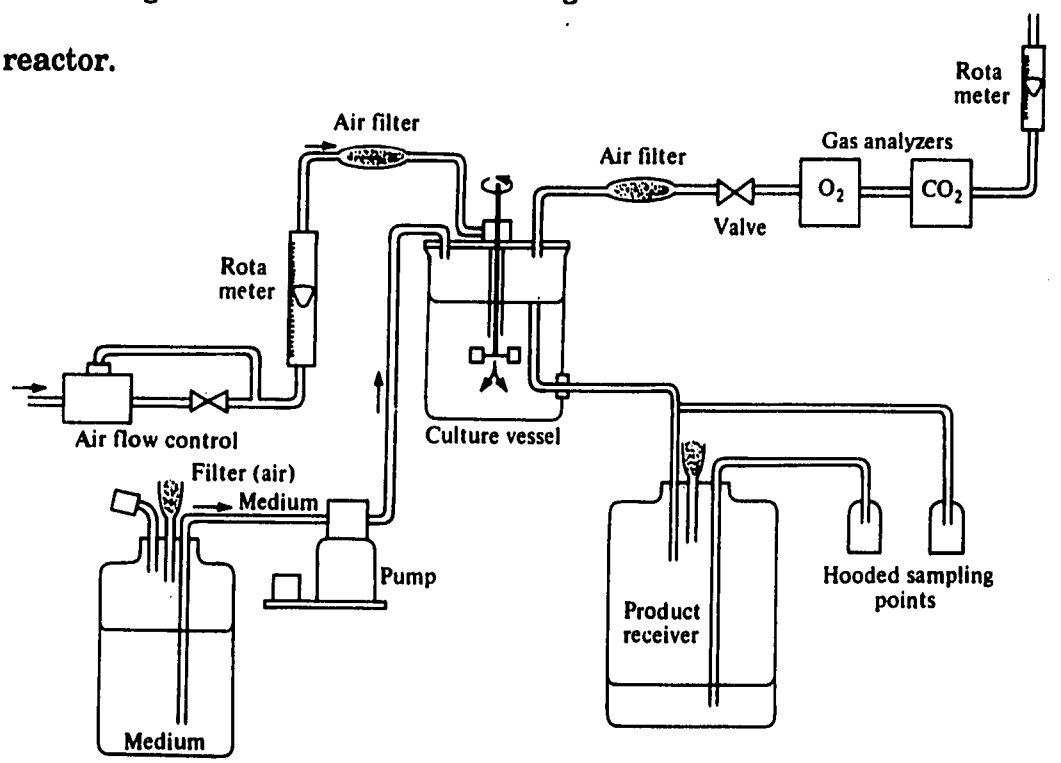


Figure 4. Schematic diagram of continuous Stirred Tank Reactor (5)

Dialysis fermenter

The dialysis fermentation technique was developed by Gallup and Gerhardt (21). This method offers preferential removal of toxic metabolites during the fermentation. The system consists of three basic sections: fermenter, dialyzer, and reservoir (Figure 5).

The medium is dialyzed against a continuous dialysate through a permeable membrane. The dialysate consists of water, which maximizes diffusion of inhibitory products (i.e., lactic acid). Relative to CSTR and batch reactors, use of this type of fermenter results in a higher rate of substrate utilization, cells multiply at a higher rate and reach a higher concentration. Additionally a dialysate effluent containing a cell-free product is produced. Friedman and Gaden (20) reported yields 8 g/l/h in a continuous dialysis culture system as contrasted with 5 g/l/h in a conventional batch fermentation without dialysis. Thus, dialysis fermentation is very effective. However, to achieve more effective dialysis, reservoir volume or volume of dialysate must be extended to more than the volume of the fermentation vessel or the fermentation broth to increase diffusion efficiency.

Electrodialysis fermenter

"Electrodialysis fermentation is defined as a novel fermentation which is able to alleviate the inhibitory effect of a microbial metabolic product on microbial metabolism and to increase the product, in addition to the effect of

electroenergizing on microbial metabolism" (24). If an inhibitory product ionizes, as lactic acid does, it is possible to remove it continuously from the fermentation broth. A schematic diagram of electrodialysis is shown in Figure 6. In this type of fermenter, culture broth is circulated through an electrodialyser which consists of a copper plate as the cathode and a platinum plate as the anode. When lactic acid is produced, the pH of the fermentation broth falls below a set value, causing direct current to be supplied to the cathode and anode. Lactic acid penetrates the anion-exchange membrane and accumulates in the concentration compartments. By this method, 82.2 g/l lactic acid has been produced which is the highest

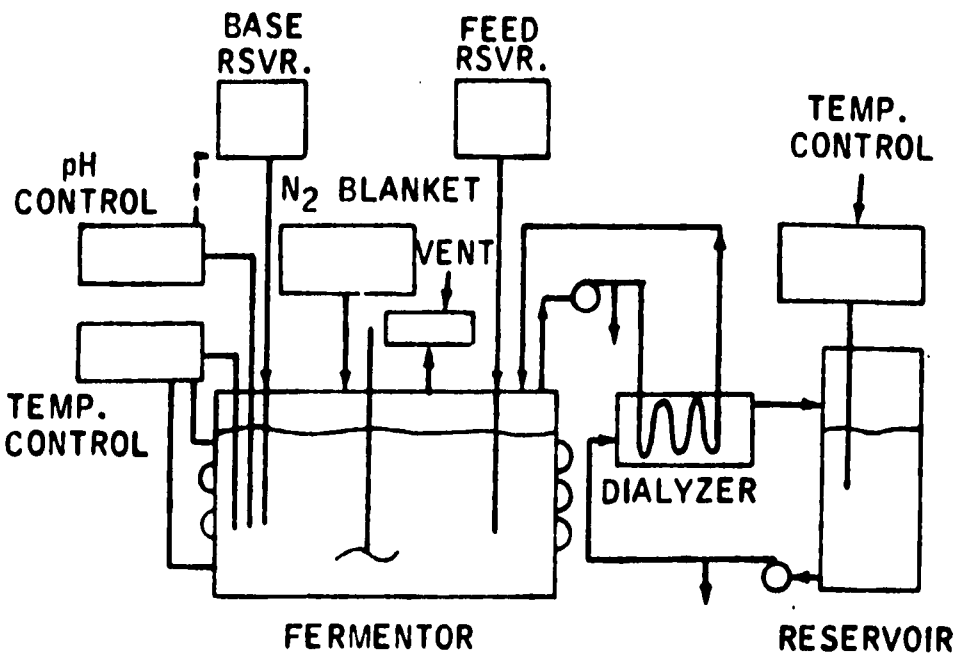


Figure 5. Schematic diagram of dialysis fermenter (20)

concentration reported in a comparison of method (25). Lactic acid can be recovered in almost pure form but in rather dilute aqueous solution by this method. However, microbial cells adhered to the anion-exchange membranes and retarded lactic acid removal.

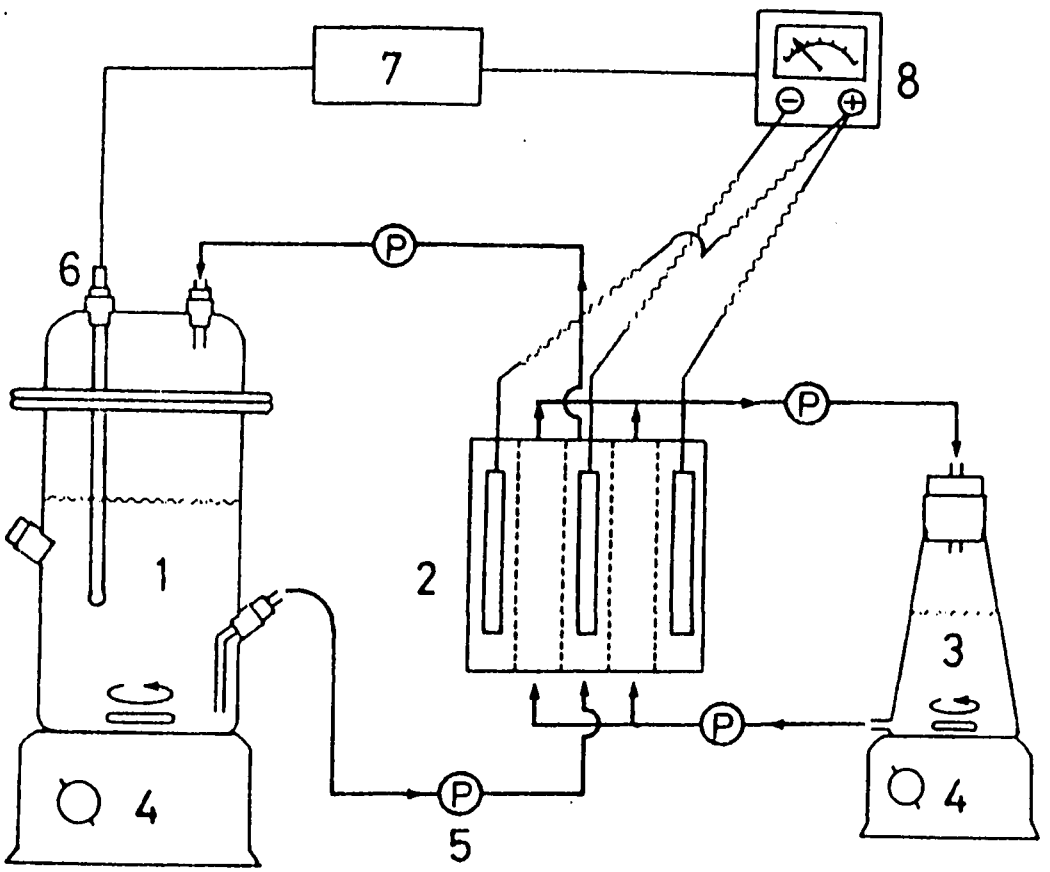


Figure 6. Schematic diagram of electrolysis fermenter: 1. Fermenter; 2. Electrodialyser; 3. Concentrated-fluid reservoir; 5. Pump; 6. pH electrode; 7. pH controller; 8. Direct-current power supply (24)

Cell-recycle fermenter

The goal of this type of fermenter is to increase the biomass concentration in the reactor during continuous fermentation by preventing cell wash-out. In this method, medium ultra-filtrate is removed from the system and the cells are recycled back into the fermenter by using either a flat membrane, or an ultrafiltration unit with tangential flow (59) (Figure 7).

Using such a system, Ohleyer et al. (46) reported a maximum lactic acid concentration below 60 g/l with only 70% conversion at dilution rate between 0.59-2.80 h^{-1}. At dilution rate 2.80 h^{-1}, a 140 g/l (wet weight) biomass concentration, 150 g/l/h volumetric productivity, and lactic acid concentration of 57 g/l were attained in the reactor. This was the greatest volumetric productivity value ever reported. However, this high productivity rate is maintained only for a short time. Although the results were impressive, the application of this technology to commercial lactic acid production seems unfeasible because of high capitol and operation costs. The system needs several pump, and, most importantly, an expensive ultrafiltration unit. Also, upon long term use, plugging of the ultrafiltration unit required disassembly and cleaning.

Hollow fiber fermenter

In this fermenter (Figure 8), microbes are gently immobilized on the outside of the hollow fibers in the device shell as medium is pumped through the inside of the fibers (27). The microporus walls of the hollow fibers allow the nutrients and

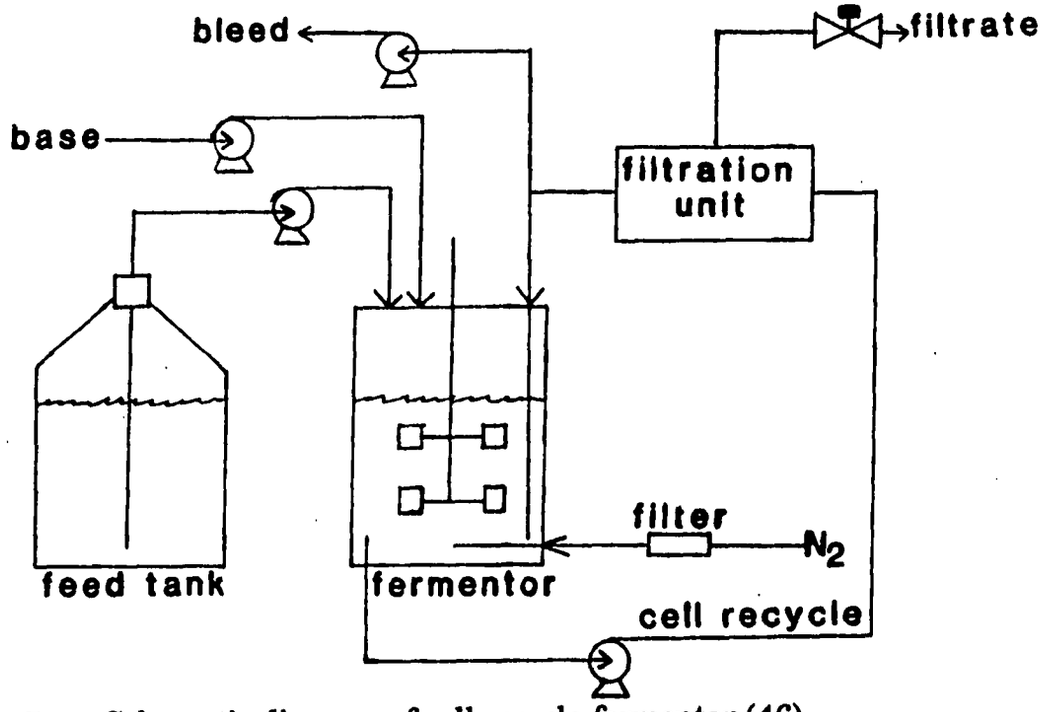


Figure 7. Schematic diagram of cell-recycle fermenter (46)

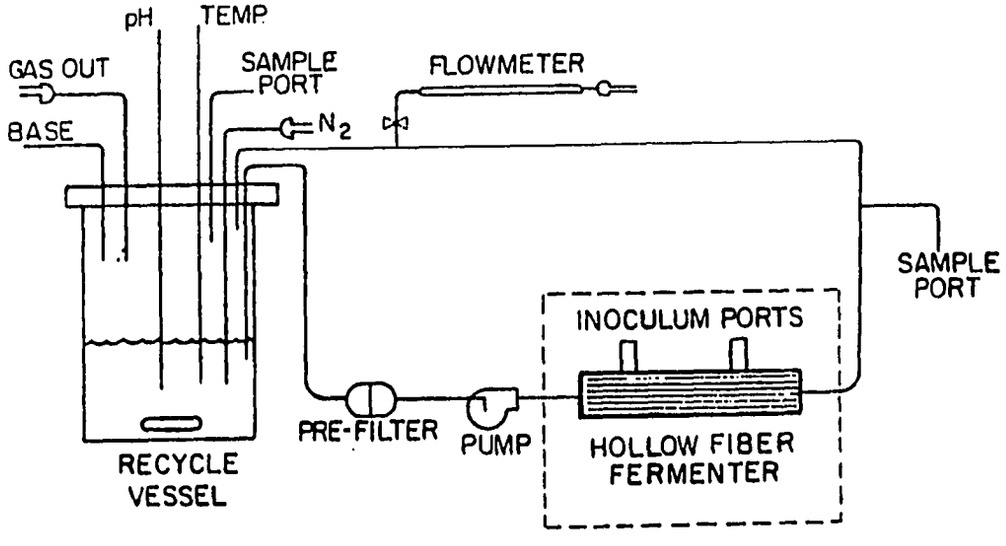


Figure 8. Schematic diagram of hollow fiber fermenter (58)

products to diffuse freely, whereas the microbes are unable to penetrate. Advantages of this kind of cell-immobilization are extremely high cell-densities in the reactor, high volumetric productivities, low susceptibility to process upsets, and a reduced requirement for feed sterilization. The hollow fiber module consists of device shell, microporous hollow fibers, and a pump. The hollow fiber module is heat sensitive; therefore, it is sterilized by ethylene oxide then wetted with a solution of 50% (v/v) ethanol prior to use. The fermenter is inoculated by injecting a growing cell suspension into the device shell space through one of the inoculation ports. Temperature and pH are controlled, and fermentation medium is continuously pumped through the hollow fibers. VickRoy et al. (58) reported that final cell densities in the fluid surrounding the fibers in the fermenter were as high as 480 g/l (wet weight), and volumetric productivity of lactic acid in the fluid moving through the fibers was 100 g/l/h lactic acid.

Artificially immobilized-cell fermenter

In this type of continuous fermentation, cell density is increased and cell wash-out is prevented by restricting cell mobility. Some advantages of immobilized-cell over free-cell fermentations include maintenance of stable and active biocatalysts, reuse of biocatalysts, accelerated reaction rates, high volumetric productivities, improved process control, and improved production efficiency (31). Two general types of cell immobilization are:

1. Attachment, where microorganisms adhere to surface by self adhesion or chemical bonding,

2. Entrapment, where the organisms are trapped in the interstices of a fibrous or porous matrix, such as a stabilized gel or membrane.

Entrapment of cells represents a type of immobilization that does not depend on cellular properties (i.e., flocculation, aggregation, appendages). In this case, cells are held either within the interstices of porous materials, such as a sponge or fibrous substance, or by the physical restraints of membranes or encapsulating gel matrices. Cell entrapment in polymeric networks is the most commonly applied method of cell immobilization. Table 5 lists some different mechanisms and polymers which have been used. The most widely used cell-entrapment method involves a layer or a bead of alginate, a natural polysaccharide. The gentleness of gelation procedure, in contrast to chemical polymerization, results in much higher initial viability of the immobilized cells. Figure 9 illustrates schematically one protocol for preparation of calcium-alginate beads containing immobilized cells (53). Strenrous et al. (53) reported that *L. delbrueckii* entrapped in calcium-alginate gel beads produced a maximum of 12 g/l lactic acid with a production rate of 0.2 g/l/h. However, some workers have reported shrinkage and decreased strength of calcium alginate beads during lactic acid production (19, 48). Lactic acid production is growth-associated so this kind of fermentation results in cell-leakage and gel disruption. Furthermore, alginate gel is stabilized by calcium ions which are displaced by lactate ions during

Table 5. Polymeric networks used for cell immobilization (18)

Network formation	Cross-links	Examples
Entrapment	Nonspecific	κ-Carrageenan, Ca-alginate polystyrene
Carrier-binding	Ionic	Al-alginates
Cross-linking	Covalent	Epoxy resin

fermentation weakening the matrix. Tuli et al. (55) 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. Audet et al. (3) used κ-carrageenan, a polysaccharide extracted from seaweed, enriched with locust bean gum to immobilize *S. thermophilus*, *L. bulgaricus*, or *S. lactis*. Acid production was increased compared with other gels and viability remained high throughout the entrapment steps and subsequent storage.

Immobilized-cell techniques enhance lactic acid production rates, and yields. Also, artificially immobilized-cell systems have the advantage of long-term storage or immediate usage. However, industrial application of type of fermenter has been limited for the following reasons:

1) Short active lives (20-40 days),

2) Toxic effects on cell viability,

3) Limited mass transfer across the beads,

4) High cost of the carrier,

5) Poor operational stability.

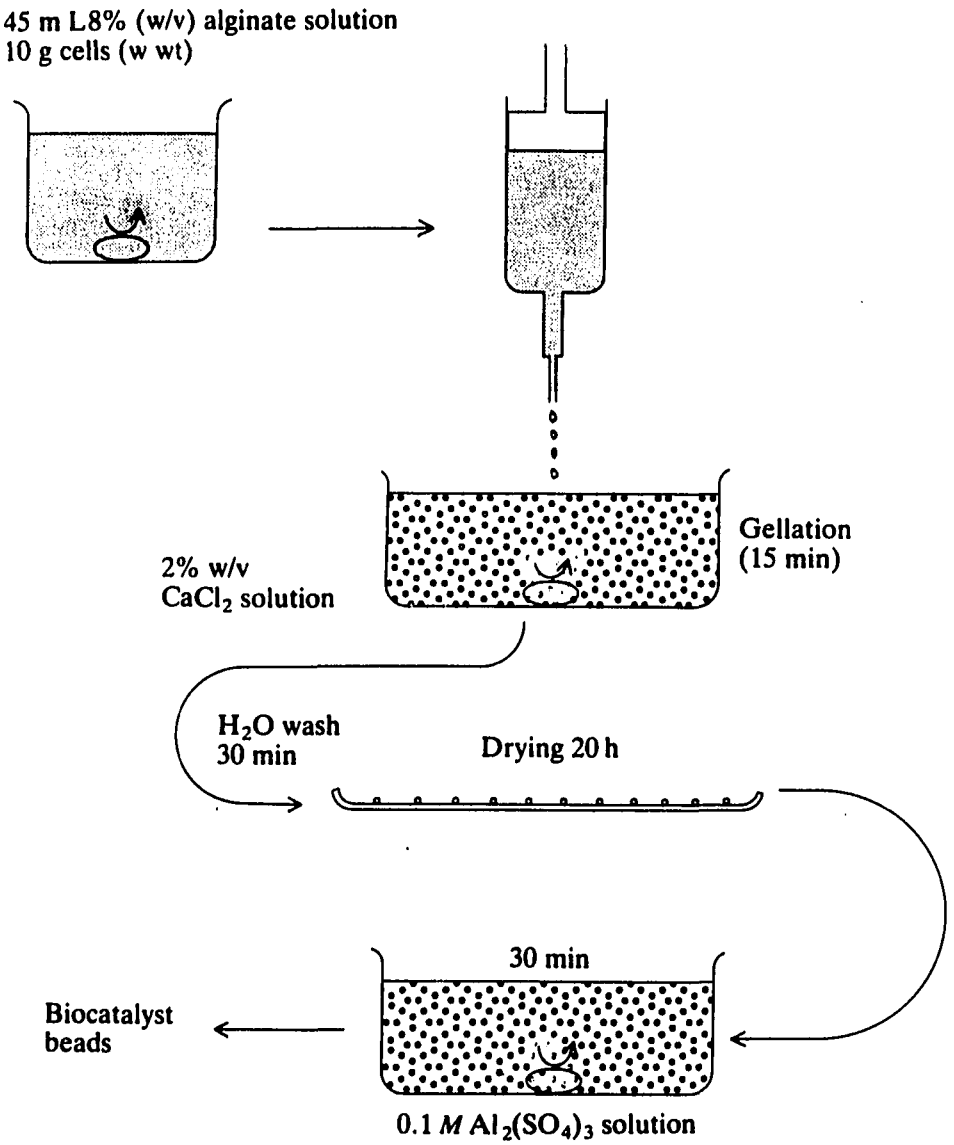


Figure 9. Summary of one protocol for entrapping cells in alginate beads (5)

Biofilm

Development of biofilm

Attachment of microorganisms to solid surfaces has attracted the attention of scientists since Zobell (61) discovered this phenomenon in 1943. A major advantage of cell attachment by biofilms is that the concentration of nutrients tends to be higher at solid surfaces then in solution which increases cell efficiency.

In general, for microbial adhesion to occur, the forces can be divided into three main categories (15).

1. Conservative forces that arise out of mutual proximity, like electrostatic and electrodynamic forces,

2. Hydrodynamic or aerodynamic forces that are determined by particle diffusion and equilibrium rates,

3. Forces caused by externally applied fields, such as electric, magnetic, hydrodynamic shear, and gravitational fields.

The interaction of microorganisms with solid surface is assumed to take place in three stages. The first stage is a weak reversible adhesion. The second is a firmer and non-specific adhesion by the fimbriae and pili, involving the formation of hydrogen and ionic bonds. The third stage would be the formation of extracellular material such as polysaccharides, by microorganisms. The physiological state of the microorganism influences the rate and possibly the extent of attachment. Bryers and Characklis (10) observed that attachment rates

were directly proportional to growth rates in a mixed-culture continuous system, and micro-rough surfaces were better than smooth surfaces for biofilm formation.

The formation of fouling films in pipes, the corrosion of heat exchangers, and the formation of bacterial films on submerged surfaces imposes both economical and safety problems. The deposition and adhesion of bacteria and other microorganisms on teeth enamel forming a layer $\leq$ 10 μm thick results in subsequent plaque formation and tooth decay. However, scientists have used microbial films in fermentation processes as a natural form of immobilized cells.

Advantages of natural biofilms include;

1) The use of inexpensive easily obtainable support material,

2) The ability to process large volumes of nutrient flow for long times, and

3) The freedom to employ mixed cultures that can mediate multiple biological conversions.

The major distinction between naturally and artificially immobilized cell systems is that the growth of cells is promoted in the former class, thus providing a constantly active system.

A major disadvantage of naturally formed biofilms is the lack of control over biofilm density and thickness, which may lead to internal mass transfer limitations.

Application of biofilms

Biofilms have been used for centuries in different applications. New application areas are being investigated in the food and non-food industries. Best known applications of biofilm reactors are:

Acetic acid production. Vinegar production is one of the earliest applications of biofilm reactor. The quick vinegar process operates in wooden vats with perforated bottoms packed with wooden chips. The substrate is an alcoholic solution, which is trickled down through the packed bed of wood chips, where an enriched biofilm of *Acetobacter* spp. develops (Figure 10). The biological conversion requires substantial amounts of oxygen (16).

Microbial leaching. Extraction of copper from ore deposits using acid solutions has been practiced for centuries, but the role of bacteria in metal dissolution was not understood until the late 1940s. Approximately 10-20% of copper mined in the U.S. is extracted by microbially-assisted processing of low grade ores (9). Microbial leaching for the recovery of other metals, such as uranium, silver, cobalt, molybdenum, nickel, and gold (38), as well as the microbial desulfurization of coal (11, 28), is also being used. Most microbial leaching depends upon microbial oxidation of metal sulfides. Aqueous environments in association with spent minerals produce very harsh conditions of low pH, high metal concentrations, and elevated temperatures, which enrich for microbial flora with very discriminating nutritional requirements.

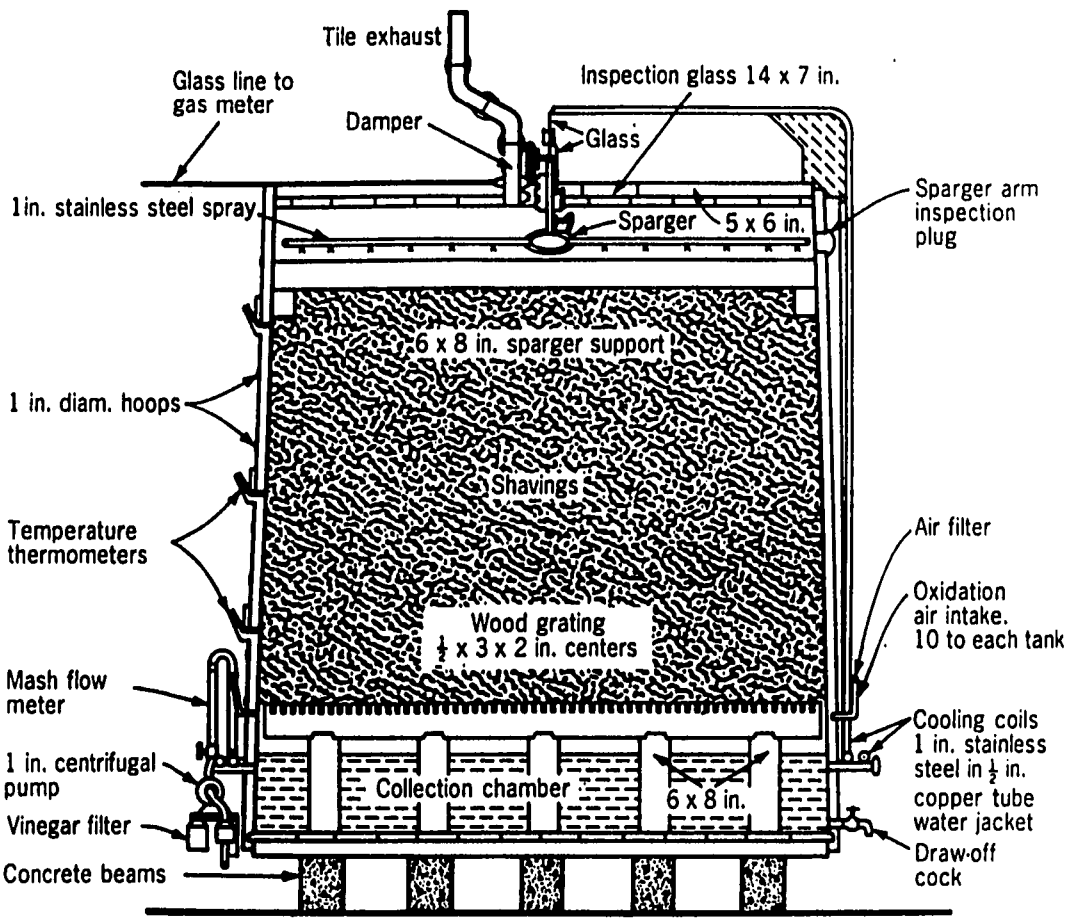


Figure 10. Schematic diagram of packed bed reactor for vinegar production (5)

Polysaccharide production. Polysaccharides occur as structural material in microbial cell walls and extracellular capsular layers. A large number of extracellular polymers have gained commercial importance because of their ability to alter the rheological properties of aqueous solutions at low concentrations. One novel approach for exploiting the microorganisms' tendency to overproduce

extracellular polysaccharide during biofilm formation is offered by Robinson and Wang (47). In this process, the mass transfer limitations inherit in biofilm systems are exploited in order to selectively retain the macromolecular products within the support. In a typical *Xanthomonas campestris* fermentation, maximum xanthan gum concentrations are apparently limited, not by feedback or other physiological inhibition, but by the corresponding decrease in mass transfer rates with increasing product concentration in the bulk phase. They immobilized living *X. campestris* on Celite, a highly porous, rigid support particle. Due to the low diffusitivities of the macromolecules, xanthan was preferentially retained within the porous matrix; nutrients and other metabolites diffused rapidly to the cells, promoting further biofilm development. Retained polysaccharide concentrations were enhanced so that overall productivity was greater in the immobilized cell system than in a classical suspended culture.

Alcohol production. Continuous fermentation techniques are used widely to produce alcohols, mainly ethanol. However, cell wash-out and low yield and low productivity are still important problems. Biofilm reactors are helping to solve these problems. Lin et al. (39) used sponges as packing in a trickle bed reactor. Recently, Koutinas and Kanellaki (32) used γ-alumina pellets in a packed bed reactor in which ethanol productivity was 23.8 g/l/h with *Zymomonas mobilis*. To improve productivity, yield and ethanol concentration, more supports need to be investigated (7).

Biofilm Reactor Designs

As has been discussed, biofilm technology brings some advantages to microbial reactors. These advantages include prevention of cell wash-out, easier product recovery, reactor stability under varying substrate rates and high substrate conversion. Microbial film reactors can be operated to exploit these advantages.

Packed bed film reactors

The best example to this kind of reactor is the trickling filter system used for production of vinegar by the 'quick vinegar' process in which a microbial population develops as a slime layer on the surface of the packing material arranged as a bed (Figure 10). A relatively thick microbial film develops which makes it difficult to predict the performance of the reactor. The major advantages of trickling filters compared with other effluent treatment systems is that no power is consumed for agitation or gas transfer so that the operating cost is low.

Fluidized bed film reactors

Solid supports used in packed film reactors are fixed in space either by gravity or by direct attachment to the reactor wall. However, the supports are kept in suspensions by drag forces exerted by the upflowing nutrient medium and/or gas (Figure 11). Fluidization also overcomes operating problems such as bed clogging, channeling of flow, and the high pressure drop common to fixed bed

reactors. Therefore, fluidized bed film reactors retain a high biomass concentrations which enable the system to operate at significantly reduced retention times. Furthermore, it has better control of biofilm thickness on the solid supports because of the continual collisions of the particals in the reactor which makes biofilms more homogenous.

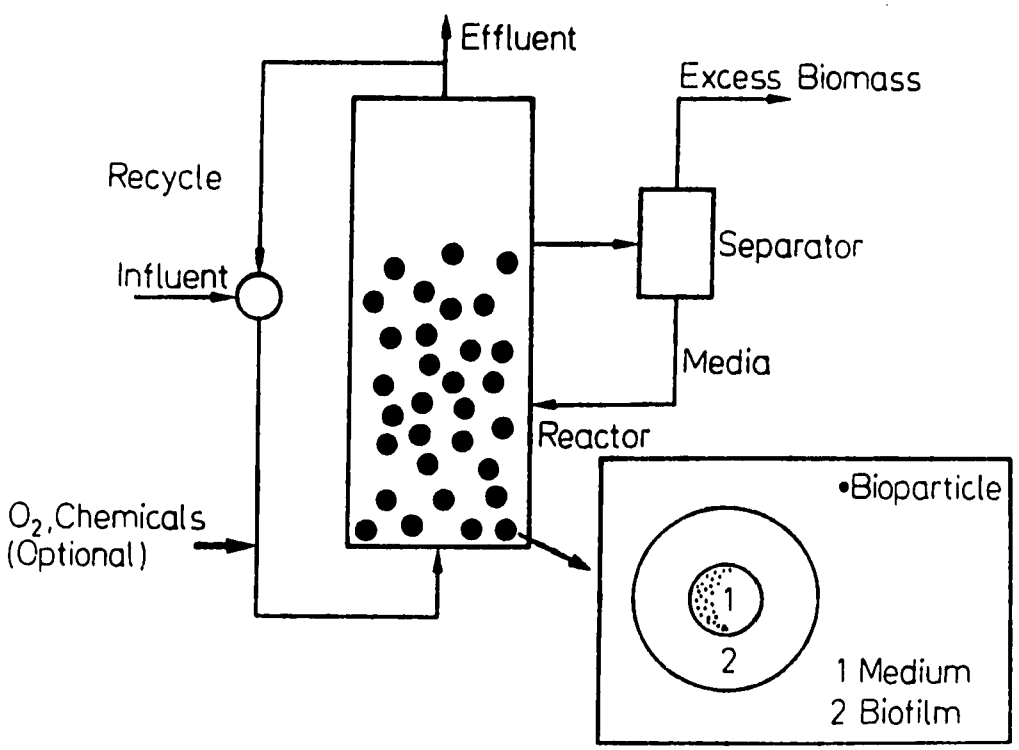


Figure 11. Schematic diagram of fluidized bed film reactor (50)

Rotating disc reactors

In this reactor, a complex microbial slime layer builds up on a series of partly submerged discs which rotate slowly in the vertical plane immersed in a culture medium (Figure 12). This system has been developed especially for waste water treatment. As the discs turn, the microbial film is exposed in succession to nutrient solution and to air. The film thickness is self regulating because excess biomass sloughs from the disc surface. The major advantages of the system are the ability to resist shock loading, short retention time, low power requirement, and excellent process control (12). Torma (54) proposed the use of this reactor for treatment of mine drainage water which is serious pollutant to receiving streams. They use *Thiobacillus ferrooxidans* which converts toxic ferrous iron to the non-toxic ferric state.

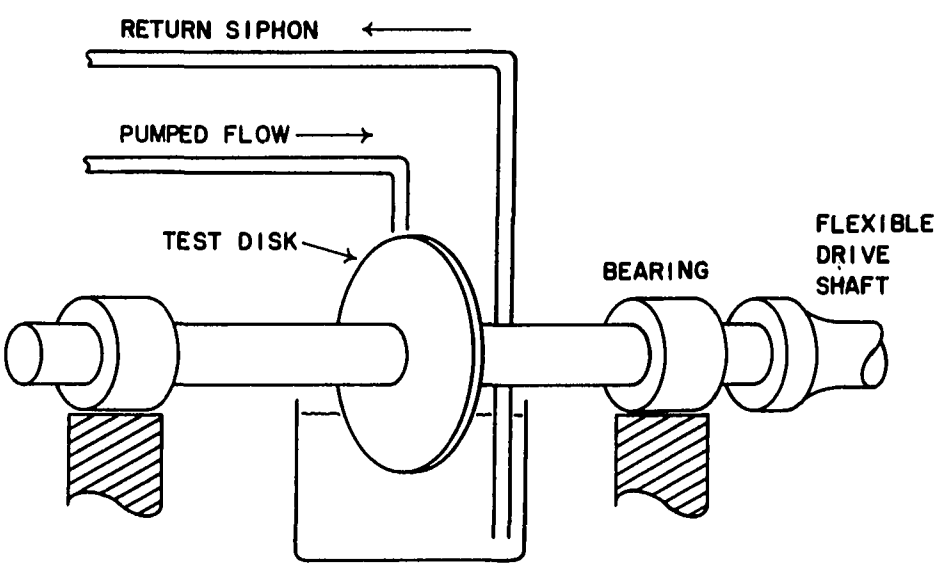


Figure 12. Schematic diagram of rotating disc reactor (12)

Completely mixed microbial film fermenter (CMMFF)

This is the microbial film version of a continuous stirred tank reactor (CSTR). To prevent wash-out and increase productivity, Atkinson and Davies (2) designed a completely mixed microbial film fermenter based upon the fluidized bed principle (Figure 13). Medium is pumped into of the bottom of a reactor containing solid supports and is taken from the top to a mixing tank where pH and temperature are adjusted, fresh medium is added, and product is removed.

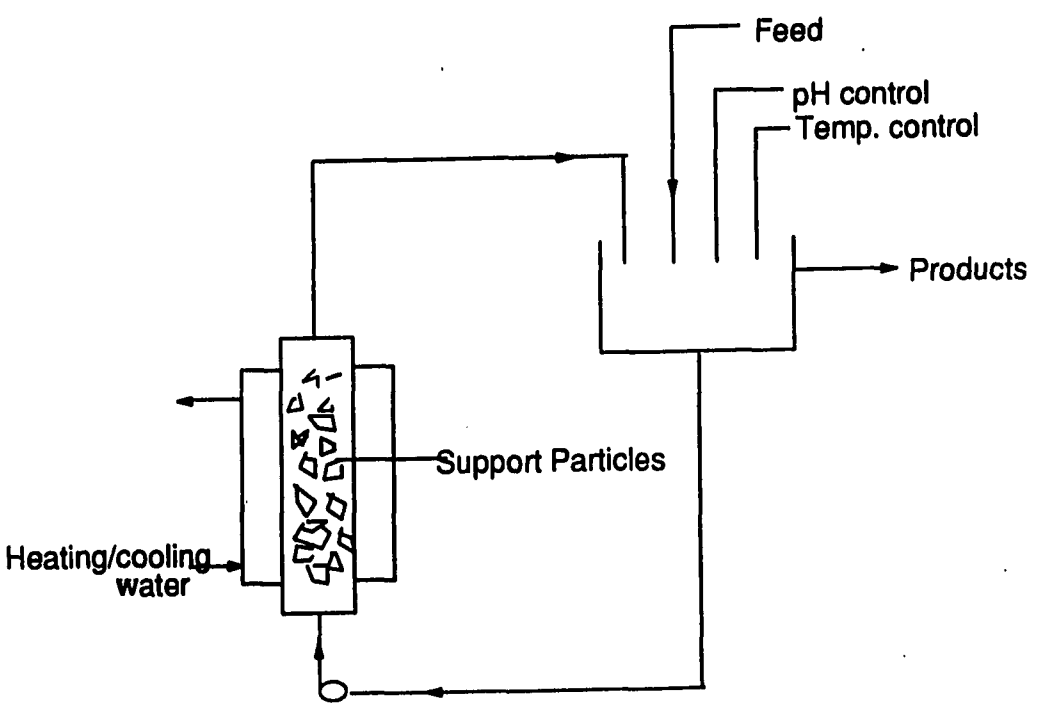


Figure 13. Schematic diagram of completely mixed microbial film fermenter (CMMFF) (2)

PAPER I. ENHANCED PRODUCTION OF D(-)-LACTIC ACID BY MUTANTS OF *LACTOBACILLUS DELBRUECKII* ATCC 9649

Enhanced Production of D(-)-Lactic Acid by Mutants of *Lactobacillus delbrueckii* ATCC 9649[1]

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1. Journal Paper No. J-14087 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Projects No. 2889 & 0178

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ABSTRACT

Chemical mutagenesis with ethyl methanesulfonate followed by selective culturing was used to develop strains of *Lactobacillus delbrueckii* (ATCC 9649) that tolerated increased lactic acid concentrations while continuously producing the acid. Three mutants (DP2, DP3, and DP4) produced higher levels of lactic acid than the wild type *L. delbrueckii* when compared in standing fermentations at various glucose concentrations. In stirred-tank-batch fermentations (pH 6; 45°C), mutant DP3 in 12% glucose, 1% yeast extract-mineral salt-oleic acid medium produced lactic acid more than two times faster than the wild type and produced 77 g/l lactic acid compared with 58 g/l for the wild type. Mutants DP2 and DP3 exhibited faster specific growth rates, shorter lag phases, greater lactic acid yields, and better lactic acid tolerance than the wild type. Concentration of lactic acid as high as 12% was obtained. Mutant DP3 was stable for over 1.5 years (stored freeze dried). Mutants with enhanced lactic acid-producing capacity were obtained each time the strain development procedure was employed.

INTRODUCTION

Lactic acid is an organic acid that can be produced chemically from coal, petroleum, and natural gas and biologically from the bioconversion of carbohydrates, agricultural and industrial wastes, and plant biomass (2, 12). Lactic acid is the smallest natural molecule to exhibit optical activity. It exists in two isomeric forms, D(-)lactic acid and L(+)lactic acid (12). Lactic acid fermentation is essential for the manufacture of cheeses, pickles, yogurt, cultured sausages, and buttermilk. Lactic acid is used as an acidulant, a flavor agent, and a preservative. It is "generally recognized as safe" for human consumption (4). Lactic acid also can be used as a feedstock for the production of plastics (5, 12), other organic acids, propylene glycol, ethanol, and acetaldehyde (4).

Lactic acid production *via* fermentation provides only about 50% of the world supply (7) because product inhibition and the difficulty of lactic acid recovery from aqueous medium limits the economic viability of the conventional fermentation. To improve production, novel fermentation techniques have been applied. These include immobilized-cell bioreactors (14), hollow-fiber reactors (8), cell-recycled reactors (7), and extractive fermentations (13). To overcome feedback inhibition problems, strain development *via* chemical and irradiation mutagenesis or molecular genetic approaches need to be employed.

In this paper, we describe a reliable chemical mutagenesis procedure that can be used to develop enhanced strains of *Lactobacillus*. Three mutants were

selected that demonstrated faster growth rates and increased product yields than the wild type in various media modifications.

MATERIALS AND METHODS

Microorganism. *Lactobacillus delbrueckii* (ATCC 9649), a homofermentative D(-)lactic acid producer, was obtained from the American Type Culture Collection (Rockville, MD), and maintained in *Lactobacillus* MRS medium (Difco Laboratories, Detroit, MI) at 4°C. Cultures were transferred to fresh MRS broth every 2-3 weeks.

Chemical mutagenesis and killing curve construction. A 1-ml inoculum from an overnight static culture in MRS medium was added to 100 ml of MRS medium in a 250-ml flask. The culture was incubated as a standing culture in a 45°C water bath until it reached stationary phase; then, 20 ml of cell suspension (10^7 cells/ml) was harvested by centrifugation at 1000 x *g* for 10 min. The supernatant was decanted, and the cell pellet was resuspended in 20 ml of fresh MRS medium containing 0.4% (w/v) caffeine (Sigma Chemical Co., St. Louis, MO), which was the maximum level that the bacterium could tolerate. After 0.4 , 0.6, 0.8, 1.0, 1.2, or 1.5 ml of ethyl methanesulfonate (EMS) or methyl methanesulfone (MMS) (Sigma Chemical Co.) was added to the tube, 1 ml of zero-time sample was taken. The tube was incubated at 45°C, and after 5, 10, 15, and 30 min, 1-ml samples were serially diluted in sterile water. Survivors were determined by using pour plates of MRS agar medium without caffeine in replicates of three after incubation at 45°C for 24-48 h.

Mutagenesis and mutant selection. The mutant selection medium contained 5% (w/v) glucose; 1% (w/v) yeast extract (Difco); 8, 12 or 16% (w/v) L(+)lactic acid (Aldrich Chemical Co., Milwaukee, WI) 0.4% caffeine (w/v) and 0.05% (w/v) oleic acid (Sigma Chemical Co.) in mineral salt solution (0.6 g of $MgSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot H_2O$, 1.0 g of sodium acetate, 0.5 g of K_2HPO_4, and 0.5 g of KH_2PO_4 per liter of deionized water, pH adjusted to 6.5). The glucose and mineral salts solutions were sterilized separately in an autoclave, cooled, and mixed; filter-sterilized oleic acid was added aseptically.

L. delbrueckii ATCC 9649 was treated with EMS to 99% kill. The cells were removed by centrifugation, washed once in 0.1 M phosphate buffer (pH 7), and resuspended in 5 ml of phosphate buffer. One milliliter of cell suspensions was transferred to 9 ml of selection medium. From culture tubes with growth, 1-ml was transferred to 9 ml of fresh MRS medium without lactic acid, and incubated standing in a 45°C water bath. Next, 1 ml was transferred to 9 ml of the mutant selection medium and incubated until visible growth was detected. The same sequential transfer procedure between MRS medium and selection medium was applied two more times for a total of four transfers in the lactic-acid-selection medium. Finally, the culture was transferred to fresh MRS medium. After overnight incubation, the culture was inoculated into 100-ml of MRS medium in a 250-ml flask, and the flask was incubated standing in a 45°C water bath until stationary phase as determined by absorbance at 620 nm. This culture was mutated a second and third time as previously described. A total of four

serial transfers into 8% lactic acid selection medium followed by MRS medium was performed after each mutagenesis treatment, except for the mutant DP4 which survived only two transfers. Mutants DP2, and DP3 were obtained by following the complete procedure described. The mutants were freeze dried in 20% skim milk powder solution and stored at -20°C.

To further stress mutant DP3, continuous fermentation was performed by using filter-sterilized medium containing 1% yeast extract, 5% glucose, mineral salt solution, 0.05% oleic acid and 8% L-lactic acid. A custom fitted 1800-ml Fleaker beaker (Corning Glass Works, Corning, NY) with a 500-ml working volume and magnetic stirrer were used at 45°C, while maintaining the pH at 6 with 7 N NH_4OH. The Inoculum was prepared from freeze-dried *L. delbrueckii* DP3 suspended in 100 ml of MRS medium and incubated for 24 h. A 50-ml inoculum was added to the reactor. After a 48-h batch fermentation, continuous fermentation was started and 0.007, 0.014, 0.024, 0.048 h^{-1} dilution rates were initiated on days 2, 5, 12, and 15, respectively. The mutant selected during this continuous fermentation was designated DP3.19.

Culture broth analysis. Glucose consumption and D(-)lactic acid production were analyzed by a Water's high performance liquid chromatograph (HPLC) (Milford, MA) equipped with Waters Model 401 refractive index detector. The separation of lactic acid, glucose, and other broth constituents was achieved on 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 a mobile phase at a flow rate of

0.8 ml/min with a 20-µl injection loop. Biomass was determined by measuring absorbance at 620 nm on a Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, NY)., which was then converted to dry cell mass (g/l) *via* a standard curve prepared with log-phase cells.

Comparison of wild type and mutants.

i) Standing culture fermentation. Growth rates and lactic acid production rates of the wild type and mutants were compared by using MRS medium containing 12% glucose and 1% yeast extract, mineral salts (0.6 g of $MgSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot H_2O$, 1.0 g of sodium acetate, 0.5 g of K_2HPO_4, and 0.5 g of KH_2PO_4 per liter of medium) and 0.05% oleic acid (1% YMO) with 12% glucose in standing culture without pH control at 45°C. After incubation for 24 h, absorbance at 620 nm and lactic acid concentration were determined.

ii) Stirred batch fermentation. A 7.5-liter Microferm fermenter (New Brunswick Scientific Co., New Brunswick, NJ), was used with a working volume of 3 liters. Three media were examined: (i) filter-sterilized MRS; (ii) 1% YMO; and (iii) 3% yeast extract, mineral salts, and 0.05% oleic acid (3% YMO). All of the media contained about 12% glucose. The 3% YMO medium required an additional 9% glucose, which was added when the residual glucose concentration decreased to $\leq$3%. The pH was maintained at 6 by adding filter-sterilized 6 N NH_4OH. The fermentation temperature was 45°C, and the agitation rate was 100 rpm. The fermentation was followed by measuring cell biomass production, glucose

consumption, and lactic acid production. All fermentation data represent at least duplicate fermentations.

RESULTS AND DISCUSSION

Chemical mutagenesis and mutant selection. As determined by the kill curve, 0.9 ml of EMS per 20 ml of cell suspension killed 99% of the original microorganisms after 15 min of incubation; this was deemed the optimum procedure. The wild type of *L. delbrueckii* would not tolerate more than 6% lactic acid, but mutants grew in the 8% lactic acid selection medium; even after repeated mutations, no growth was present in tubes containing 12 or 16% lactic acid. Transfer of cultures from the 8% lactic acid selection medium to MRS medium and back to lactic acid again for a total of four transfers into 8% lactic acid selection medium was essential for securing stable mutants. Mutant DP4 was treated with EMS three times, as were DP2 and DP3. However, mutant DP4 did not grow in the third 8% lactic acid transfer-culture medium after the third mutagenesis treatment, and its acid-producing abilty was different from mutants DP2 and DP3 (Table 1).

L. delbrueckii cells treated with MMS to a 99% kill did not grow in any of the lactic acid selection media. The main difference between the two chemical mutagens, MMS and EMS, is that 7-methyl guanosine produced during MMS treatments is not as stable as 7-ethyl guanosine, which is produced by EMS (10).

Another factor that could affect mutant selection and survival is the growth stage of the cells to be mutated. Hince and Neale (3) and Matijasavic and Zeiger (6) reported that mutagenesis was most effective if applied to stationary-phase

cells. We observed that, when log-phase cells were treated with EMS, they would not grow in any of the selection media. Therefore, mutagenesis was routinely performed with cells in early stationary phase.

Caffeine was added to the mutagenesis MRS medium and to the 8% lactic acid selection medium. Caffeine is a mutation stabilizer which causes error-prone SOS repair of DNA (9). Caffeine also slows down an error-prone, postreplicative DNA repair mechanism without changing the mutation frequency (11). It was determined that the highest concentration of caffeine that *L. delbrueckii* could tolerate was 0.4%. Furthermore, to avoid the selection of crippled mutants, a minimal medium was used for the 8% lactic acid selection medium.

Fermentation characteristics. Mutants DP2, DP3, and DP4 were evaluated initially by standing cultures in MRS-12% glucose medium without pH control. All the mutants culture became turbid more rapidly than wild-type cultures. Figure 1 illustrates the shorter lag time and faster growth rate for one of the mutants (DP2) compared with wild type. All mutants produced more lactic acid than the wild type in 12% glucose with either MRS or 1% YMO media (Table 1). In 1% YMO medium, mutants DP2 and DP3 produced about four times more lactic acid in 24 h than the wild type, whereas mutant DP4 produced only three times more. This difference between mutant DP4 and the other mutants illustrates the importance of mutant survival in the complete selection scheme.

Stressing the mutant. Continuous cultivation of mutant DP3 in a medium containing 8% L(+)lactic acid was performed in an effort to stress further

the mutant. During this continuous fermentation mutant DP3.19 was the last survivor; it was obtained at the dilution rate of 0.024 h^{-1} on the 13th day of the fermentation. This lengthy treatment resulted in an organism less vigorous than DP3 that exhibited an extended lag phase in MRS medium.

pH-Controlled batch fermentations. In a 1% YMO with 12% glucose medium, the wild type and mutant DP3 produced 58 and 77 g/l D(-)lactic acid, respectively, in 100 h (Table 2). Performance of the wild type was similar to that detailed in a previous report for lactic acid production under similar conditions (7). The yield was about 80% for both the wild type and mutant DP3. Mutant DP3 grew about 40% faster than the wild type. All mutants tested, except DP3.19, demonstrated shorter lag times and faster growth rates than did the wild type. The maximum lactic acid production rate for mutant DP3 was more than two times faster than that of the wild type. This advantage in production rate for DP3 decreased with fermentation time and increased volumetric productivity. Mutant DP3.19 showed higher lactic acid concentration, yield, and production rate than wild type (Table 2).

In 3% YMO medium, mutant DP3 produced 117 g/l lactic acid compared with 67 g/l lactic acid by the wild type (Table 3, Figure 2). Furthermore, mutant DP3 exhibited maximum growth and production rates that were two and five times higher, respectively, than those of the wild type. These higher growth and lactic acid production rates in 3% YMO as compared to 1% YMO, indicated the importance of yeast extract for *L. delbrueckii*. This agrees with the observation of

Aeschlimann and von Stockar (1), who reported that high yeast extract concentrations resulted in increased production rate and final lactic acid concentration by *L. helveticus*. Mutant DP3.19 produced 89 g/l lactic acid with a 99% yield whereas the wild type produced 67 g/l lactic acid with a 75% yield (Table 3). However, in either 1% or 3% YMO media, the maximum growth rates of DP3.19 were three times slower than that of the wild type (Table 2 and 3). Thus, continuous stressing with a high level of lactic acid altered the physical characteristics of DP3 negatively, at least in this instance. Finally, after 22 months of storage at -22 °C, freeze-dried cultures of mutant DP3 were stable. They produced 117 g/l lactic acid at almost the same yield and production rate as the original DP3 mutant (Table 3). Although the maximum growth rate of the freeze-dried culture returned to that of the wild type, this did not affect the lactic acid production rate. On the other hand, subculturing the mutants in MRS medium resulted in a reduction in lactic acid production rates and final lactic acid concentration for all the mutants after about 6 transfers, probably because of the DNA repair mechanism in the cell during repeated unstressed growth.

CONCLUSION

To our knowledge this is the first report of a successful strain development protocol to increase lactic acid production by *Lactobacillus* sp. Stable enhanced mutants were obtained rapidly. Mutants, such as mutant DP3, could be used to reduce lactic acid production costs because of the faster production rates, and higher concentrations attained. The proposed mutagenesis and selection protocol developed might have applications to enhance lactic acid production by other lactic acid bacteria, particularly those that are not as fastidious as *L. delbrueckii*.

ACKNOWLEDGMENTS

This research was supported by the Center for Crops Utilization Research, by a scholarship to Ali Demirci from the University of Gaziantep, Turkey, by the Iowa Corn Promotion Board and by the Iowa Agriculture and Home Economics Experiment Station.

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Table 1. D(-)Lactic acid production (g/l) by wild type and mutant strains of *Lactobacillus delbrueckii* grown in standing culture at 45°C for 24 h without pH control

Strain	12% glucose-MRS medium	12% glucose-1% YMO[a] medium
Wild	34	1.8
DP2	39	9.1
DP3	41	8.1
DP4[b]	38	5.6

[a]The 1% YMO is 1% yeast extract, mineral salts, and 0.05% oleic acid medium.

[b]Mutant DP4 was transferred into 8% lactic acid selection medium two times instead of four times.

Table 2. Physiological differences between wild type *Lactobacillus delbrueckii*, and mutants DP3 and DP3.19 in pH controlled fermentation in 12% glucose-1% YMO medium at 45°C [a]

DESCRIPTION	WILD TYPE	MUTANT DP3	MUTANT DP3.19
D(-)lactic acid produced (g/l)	58	77	68
Glucose consumed (g/l)	73	95	75
Percent yield ($Y_{p/s}$)[b]	80	81	91
Max. growth rate $(dx/dt)_{max}$ (g/l/h)	0.09	0.12	0.03
Max. production rate $(dp/dt)_{max}$ (g/l/h)	0.72	1.72	1.17
Max. consumption rate $(ds/dt)_{max}$ (g/l/h)	0.96	1.99	1.29

[a]The total fermentation time was 100 h. All values are averages of two replicates. The 1% YMO is 1% yeast extract, mineral salts, and oleic acid medium.

[b]Yield is defined as the concentration of lactic acid produced divided by the amount of glucose consumed times 100.

Table 3. Physiological difference between wild type *Lactobacillus delbrueckii*, and mutant DP3 and DP3.19 in pH-controlled fermentation in 12% glucose-3% YMO medium at 45°C [a]

DESCRIPTION	WILD TYPE	MUTANT DP3		MUTANT DP3.19
		Initial Culture	Freeze Dried[b]	
D(-)lactic acid produced(g/l)	67	117	117	89
Glucose consumed (g/l)	88	152	160	89
Percent yield ($Y_{p/s}$)[c]	75	76	73	99
Max. growth rate $(dx/dt)_{max}$ (g/l/h)	0.15	0.34	0.17	0.05
Max. production rate $(dp/dt)_{max}$ (g/l/h)	1.35	6.46	5.57	2.25
Max. consumption rate $(ds/dt)_{max}$ (g/l/h)	1.86	7.82	7.16	1.70

[a]The total fermentation time was 100 h. All values are averages of two replicates. The 3% YMO is 3% yeast extract, mineral salts, and 0.05% oleic acid medium.

[b]Freeze dried DP3 which was stored at -20 °C for 22 months.

[c]Yield is defined as the concentration of lactic acid produced divided by the amount of glucose consumed times 100.

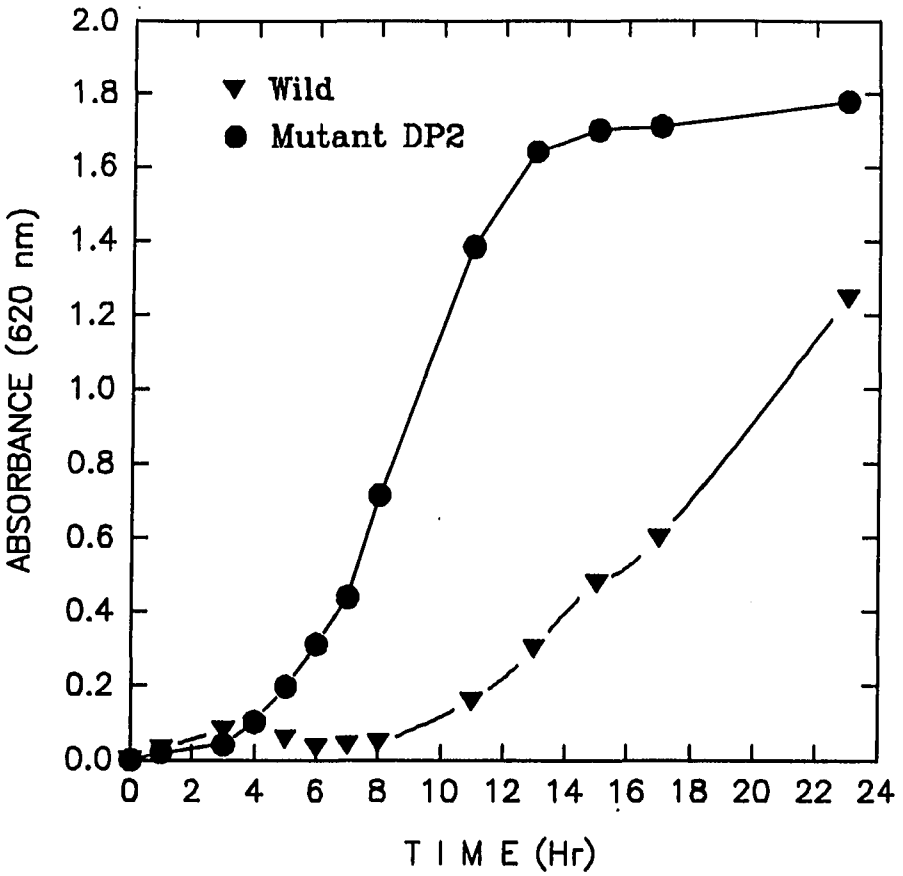

Figure 1. Growth curve of wild type *L. delbrueckii* and mutant DP2 in MRS medium stationary-culture at 45°C without pH control

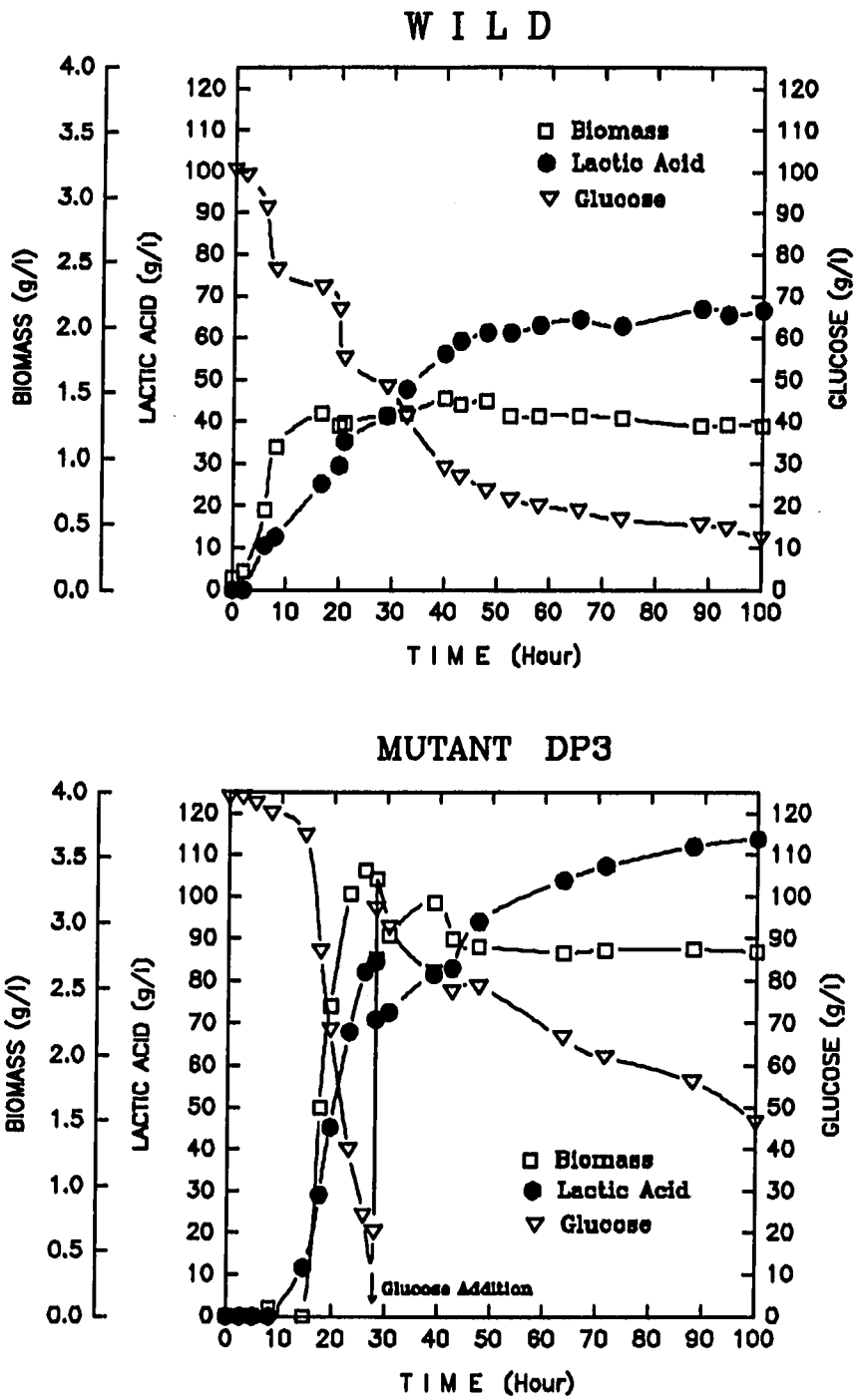

Figure 2. Wild type and mutant DP3 *L. delbrueckii*; cell mass production, D(-)lactic acid production, and glucose consumption in pH-controlled, stirred-tank fermentation in 3% YMO medium

PAPER II. BIOFILM REACTOR SOLID SUPPORT EVALUATION FOR MIXED CULTURE LACTIC ACID PRODUCTION

Biofilm Reactor Solid Support Evaluation for Mixed Culture Lactic Acid Production[1]

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1. Journal Paper No. J-14840 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Projects No. 2889 & 0178

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ABSTRACT

Combination of *Lactobacilli* and biofilm-forming bacteria were evaluated in continuous fermentations for lactic acid production using various supports. Twelve different bacteria, including species of *Bacillus, Pseudomonas, Streptomyces, Thermoactinomyces, Thermomonospora,* and *Thermonospora*, were tested for biofilm-forming capabilities. Solid supports that were evaluated in either batch or continuous fermentations were pea gravels, porcelain berl saddles, 3M-macrolite ceramic spheres, and polypropylene mixed with 25% various agricultural materials (e.g. corn starch, oat hulls) and extruded to form chips (pp-composite). Biofilm formation was evaluated by the extent of clumping of solid supports, weight gain and (in some instances) gram stains of the supports after drying overnight at 70°C. The supports consistently producing the best biofilm were pp-composite chips followed by 3M-Macrolite spheres then by pea gravels. The best biofilm formation was observed with *Pseudomonas fragi* (ATCC 4973), *Streptomyces viridosporus* T7A (ATCC 39115), and *Thermoactinomyces vulgaris* (NRRL B-5790), grow at 25, 37, and 45°C, respectively, on various pp-composite chips. Lactic acid bacteria used in the fermentations were *Lactobacillus amylophilus* (NRRL B-4437), *Lactobacillus casei* (ATCC 11443), and *Lactobacillus delbrueckii* mutant DP3; these grow optimally at 25, 37, and 45°C, respectively. Lactic acid and biofilm bacteria with compatible temperature optima were inoculated into 50-ml reactors containing sterile pp-composite supports. Lactic

acid production and glucose consumption were determined by HPLC at various flow rates from 0.06 to 1.92 ml/min. Generally, mixed-culture biofilm reactors produced higher levels of lactic acid than lactic acid bacteria alone. *S. viridosporus* T7A and *L. casei* on pp-composite chips were the best combination of those tested, and produced 13.0 g/l lactic acid in the reactors without pH control. *L. casei* produced 10.3 g/l lactic acid under similar conditions.

INTRODUCTION

Lactic acid is an organic acid ($C_3H_6O_3$) that can be produced both biologically and chemically (7). In 1985, 50 million lb of lactic acid were produced in the U.S. (9). Lactic acid is used in the food industry as an acidulant and preservative. Lactic acid and its derivatives have potential industrial uses in the nonfood industry (7). Polyesters of lactic acid form a plastic with good tensile strength, thermoplasticity, fabricability, and biodegradability (8). To produce such polymers, extremely pure L- or D-lactic acid is required. Polylactics are currently used for resorbable sutures, prosthetic devices, and slow-release carriers of herbicides and pesticides. Lactic acid can also be used as a feedstock for the chemical and biological production of other organic acids, such as propionic, acrylic, acetic acids, propylene glycol, ethanol, and acetaldehyde.

To meet a growing need for more lactic acid and make the price of lactic acid more attractive, the efficiency of microbial fermentation and recovery must be improved. Methods used to increase cell density and rate of lactic acid production include hollow-fiber reactors (12), cell-recycle reactors (10), and immobilized-cell reactors (2). In immobilized-cell reactors, entrapment is commonly achieved with calcium alginate or κ-carrageen. Stenrous et al. (13) reported that lactic acid production rates which are growth associated, are low in immobilized reactors because entrapped cells grow slowly if at all. Also, long-term use of immobilized cells results in gel bead swelling and rupture (5).

Biofilm is a natural form of cell immobilization that results from microbial attachment to solid supports (3). In nature, mixed-culture biofilms are commonly found on the surfaces of underwater rocks, buried metal surfaces and on decaying organic matter (3). Aktinson and Davies (1) reported that, in continuous stirred tank fermentors, biofilms maximized substrate utilization at high flow rates and minimized loss of microbial. Biofilm reactors have found application in waste treatment plants, production of vinegar by the 'quick vinegar' process (4), and alcohol production (14). In this paper, we evaluate various solid supports, and biofilm-forming bacteria and *Lactobacillus* species for lactic acid production in pure- and mixed-culture systems.

MATERIALS AND METHODS

Microorganisms. Bacteria were obtained from either the American Type Culture Collection (ATCC) (Rockville, MD) or the Northern Regional Research Laboratory (NRRL) (Peoria, IL) (Table 1). *Lactobacillus delbrueckii* DP3 is a mutant developed in our laboratory (6).

Solid supports. The supports evaluated were porcelain berl saddles (Aldrich Chemical Company, Milwaukee, WI), local pea gravels, 3M-macrolite ceramic spheres (Aluminum oxide) (Industrial Mineral Products Division/3M, St. Paul, MN), and polypropylene (pp) composite chips containing 25% (w/w) of agricultural materials listed in Table 2. To stimulate biofilm development on the porcelain berl saddles, they were subjected to pretreatment with acid or base (2 N H_2SO_4, 2 N HCl, or 2 N NaOH) for 48 h at 25°C. The pp-composite chips were prepared by high-temperature extrusion of the polypropylene (Quantum USI Division, Rolling Meadows, IL) and various agricultural materials in a Brabender PL2000 twin-screw extruder (C. W. Brabender Instruments, Inc., South Hackensack, NJ). The barrel temperatures were 200, 210, and 220°C, die temperature was 220°C, and the screw speed was 20 rpm. The agricultural products used (Table 2) were: carboxy methyl cellulose (Sigma Chemical Co., St Louis, MO), cellulose (Sigma Chemical Co.), ground (20 mesh) oat hulls (National Oats Co., Cedar Rapids, IA), ground (20 mesh) soybean hulls (ISU Center for Crops Utilization Research, Ames, IA), soybean flour (Archer Daniels Midland

Company, Decatur, IL), zein (Sigma Chemical Co.), and xylan (Sigma Chemical Co.). Each material was vacuum dried for 48 h at 110°C. Polypropylene chips were compounded with different levels and blends of agricultural materials extruded as a 3 mm diameter rod, air cooled, then pelletized into a 2-3 mm long chips. All chips compounded with protein were difficult to produce and chared by the high temperatures employed.

Biofilm formation in batch reactors. Solid supports were weighed, placed in 20 x 150 mm test tubes fitted with bubbler tube units (11) containing 10 ml of culture medium as indicated in Table 1, was added to each tube. The culture apparatus was sterilized at 121°C for 15 min, and aseptically inoculated with 0.1 ml of 24-h culture. Incubation was at the optimum temperature of the bacteria (Table 1) with continuous aeration using CO_2-free air. Corresponding control tubes were prepared and incubated in the same manner but were not inoculated. After one week incubation, the culture medium was drained and the solid supports were rinsed with deionized water, placed into a preweighed 250-ml flask, and dried at 70°C overnight. After cooling in a desiccator, the flasks were reweighed. Before and after drying, the supports were visually evaluated for cell mass accumulation and clumping of the supports.

Biofilm formation in continuous reactors. Fifty-milliliters of solid supports were weighed, and placed in a 50-ml plastic syringe fitted with a silicone stopper. A 10-liter carboy containing 4 liters of culture medium (Table 1) was connected to a T-shaped tubing connector (Figure 1). One arm of the T was

connected by silicon tubing to a syringe at the hypodermic needle port, and the second arm to an air line containing a cotton plug to supply filter-sterilized, CO_2-free air. The barrel-mouth of the syringe was fitted with a silicon stopper that was penetrated by two glass connecting tubes. One tube was covered with a septum for inoculation. The other tube was used as a medium exit line. The complete system was sterilized in an autoclave at 121°C for 1 h. After cooling, the reactors were placed in water baths set at appropriate temperatures (Table 1). Culture medium was pumped into the reactor to fill it, and the reactor was inoculated with a 24-h culture. The reactors were incubated as batch cultures for 24 h with continuous aeration. Culture medium was then continuously pumped into the reactor (working volume 20-25 ml) at 0.06 ml/min flow rate for 6 weeks. The supports were evaluated as before, and by gram staining of pp-composite chips only. For gram-staining, small portions of the pp-composite chips were taken before and after the fermentation, then placed in a test tube and gram-stained by submersion. The chips were washed with alcohol and water until all excess color was removed, dried at 70°C overnight, and then visually evaluated for increased blue color.

Continuous lactic acid fermentation. The biofilm-forming bacteria selected for mixed-culture fermentations were *Pseudomonas fragi* (ATCC 4973), *Streptomyces viridosporus* T7A (ATCC 39115), and *Thermoactinomyces vulgaris* (NRRL B-5790), which grow optimally at 25, 37 and 45°C, respectively. For both pure- and mixed-culture lactic acid fermentations, the lactic acid bacteria used

were *Lactobacillus amylophilus* (NRRL B-4437), *Lactobacillus casei* (ATCC 11443), and *Lactobacillus delbrueckii* mutant DP3 (6), which have matching optimum temperatures with the biofilm formers, respectively. Mixed- and pure-culture fermentations were performed on various pp-composite chips described in Table 3. In mixed-culture fermentations, biofilm formers were used to "capture" the lactic acid bacteria which do not form biofilms. Therefore, biofilm-forming bacteria were initially grown in the continuous reactor with a flow rate of 0.06 ml/min for 15 days. Then heat-sterilized MRS *Lactobacillus* broth was added, and specific lactic acid bacteria were inoculated. In pure-culture fermentations, lactic acid bacteria were inoculated without a biofilm former. Medium was pumped at various flow rates (0.06, 0.12, 0.24, 0.48, 0.96, 1.92 ml/min). Each was held constant for 24 h. Samples were taken every 4 or 5 h. The pH, optical density (620 nm), % lactic acid, and % glucose in the effluents were analyzed by using a pH-meter, Spectronic 20 spectrophotometer (Milton Roy Co, Rochester, NY), and a Water's high performance liquid chromatograph (HPLC) (Milford, MA), equipped with Water's Model 401 refractive index detector, respectively. The HPLC separation of lactic acid, glucose and other broth constituents was achieved on a Bio-Rad Aminex HPX-87H column (300 X 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using a 20-µl injection loop and 0.012 N H_2SO_4 as a mobile phase at a flow rate of 0.8 ml/min.

RESULTS AND DISCUSSION

Porcelain berl saddles in batch fermentations. No biofilm formation was observed on untreated saddles for any bacteria. Therefore, pretreatments were used on these supports to stimulate biofilm formation. Saddles were immersed in 2 N H_2SO_4, HCl, or NaOH, washed in water before being sterilized in autoclave in various culture media or water. Compared with the water control, a slight increase in weight by soluble-solid binding was observed for only the treated saddles with 2 N HCl. However, biofilm formation was not affected by this pretreatment. Calcium ion addition to the medium also had no effect on biofilm formation.

Pea gravels and 3M-macrolite spheres in batch fermentations. Some bacteria formed biofilms on both pea gravels and 3M-macrolite spheres (Table 4). *Pseudomonas fragi* and *Pseudomonas amyloderamosa* formed films on pea gravels and macrolite spheres, respectively. The three filamentous bacteria on pea gravels and six on macrolite spheres formed detectable films. No detectable weight gain was observed on either support. Pea gravels are a mixture of different stones making their continued use unpredictable. The 3M-macrolite spheres were unpredictable, and the 3M-spheres floated plugged the exit tubes.

Polypropylene composite chips in continuous fermentations. Some weight loss occurred for all the pp-composite chips evaluated for biofilm formation with *Streptomyces viridosporus* T7A, *Thermoactinomyces vulgaris,* and *Pseudomonas fragi.* This would be expected because of biodegradation or leaching of the agricultural materials from the chips. Clumping of the pp-composite chips was observed during the fermentation and after the chips were harvested, washed, and dried (Table 5). Gram stains of the chips also confirmed that biofilms were formed because significant color differences existed between zero-time chips and those after incubation with culture. The best biofilm formers were *Pseudomonas fragi, Streptomyces viridosporus* T7A, and *Thermoactinomyces vulgaris* on pp-composite chips (Table 3).

Optimization of biofilm formation on selected supports. The time required for biofilm formation on the various pp-composite chips was determined for the three best bacteria by harvesting after 4, 8, and 15 days of continuous fermentation (Table 3). Substantial biofilms were formed by all three bacteria after 15 days of incubation.

Mixed- and pure-culture lactic acid fermentations. *P. fragi, S. viridosporus* T7A, and *T. vulgaris* had temperature optima of 25, 37, and 45°C, respectively, permitting evaluation of lactic acid bacteria with similar temperature optima in mixed-culture fermentations. Fermentations were evaluated in bioreactors containing selected pp-composite chips with mixed-cultures (biofilm formers and lactic acid bacteria) and pure-culture (lactic acid bacteria alone).

i. *P. fragi* and *L. amylophilus* (25°C). The mixed-culture and the pure-culture (*L. amylophilus* alone) fermentations were similar in lactic acid production when pp-composite chips containing oat hulls-zein and oat hulls-soy flour were used (Figure 2). The best lactic acid productions were observed at flow rates of 0.06 to 0.48 ml/min; 2.28 to 3.97 (g/l) lactic acid was produced by the pure-culture on oat hull-zein and 2.92 to 4.82 (g/l) lactic acid was produced by the mixed-culture on oat hull-soy flour. Stable lactic acid production was observed over a broader dilution rate for the mixed culture than the pure-culture (Figure 2). Figure 3 shows the percentage yields at various dilution rates which were defined as percent lactic acid produced (g/l) per consumed glucose (g/l). At the highest lactic acid concentrations, the percentage yields were consistently lower for the mixed-culture than for the pure-culture fermentations. For example, at the same flow rate (0.48 ml/min), the effluent lactic acid concentration was about the same for pure- and mixed-culture fermentations (3.97 g/l on oat hulls-soy flour and 3.59 g/l on oat hulls-zein, respectively), whereas percentage yields were significantly different at 100% and 81%, respectively. Approximately twice the level of cell mass in the effluent was observed for mixed-culture fermentation on oat hulls-zein compared to oat hulls-soy flour. The former produced the highest lactic acid concentration.

ii. *S. viridosporus* T7A and *L. casei* (37°C). pp-Composite chips made with both soy hulls-zein and soy hulls-soy flour pp-composite chips produced

consistently high levels of lactic acid in mixed- and pure- (*L. casei* alone) culture fermentations (Figure 2). At almost every flow rate, lactic acid production in mixed-culture fermentation was higher than in pure-culture. This slightly better performance for mixed-culture fermentations could be the result of better *Lactobacillus* immobilization. For these supports, yields were good for both mixed- and pure-culture fermentation. Both resulted in 100% yields at the faster flow rates (Figure 3), maximum lactic acid concentrations of 13.0 g/l for the mixed-culture on soy hulls-zein and 10.2 g/l for *L. casei* alone on soy hulls-soy flour. Both pure- and mixed-culture fermentations produced a constant or increasing level of lactic acid until 0.48 ml/min.

iii. *T. vulgaris* and *L. delbrueckii* DP3 (45°C). Mixed- and pure- (*L. delbrueckii* alone) culture fermentations demonstrated that no preferences existed for different pp-composite supports (Figure 2). All three pp-composite chips exhibited almost the same concentrations of lactic acid (2-5 g/l) in the mixed-culture fermentations which were higher than those in the pure-culture fermentation (2-3 g/l) at the slower flow rates. Furthermore, cell mass and lactic acid production in the effluent were stable for the pure-culture on oat hull supports where lactic acid levels were 2-3 g/l. Lactic acid production by the pure-culture on oat hulls and the mixed-culture on zein pp-composite chips were stable over the different flow rates, thereby, increasing the overall volumetric production (g/l/h). Yields were distinctly different; pure-culture generally demonstrated the best yields on zein supports (Figure 3).

The lower levels of lactic acid production by *L. amylophilus* and *L. delbrueckii* DP3 probably were due to their sensitivity to reduced pH. Neither will grow at pH values below 6, whereas *L. casei,* which produced the highest level of lactic acid, is less sensitive and grows at pH > 4.5. With a pH-controlled bioreactor *L. amylophilus* or *L. delbrueckii* DP3 would probably produce higher levels of lactic acid. However, both *L. amylophilus* and *L. delbrueckii* DP3 produced significantly higher levels of lactic acid in mixed-culture fermentation than in pure-culture (Figure 2). This suggests that mixed-culture biofilms provided some type of pH protection or stabilization for these fastidious bacteria. These results should not be compared with the data in the literature for continuous lactic acid production because the pH was not controlled and overall performance of the reactors would be expected to be low.

CONCLUSION

The extent biofilm formation on various solid supports was difficult to measure, but gram staining and clumping of the supports were adequate indicators of biofilm formation. *L. casei* and *S. viridosporus* T7A combination performed the best in a mixed-culture system. Continuous lactic acid fermentation can be enhanced by using these biofilm reactors containing supports of pp-composite chips blended with various agricultural materials. For future research, pH-controlled fermentations should be performed. The proper combination of solid support and biofilm-forming bacteria is critical for this type of immobilized-cell fermentation. Polypropylene chips have several advantages over other solid supports because of their light weight, but with adequate density, and ability to be blended with almost any kind of material. Their placticity permits the production of various shapes. Finally, the ability of mixed-culture bioreactors to enhance the production of a desired product was demonstrated.

ACKNOWLEDGMENTS

This research was supported by the ISU Center for Crops Utilization Research, by a scholarship to Ali Demirci from University of Gaziantep, Turkey, by the Iowa Corn Promotion Board, and by the Iowa Agriculture and Home Economics Experiment Station. We also acknowledge Dr. John Strohl technical assistant for HPLC analysis.

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Table 1. The potential biofilm-forming and lactic acid bacteria, media and incubation temperatures used in the study.

Microorganism	Medium[a]	Incubation temperature (°C)
Biofilm formers:		
Bacillus licheniformis (NRRL NRS 1264)	SSB	30
Bacillus stearothermophilus (NRRL B 1172)	SSB	45
Pseudomonas amyloderamosa (ATCC 21262)	3	30
Pseudomonas fragi (ATCC 4973)	3	25
Pseudomonas thermocarboxydovorans (ATCC 35961)	1492	45
Streptomyces badius 252 (ATCC 39117)	196	37
Streptomyces setonii 75Vi2 (ATCC 39116)	19	37
Streptomyces viridosporus T7A (ATCC 39115)	196	37
Thermomonospora curvata (ATCC 19995)	1489	45
Thermomonospora fusca (ATCC 27730)	74	45
Thermoactinomyces vulgaris (ATCC 43649)	18	45
Lactic Acid Bacteria:		
Lactobacillus amylophilus (NRRL B 4437)	MRS	25
Lactobacillus casei (ATCC 11443)	MRS	37
Lactobacillus delbrueckii DP3	MRS	45

[a]Medium composition:
SSB: 10 g/l Soluble Starch, 5 g/l Difco Yeast Extract in nitrogen-free salt solution
MRS: 55 g/l Lactobacillus MRS Broth
Medium 3: 3 g/l Beef Extract, 5 g/l Peptone
Medium 18: 30 g/l Trypticase Soy Broth
Medium 196: 6 g/l Yeast Extract in nitrogen-free salt solution
Medium 741: 3 g/l Tryptone, 3 g/l Difco Yeast Extract, 3 g/l Glucose, 1 g/l K_2HPO_4
Medium 1489: 10 g/l Dextrin, 2 g/l Tryptone, 1 g/l Beef Extract, 1 g/l Difco Yeast Extract, 2 mg/l $CoCl_2$
Medium 1492: 2 g/l pyruvate and mineral solution.
All numbered media are from the American Type Culture Collection Catalogue of Bacteria and Bacteriophages, 17th ed. (1989)

Table 2. Polypropylene composite support formulation[a]

pp-Composite chip	Agricultural Product (%)	Minor Agricultural Product (5%)
Polypropylene	NA[b]	NA
Carboxy Methyl Cellulose	25	NA
Cellulose	25	NA
Cellulose-Soy Flour	20	Soy Flour
Cellulose-Zein	20	Zein
Oat Hulls	25	NA
Oat Hulls-Soy Flour	20	Soy Flour
Oat Hulls-Zein	20	Zein
Soy Flour	25	NA
Soy Hulls	25	NA
Soy Hulls-Soy Flour	20	Soy Flour
Soy Hulls-Zein	20	Zein
Zein	25	NA
Xylan	25	NA

[a]pp-Composite chips consisted of 75% of polypropylene (w/w)
[b]NA is for not added

Table 3. Select biofilm forming bacteria, the best pp-composite support, and the optimum incubation time for biofilm formation

Biofilm Forming Bacteria (Medium and incubation temperature)	Best pp-Composite Support	Biofilm Formation[a]		
		4 days	8 days	15 days
Pseudomonas fragi	20% oat hulls plus 5% zein	-	-	++
(0.8% nutrient broth at 25°C)	20% oat hulls plus 5% soy flour	-	-	++
Streptomyces viridosporus T7A	20% soy hulls plus 5% zein	+	++	+++
(0.6% yeast extract broth at 37°C)	20% soy hulls plus 5% soy flour	+	++	++
Thermoactinomyces vulgaris	25% oat hulls	+	++	++
(3% Trypticase Soy Broth at 45°C)	20% oat hulls plus 5% zein	+	+	++
	25% zein	+	+	+++

[a]Values are based on visual clumping of chips. Minus (-) means no detectable biofilm, pluses (+) mean the strength of biofilm observed

Table 4. Biofilm formation in batch fermentation on different solid supports in batch fermentation[a]

Microorganisms	Pea Gravels	3M-Macrolite Spheres
B. licheniformis	nd	-
B. stearothermophilus	nd	-
L. amylophilus	nd	-
P. amyloderamosa	nd	+
P. fragi	+	nd
P. thermocarboxydovorans	-	-
S. badius 252	-	+
S. setonii 75Vi2	+	+
S. viridosporus T7A	+	+
T. curvata	-	+
T. fusca	+	+
T. vulgaris	-	+

[a]Minus (-) means no biofilm, plus (+) means biofilm present as determined by clumping characteristics, and nd means not determined

Table 5. Biofilm formation on pp-composite chips as determined by clumping after a 6-week continuous fermentation[a]

PP-Composite Chips	Biofilm Formation		
	P. fragi	*S. viridosporus* T7A	*T. vulgaris*
Carboxyl Methyl Cellulose	nd	-	nd
Cellulose	-	+	+
Cellulose-Soy Flour	++	++	+++
Cellulose-Zein	+	+	+++
Oat Hulls	+	+++	+++
Oat Hulls-Soy Flour	++	+++	+
Oat Hulls-Zein	++	+	+++
Soy Flour	nd	nd	-
Soy Hulls	-	++	++
Soy Hulls-Soy Flour	+	+++	+
Soy Hulls-Zein	+	++	+
Zein	+	+	+++
Xylan	nd	-	nd

[a]Strength of clumping (+), no biofilm formation (-), not determined (nd)

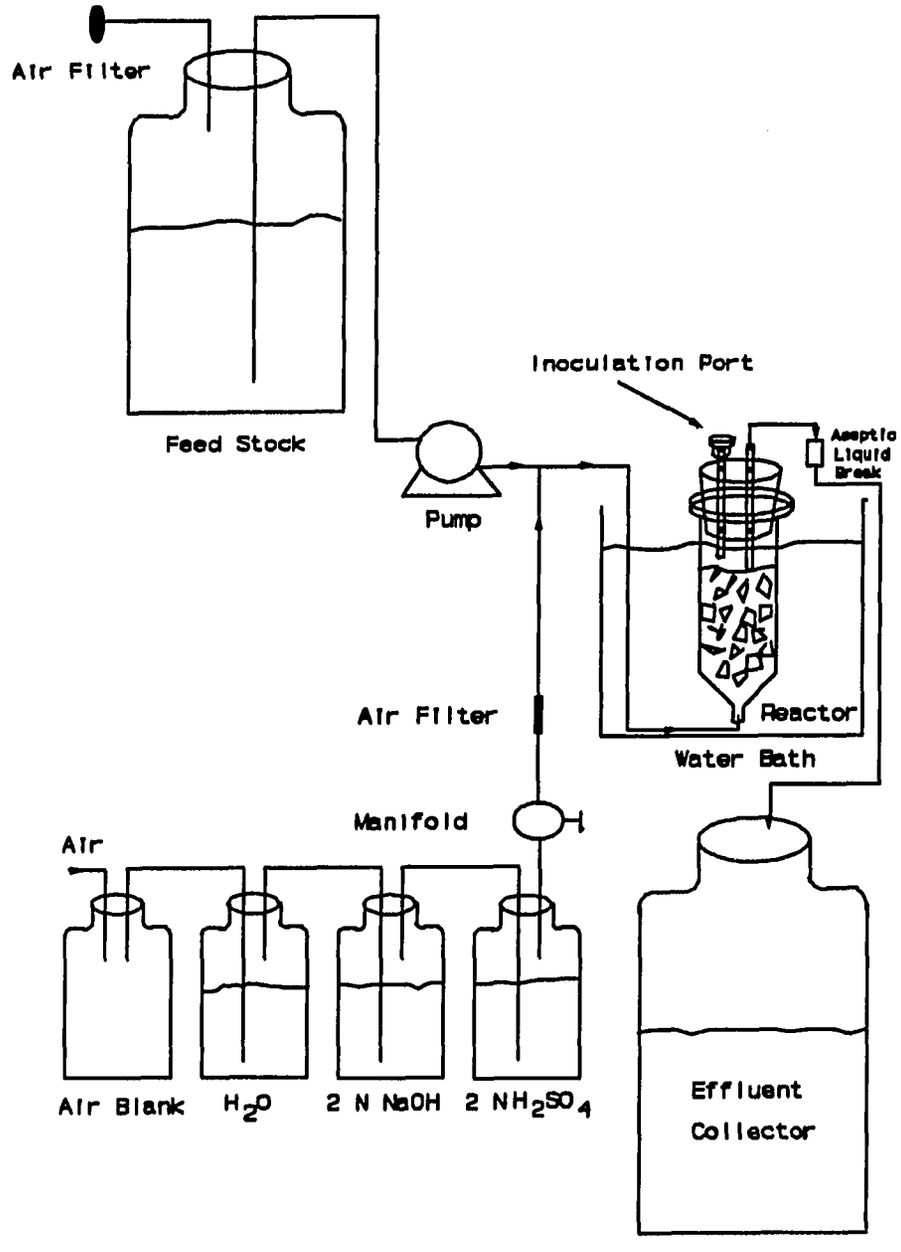

Figure 1. Schematic diagram of the biofilm reactor

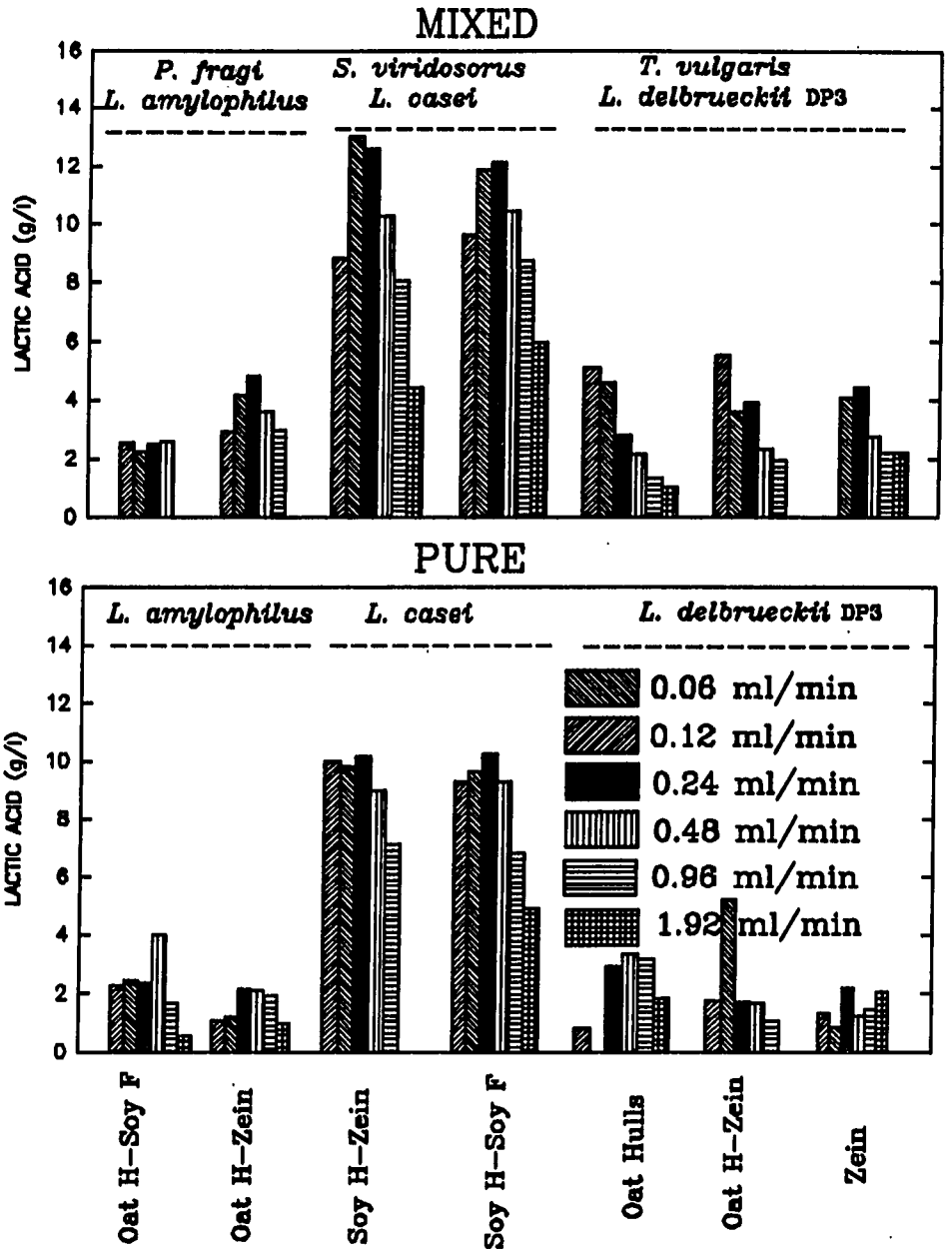

Figure 2. Lactic acid production in mixed- and pure-culture fermentations on various pp-composite chips

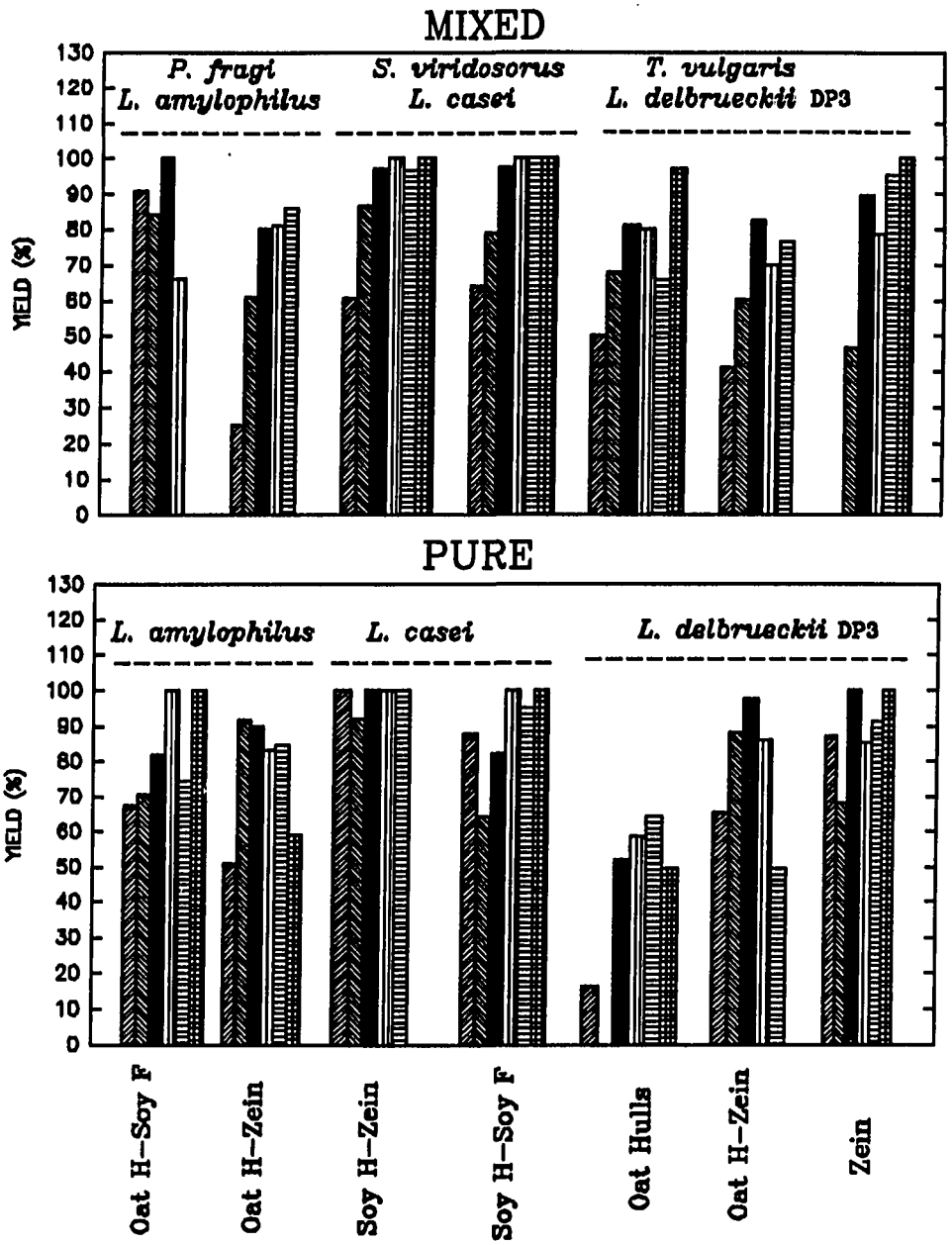

Figure 3. Percent yields in mixed- and pure-culture fermentations on various pp-composite chips (defined as percent produced lactic acid g/l per consumed glucose g/l)

PAPER III. LACTIC ACID PRODUCTION IN A MIXED CULTURE BIOFILM REACTOR

Lactic Acid Production in a Mixed Culture Biofilm Reactor[1]

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1. Journal Paper No. J-14839 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Projects No. 2889 & 0178

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ABSTRACT

Novel solid supports, consisting of polypropylene (pp-composite) blended with various agricultural materials, were evaluated as supports for pure- and mixed-culture continuous lactic acid fermentations in biofilm reactors. *Streptomyces viridosporus* T7A (ATCC 39115) was used to form a biofilm and *Lactobacillus casei* (ATCC 11443) was used for lactic acid production. For mixed-culture fermentations, a 15-day continuous fermentation of *S. viridosporus* was performed initially to establish the biofilm. Culture medium was then inoculated with *L. casei*. For pure-culture fermentation, *L. casei* was inoculated directly into the reactors containing sterile pp-composite chips. The biofilm reactors containing various pp-composite chips were compared to a biofilm reactor containing pure polypropylene chips and with a suspension culture. Continuous fermentation was started and each flow rate (0.06 - 1.92 ml/min) was held constant for 24 h; steady state was achieved after 10 h. Lactic acid production was determined throughout the 24-h period by high-performance liquid chromatography. Production rates that were two to five times faster than those of the suspension culture (control) were observed for the pure- and mixed-culture bioreactors. Both lactic acid production rates and lactic acid concentrations in the culture medium was consistently higher in mixed-culture than in pure-culture fermentations. Biofilm formation on the chips was detected at harvest by chip clumping and gram-

staining. To our knowledge this is the first report of this type of pure- and mixed-culture lactic acid fermentation.

INTRODUCTION

Lactic acid and its derivatives have found many applications in the food and nonfood industries (11). In the nonfood area, polyesters of lactic acid can be made into degradable plastics with good tensile strength, thermo-plasticity, fabricability, and biodegrability (14). Such plastics have an estimated potential yearly market of 9 billion pounds. Lactic acid can also be used as feedstock for the chemical and biological production of other organic acids such as propionic, acrylic, acetic acids, propylene glycol, ethanol, and acetaldehyde (14). In 1985, only 50% of the annual world-wide lactic acid production (24 to 28 x 10^6 kg) (20) was produced from renewable sources by fermentation. The rest was obtained from petroleum. The cost of lactic acid is currently $1.03/lb (15). If the price of lactic acid could be reduced, the market should be expanded substantially. Some methods that can be used to reduce the cost of production are microbial strain development, the design of novel bioreactors and improved recovery processes.

Hollow-fiber, cell-recycled, artificially immobilized-cell, and biofilm reactors maintain high cell density. Such reactors can generate increased volumetric productivity rates (g/l/h). However, the use of hollow-fiber and cell-recycled fermenters is limited by high start-up cost and membrane fouling during the fermentation (17, 15). In reactors with artificially immobilized-cells, production rates and yields are low because of limited diffusion rates, cell leakage, and poor cell reproduction in the beads (18). In biofilm reactors, microorganisms are

immobilized by a natural attachment to solids while continuously growing (21). Some industrial applications of biofilm reactors include biological oxidation or reduction of industrial wastes (13), the "Quick" vinegar production (8), animal tissue culture (19), and ore treatments (8). Reviews of biofilms have been published (1, 2, 4-7).

In this paper, the use of polypropylene chips blended with various agricultural materials as supports for biofilms was evaluated in a continuous lactic acid fermentations using both pure- and mixed-culture. Biofilm formation on the chips was evaluated by the clumping of the chips and gram-stains. The biofilm and lactic acid producers were *Streptomyces viridosporus* T7A, and *Lactobacillus casei*, respectively. Three- to five-fold increases in lactic acid production were observed in selected biofilm reactors, compared with results of suspension culture fermentations.

MATERIAL AND METHODS

Microorganisms and media. A biofilm producer, *Streptomyces viridosporus* T7A (ATCC 39115) was maintained on agar slants at 4°C for 3 to 6 weeks (16). The lactic acid bacterium, *Lactobacillus casei* (ATCC 11443), was maintained in Lactobacillus MRS broth (Difco Laboratories, Detroit, MI) at 4°C and subcultured every 4 weeks. For continuous fermentations, a 0.6% yeast extract medium (Difco) (pH 7), and Lactobacillus MRS Broth (Difco) (pH 6.5), were used for *S. viridosporus* T7A and *L. casei*, respectively.

Solid supports. Polypropylene (pp) composite chips containing 25% (w/w) agricultural materials were used as solid supports (Table 1). The pp-composite chips were prepared by high-temperature extrusion of the polypropylene (Quantum USI Division, Rolling Meadows, IL) and agricultural materials (Table 1) in a Brabender PL2000 twin-screw extruder (C. W. Brabender Instruments, Inc., South Hackensack, NJ). The barrel temperatures were 200, 210, and 220°C, the die temperature was 220°C, and the screw speed was 20 rpm. The agricultural products used were cellulose (Sigma Chemical Co., St. Louis, MO), ground (20 mesh) corn fiber (Penford Products Co., Cedar Rapids, IA), corn starch (American Amize-Products Co., Chicago, IL), ground (20 mesh) oat hulls (National Oats Co., Cedar Rapids, IA), soybean flour (Archer Daniels Midland Company, Decatur, IL) , ground (20 mesh) soy hulls (ISU Center for Crops Utilization Research, Ames, IA), and zein (Sigma Chemical Co.). Each agricultural material was vacuum dried for

48 h at 110 °C prior to use. Polypropylene was compounded with different levels and blends of agricultural materials, and extruded as 3 mm diameter rods, air cooled, then cut into 2-3 mm chips in length. All chips compounded with protein were difficult to produce and chared by the high temperatures employed.

Biofilm evaluations. Biofilm formed on the solid supports was evaluated by weight change, by clumping of the supports after drying at 70°C overnight, and by gram staining of the pp-composite chips at the end of continuous fermentations. Gram stains developed on stained pp-composite chips after fermentations were compared with those of uninoculated supports. For gram-staining, chips were stained by submersion, washed with alcohol and water until excess color was removed, dried at 70°C overnight, and evaluated visually for blue color.

Continuous lactic acid fermentation. Fifty-milliliters of pp-composite chips were weighed, and placed in a 50-ml plastic syringe fitted with a silicone stopper. A 10-liter carboy containing 4 liters of specific medium for feeding was connected to a T-shaped tubing connector (Figure 1). One arm of the T was connected by silicon tubing to the syringe at its hypodermic needle port. The second arm of the T connected to an air line fitted with a cotton plug to supply filter-sterilized CO_2-free air. The barrel-mouth of the syringe was fitted with a silicon stopper that was penetrated by two glass connecting tubes. One tube was covered with a septum for bioreactor inoculation. The other tube was used as a medium exit line. The complete system was sterilized in an autoclave at 121°C for 1 h. After cooling, the 50-ml reactors were placed in a water bath at 37°C. For

mixed-culture fermentations, 0.6% yeast extract medium was pumped through the reactors containing sterile dry pp-composite chips. Addition of 1 ml of inoculum from a 24-h *S. viridosporus* suspension culture was followed by 24 h batch and 15 days of continuous fermentation with a flow rate of 0.06 ml/min at 37°C for biofilm formation. The medium was then changed to heat-sterilized MRS *Lactobacillus* broth, and each reactor was aseptically inoculated with 1 ml of a 24-h *L. casei* culture, and incubated as a batch culture for 24 h at 37°C before continuous fermentation. For pure-culture fermentation, lactic acid bacteria only were inoculated into the reactors containing pp-composite chips. A 25-ml suspension culture reactor was used as the control.. Medium was pumped at various rates (0.06, 0.12, 0.24, 0.48, 0.96, 1.92 ml/min). Each flow rate was held constant for 24 h. The pH, optical density (620 nm), % lactic acid, and % glucose in the effluents were analyzed every 4 or 5 h by using a pH meter, Spectronic 20 spectrophotometer (Milton Roy Co, Rochester, NY), and a Water's high performance liquid chromatograph (HPLC) (Milford, MA), equipped with Water's Model 401 refractive index detector, respectively. The HPLC separation of lactic acid, glucose and other broth constituents was achieved on a Bio-Rad Aminex HPX-87H column (300 X 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using a 20-µl injection loop and 0.012 $\underline{N}$ H_2SO_4 as a mobile phase at a flow rate of 0.8 ml/min.

RESULTS AND DISCUSSION

Percent yield. The percent yield (%) is a measure of the efficiency of bioconversion of glucose to lactic acid (Table 2), and is defined as produced lactic acid (g/l) divided by consumed glucose (g/l). In these continuous fermentations, yields ranged from 44% (cellulose-zein, mixed-culture) to 100%. For both pure- and mixed-culture fermentations, % yield patterns were irregular with usually the higher values correlating with the faster flow rates. Generally, % yields were lower for mixed-cultures compared to the pure-cultures at the same flow rate. Biofilm former, *S. viridosporus* T7A, evidently consumed more glucose at slower flow rates of fermentation, and this consumption apparently decreased at faster flow rates, resulting in the higher yields.

Productivity rates. The productivity rate (g/h) is a measure of lactic acid production per hour (Table 2). Productivity rates for several pure- and mixed-culture fermentations on pp-composite supports were 2 to 3 times higher when compared with the suspension culture reactor (control) at the same flow rates. The lower flow rates (0.06 and 0.12 ml/min) had productivity rates very close to those of the suspension culture, whereas the higher flow rates (0.24 to 1.92 ml/min) generally had significantly higher productivity rates for both the pure- and mixed-culture fermentations. With an estimated working volume of 25 ml, the highest volumetric productivities for all chips were about 30 g/l/h which agrees

with a previous study involving *Lactobacillus helveticus* cells immobilized in calcium alginate (3).

Lactic acid production. Lactic acid concentrations were analyzed every 4 or 5 h for 24 h to determine the steady state values. After 10 h of continuous fermentation, lactic acid concentrations were steady. Therefore, the lactic acid concentration measured at 24 h of each flow rate represented lactic acid concentration at steady state for 14 h for each flow rate. Lactic acid levels were consistently higher for pp-composite supports for both pure- and mixed-culture fermentations compared with both cell suspension culture and pure polypropylene, particularly at the three fastest flow rates (Figure 2). Furthermore, mixed-culture fermentations using almost every support produced substantially higher levels of product compared with the corresponding pure-culture fermentation. These data illustrate the benefits of mixed-culture biofilm reactors for enhanced lactic acid production. Moreover, these results suggest that a higher cell density was present on each support for both pure- and mixed-culture fermentations, generating a net increase in lactic acid production.

Cell immobilization. Higher production rates in immobilized cell cultures are the result of higher cell densities in the bioreactors (9). Compared with the starting pp-composite chips, gram-staining of each chip after fermentation demonstrated an increased gram-positive color density and corresponding increase in clumping of the chips together (Figure 3). These data along with significant increases in lactic acid production, when chips were used, strongly suggest that

higher cell densities existed in each of the different pp-composite support bioreactors. Furthermore, the benefit of the mixed-culture fermentation on cell immobilization is dramatically illustrated by the comparison of the pure- and mixed-culture fermentation on pure polypropylene chips (Figure 2). Finally, cell concentration, as determined by optical density in each reactor's effluent, indicated that of suspended cells were continuously present, in addition to cells contained in the biofilm. Both *Streptomyces* and *Lactobacillus* cells were observed in the mixed-culture effluent *via* microscopic examination. However, all pp-composite chips at harvest had lost weight, probably because of agricultural material being leached or biodegraded.

Soy flour or zein addition to the chips improved the retention of *L. casei* in the pure-culture reactors for cellulose and oat hulls (Table 2, Figure 2). The addition of soy flour to pp-composite chips generally enhanced lactic acid production for the mixed-culture fermentations. The composition of the chips seemed to play an important role in bioreactor performance, indicating a need to screen different microorganisms on various pp-composite supports. Some of the added agricultural materials might be acting as a carbon and energy source. However, the data suggest that certain agricultural materials have an affinity for specific bacteria, resulting in formation of the desired biofilm. Finally, experiments involving several of the pp-composite bioreactors were repeated and every time very similar results were obtained.

Criteria for pp-composite support selection. High lactic acid concentrations, productions rates, and yields in the effluent, particularly at the faster flow rates, were the criteria used to choose the best pp-composite for pure- and mixed-culture reactors (Table 2 and Figure 1). Oat hulls-zein and oat hull-soy flour chips met these criteria for pure- and mixed-culture fermentations, respectively.

CONCLUSION

The biofilm-forming bacterium, *Streptomyces viridosporus* T7A, was definitely acting as a natural immobilizer of the *Lactobacillus casei.* Pure-culture interaction by *L. casei* with the different agricultural materials also resulted in cell immobilization and improved lactic acid production. Lignocellulosic materials (corn fiber, oat hulls, and soy hulls) generally performed better than the single polymer (cellulose or starch). Productivity obtained in these biofilm reactors (30 g/l/h) was similar to the observations in a calcium alginate immobilized-cell reactors (3). Long-term studies that compare different immobilized cell systems such as entrapped cells (calcium alginate-immobilized cells) and biofilm reactors need to be done under pH-controlled conditions. Biofilm reactors have the potential of increasing production rates for many fermentations.

ACKNOWLEDGMENTS

This research was supported by the ISU Center for Crops Utilization Research, by a scholarship to Ali Demirci from University of Gaziantep, Turkey, by the Iowa Corn Promotion Board, and by the Iowa Agriculture and Home Economics Experiment Station. We also acknowledge Dr. John Strohl for technical assistance in HPLC analysis.

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Table 1. Percentage composition of polypropylene composite supports[a]

pp-Composite chip	Agricultural Product (%)	Minor Ag. Product (5%)
Polypropylene	----	----
Cellulose	25	----
Cellulose-Soy Flour	20	Soy Flour
Cellulose-Zein	20	Zein
Corn Fiber	25	----
Corn Fiber-Soy Flour	20	Soy Flour
Corn Fiber-Zein	20	Zein
Corn Starch	25	----
Corn Starch-Soy Flour	20	Soy Flour
Corn Starch-Zein	20	Zein
Oat Hulls	25	----
Oat Hulls-Soy Flour	20	Soy Flour
Oat Hulls-Zein	20	Zein
Soy Hulls	25	----
Soy Hulls-Soy Flour	20	Soy Flour
Soy Hulls-Zein	20	Zein

[a]Seventy-five percent of each chip consisted of polypropylene

Table 2. Percent yield and productivity (g/h) for lactic acid production by *L. casei* in pure- and mixed-culture continuous fermentation with different pp-composite chips

	Cellulose				Cellulose - Soy Flour			
Flow Rate	Pure		Mixed		Pure		Mixed	
(ml/min)	Yld[a]	Prod[b]	Yld	Prod	Yld	Prod	Yld	Prod
0.06	81.7	0.04	53.3	0.04	76.9	0.05	61.6	0.04
0.12	100	0.07	72.9	0.09	85.5	0.08	63.3	0.09
0.24	100	0.12	78.0	0.18	93.1	0.12	75.1	0.19
0.48	70.5	0.15	72.4	0.29	75.4	0.19	73.1	0.31
0.96	68.8	0.22	68.2	0.44	73.1	0.38	67.3	0.48
1.92	61.0	0.38	68.2	0.68	76.2	0.81	66.1	0.75

	Cellulose - Zein				Corn Fiber			
Flow Rate	Pure		Mixed		Pure		Mixed	
(ml/min)	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Pro
0.06	80.6	0.04	44.2	0.03	81.5	0.04	45.9	0.03
0.12	84.9	0.07	42.7	0.06	81.3	0.04	54.5	0.08
0.24	87.6	0.12	64.6	0.15	84.9	0.07	70.0	0.19
0.48	78.0	0.22	58.9	0.19	89.3	0.14	78.7	0.33
0.96	70.0	0.35	61.8	0.30	93.5	0.26	86.4	0.50
1.92	69.0	0.59	73.7	0.46	100	0.43	84.3	0.76

Table 2. (continued)

Flow Rate (ml/min)	Corn Fiber - Soy Flour				Corn Fiber - Zein			
	Pure		Mixed		Pure		Mixed	
	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod
0.06	61.3	0.04	59.5	0.04	82.2	0.03	53.2	0.03
0.12	77.6	0.04	68.5	0.09	86.8	0.04	58.6	0.08
0.24	81.0	0.08	75.2	0.19	97.0	0.07	67.0	0.17
0.48	85.9	0.14	86.4	0.32	91.1	0.15	75.6	0.33
0.96	86.9	0.19	89.2	0.50	93.0	0.25	83.1	0.52
1.92	50.0	0.31	100	0.82	ND[c]	ND	ND	ND

Flow Rate (ml/min)	Corn Starch				Corn Starch - Soy Flour			
	Pure		Mixed		Pure		Mixed	
	Yld	Prod	Yld	Pro	Yld	Prod	Yld	Prod
0.06	100	0.03	60.1	0.04	85.9	0.04	50.0	0.04
0.12	100	0.03	69.0	0.08	91.3	0.03	100	0.08
0.24	92.7	0.06	77.4	0.15	90.2	0.06	67.7	0.14
0.48	100	0.12	88.1	0.28	98.3	0.13	79.6	0.27
0.96	100	0.19	69.4	0.38	94.7	0.25	86.5	0.49
1.92	100	0.26	100	0.87	100	0.39	88.0	1.04

Table 2. (continued)

	Corn Starch - Zein				Oat Hulls			
Flow Rate	Pure		Mixed		Pure		Mixed	
(ml/min)	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod
0.06	83.0	0.04	67.6	0.05	77.1	0.03	54.1	0.03
0.12	91.5	0.07	72.5	0.10	71.4	0.05	42.9	0.06
0.24	94.0	0.13	85.5	0.18	84.4	0.11	64.2	0.14
0.48	76.1	0.24	74.3	0.30	94.2	0.24	71.4	0.29
0.96	72.2	0.33	64.4	0.32	93.5	0.40	82.1	0.50
1.92	68.2	0.56	66.8	0.61	90.6	0.64	76.8	0.81

	Oat Hulls - Soy Flour				Oat Hulls - Zein			
Flow Rate	Pure		Mixed		Pure		Mixed	
(ml/min)	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod
0.06	62.6	0.03	69.1	0.04	73.8	0.04	56.2	0.04
0.12	60.4	0.06	59.5	0.08	85.7	0.05	59.5	0.08
0.24	76.6	0.13	67.6	0.18	89.5	0.15	74.3	0.17
0.48	100	0.24	81.7	0.35	100	0.29	80.7	0.29
0.96	100	0.52	82.7	0.60	97.0	0.41	89.0	0.51
1.92	96.5	0.72	83.1	0.93	100	0.69	75.1	0.68

Table 2. (continued)

Flow Rate (ml/min)	Soy Hulls				Soy Hulls - Soy Flour			
	Pure		Mixed		Pure		Mixed	
	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod
0.06	77.9	0.03	58.6	0.04	83.9	0.04	63.0	0.04
0.12	69.6	0.06	61.7	0.09	88.9	0.07	67.9	0.08
0.24	83.5	0.11	73.3	0.19	98.6	0.14	87.5	0.22
0.48	88.3	0.27	83.5	0.33	90.6	0.27	75.8	0.28
0.96	90.5	0.51	87.2	0.59	73.6	0.39	100	0.54
1.92	93.6	0.80	89.5	0.88	69.9	0.60	68.3	0.58

Flow Rate (ml/min)	Soy Hulls - Zein				Polypropylene			
	Pure		Mixed		Pure		Mixed	
	Yld	Prod	Yld	Prod	Yld	Prod	Yld	Prod
0.06	78.4	0.03	69.1	0.04	100	0.02	62.2	0.04
0.12	77.1	0.06	60.6	0.09	85.0	0.04	67.8	0.09
0.24	85.5	0.11	71.4	0.16	96.1	0.09	83.4	0.17
0.48	97.8	0.24	100	0.31	96.1	0.18	90.1	0.25
0.96	95.8	0.48	88.9	0.54	100	0.20	82.6	0.38
1.92	100	0.72	95.2	0.71	90.8	0.33	86.1	0.47

Table 2. (continued)

	Control[d]	
Flow Rate	**P u r e**	
(ml/min)	**Yld**	**Prod**
0.06	78.3	0.03
0.12	86.0	0.05
0.24	86.8	0.07
0.48	100	0.06
0.96	100	0.12
1.92	100	0.22

[a]Yld (yield in percent) was calculated by produced lactic acid (g/l) divided by consumed glucose (g/l) times 100%.

[b]Prod (productivity in g/h) was calculated by lactic acid per hour calculated as produced lactic acid (g/l) times flow rate (l/hr).

[c]ND is for not determined.

[d]Control represents the value for a 25-ml suspension culture continuous lactic acid fermentation with *L. casei.*

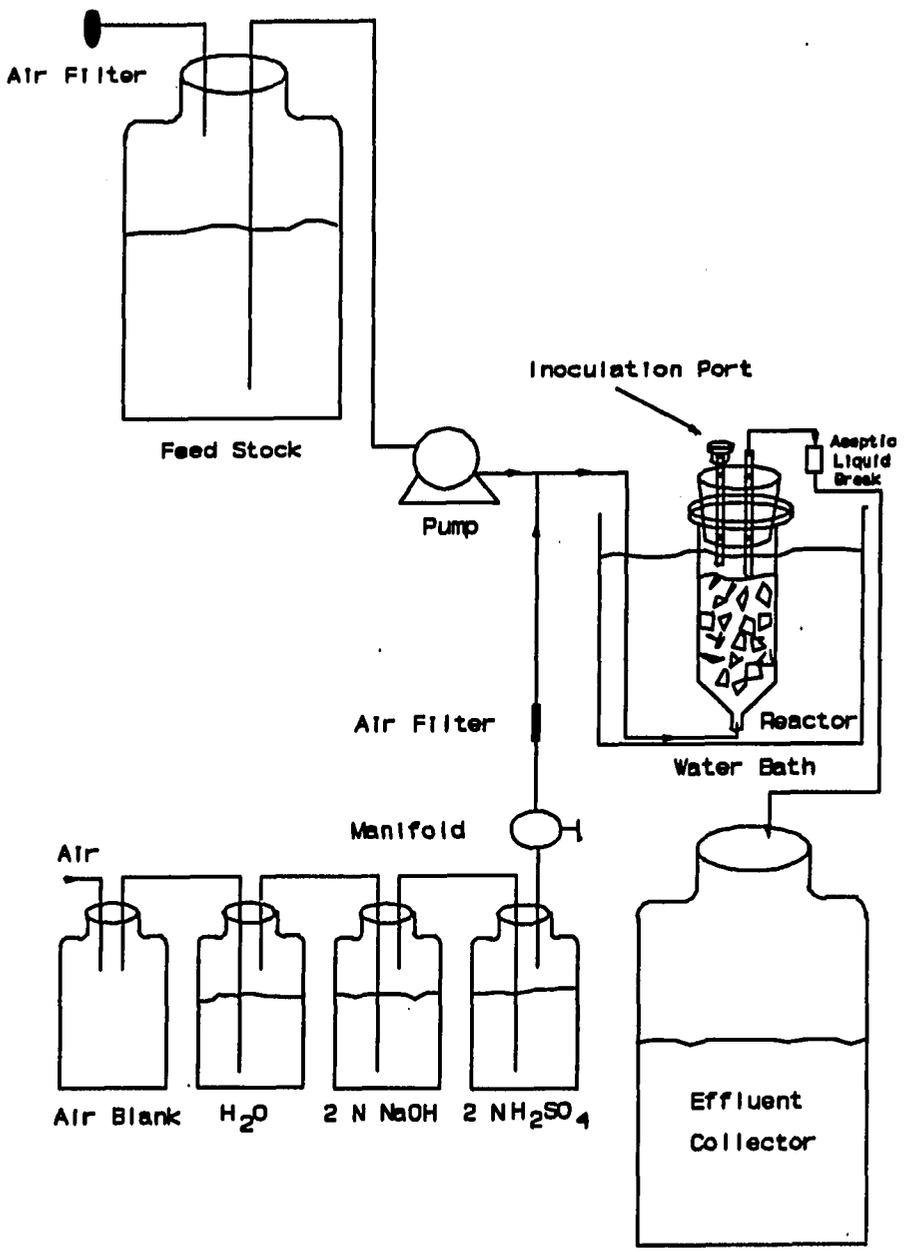

Figure 1. Schematic diagram of the biofilm reactor

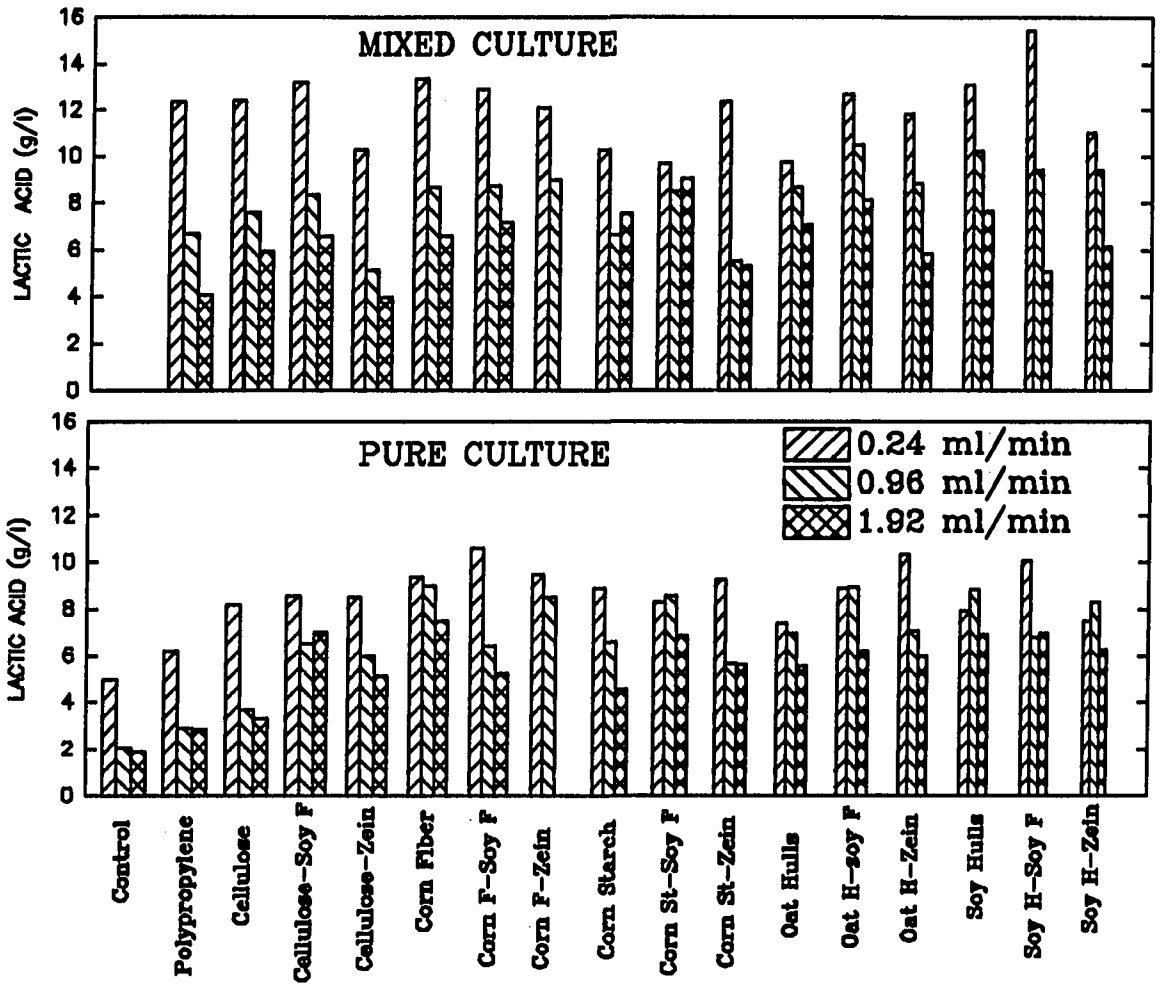

Figure 2. Lactic acid concentrations at three flow rates on chips containing various agricultural materials compared to the suspension culture and polypropylene chips controls

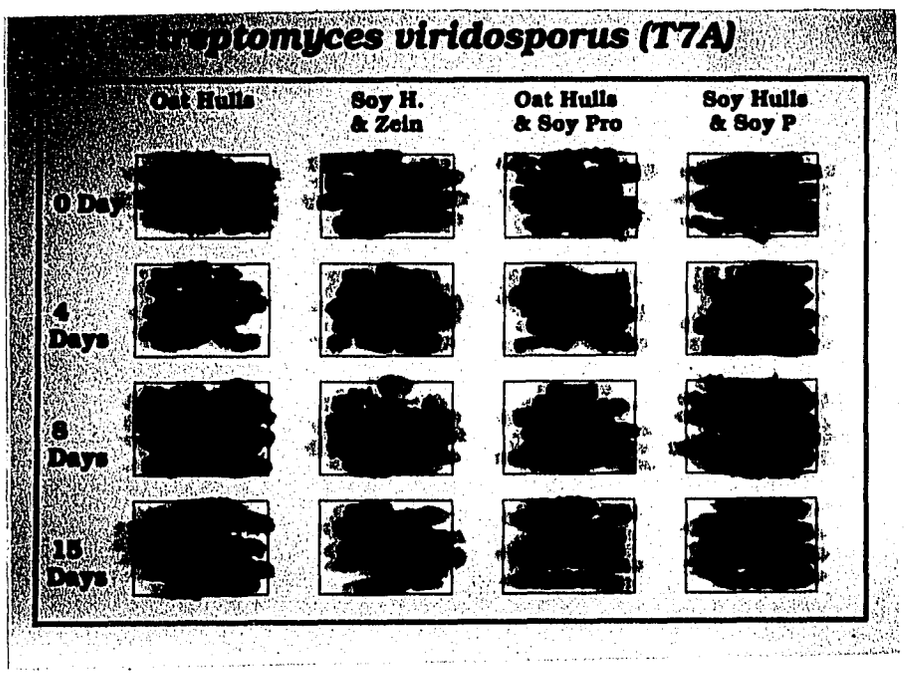

Figure 3. Gram stain of several different pp-composite supports after continuous fermentation

SUMMARY AND CONCLUSION

Enhanced lactic acid production by strain development and by a novel biofilm reactor was performed:

Strain development was done by chemical mutagenesis in which ethyl methanesulfonate was used as mutagen. The developed strain *L. delbrueckii* DP3 produced 117 g/l lactic acid compared with 67 g/l lactic acid by the wild type in pH-controlled (pH 6) batch fermentation at 45°C. Mutant DP3 had a maximum growth and a production rate which were two, and five times higher than those of the wild type, respectively. Repeated batch fermentation after 22 months of storage of freeze-dried mutant DP3 showed the stability of this mutant over time by giving similar fermentation results.

In novel biofilm reactors, various biofilm-forming bacteria including some species of *Bacillus, Pseudomonas, Streptomyces, Thermoactinomyces*, and *Thermomonospora*, and several different inert supports were evaluated after either batch or continuous fermentation. The supports evaluated include pea gravels, porcelain berl saddles, 3M-macrolite ceramic spheres, and polypropylene composite [pp-composite] chips made with various agricultural materials. Extent of biofilm formation was determined by weight changes, clumping of the inert supports, and gram staining of pp-composite chips after the fermentations. Weight-gain data were inconclusive because of very small amount of biofilm on the supports, or in the case of pp-composite chip's

leaching or microbial consumption of agricultural material from the supports. However, clumping and gram staining illustrated the formation of biofilm on the selected supports. The best biofilm-forming bacteria on pp-composite chips were *P. fragi*, *S. viridosporus* T7A, and *T. vulgaris* which grow optimally at 25, 37, and 45°C, respectively. For mixed-culture continuous lactic acid fermentation, the lactic bacteria *L. amylophilus*, *L. casei*, and *L. delbrueckii* DP3 were incubated with *P. fragi*, *S. viridosporus* T7A, and *T. vulgaris*, respectively, because of their matching growth temperature. Also, pure culture fermentations were performed by using only lactic acid bacteria in the reactor. *L. amylophilus*, and *L. delbrueckii* DP3 in pH uncontrolled reactors did not performed well because of their intolerance to pH reduction caused by lactic acid production. However, *L. casei* was not as pH sensitive, and produced higher levels of lactic acid in both pure- and mixed-culture fermentations. In the mixed-culture, *L. casei* produced about 13 g/l lactic acid at flow rate 0.24 ml/min on some pp-chips, whereas suspension-cell culture (control) produced only 4.5 g/l lactic acid at the same flow rate. When flow rate was increased, the biofilm reactors continued to produce high levels of lactic acid whereas the suspension culture did not. Also, some biofilm reactors did not show any decrease or only slight decrease in product concentration at the faster flow rates. Mixed-culture systems at every flow rate produced more lactic acid than pure-culture systems because of probably more cell-immobilization on the supports. Composition of pp-composites apparently played important role,

because every reactor containing different pp-composite chips showed different performance. Oat hulls-soy flour, and oat hulls-zein were chosen best for pure- and mixed-culture, respectively, based on the % yields, productivities, lactic acid concentrations, and stabilities over increase in flow rates. Under these pH-uncontrolled fermentations, lactic acid concentrations are not high compared to pH controlled-fermentation studies. The purpose of this study was to determine best biofilm former, lactic acid bacteria, and solid support combination under the conditions used. Finally, pH controlled-fermentations need to be done for extended periods to prove stability of this type of cell-immobilization.

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ACKNOWLEDGEMENTS

Sincere gratitude is expressed to my major professor, Dr. Anthony L. Pometto III. His constant support, guidance, and encouragement made this research possible. He has been more than an academic adviser to me; he has been a friend, too.

I wish to thank to Dr. Earl G. Hammond, Dr. Paul A. Hartman, Dr. Zivko Nikolov, and Dr. Richard C. Seagrave for serving on my committee, and for their excellent comments throughout my research. Also, Dr. Seagrave's guidence in obtaining my minor in Chemical Engineering.

I am forever grateful to my parents, sisters and brother for their endless support and love. Also, I cannot pay back to my sister, Yurdagul, for coming to the U.S., and taking care of our baby during my study.

I also won't forget my in-laws for their encouragement.

I would like to express my deep indebtedness to my beautiful, loving wife, Ann. I don't think I could have done so much without her. Her support and encouragement, most importantly her love, cannot be stated enough. She was always next to me, when my media contaminated resulting in several lost weeks of research. I, also, appreciate her patience in waking me up to take sample from the reactors in the middle of night. I also would like to thank her for giving birth to our son, Bayu Kerem, during this period.

I wish to extend my thanks Dr. John Sthrol for his help with HPLC analysis, Ken Johnson for technical help, and my other graduate colleagues Byungtae, Erwin, Mahipal, Meera, and Mohammed for their cooperation.

I will not forget the help and smiling faces of our secretaries Nancy Holmcomb, Barbara Malchow, Nan Morain, and Marylou Weigel.

I am very grateful to a wonderful couple, Monica and Peeyush Mahishwari for being with me in my good and bad days.

A special thanks to my cousin Dr. Mufit Akinc, and his family for helping me since my first day in the U.S.

I also would like to thank my other cousin Ferhan, his family, and my friends in the U.S. and Turkey.

I want to express my thanks to Center for Crops Utilization Research (CCUR), and Iowa Corn Promotion Board (ICPB) for financial support during this research. Also, scholarship given to me by University of Gaziantep, Turkey is very much appreciated.