1-1-2000

Evaluation of continuous lactic acid fermentation using a plastic composite support biofilm reactor and lactic acid recovery using emulsion liquid membrane extraction

Julie C. Cotton
Iowa State University

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by

Julie C. Cotton

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Major Professor: Anthony L. Pometto III

Iowa State University

Ames, Iowa

2000

Graduate College
Iowa State University

This is to certify that the Master's thesis of

Julie C. Cotton

has met the requirements of Iowa State University

Signatures have been redacted for privacy

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GENERAL INTRODUCTION

Introduction

Lactic acid (2-hydroxypropanoic acid) exists in two optically active enantiomers, L(+) and D(-), and it is widely used by the food, cosmetic, pharmaceutical and plastics industries (56). The organic acid is produced by chemical synthesis and microbial fermentation. Microbial fermentation has the advantage of being able to produce optically pure lactic acid. However, for fermentation processes to be competitive, improvements are needed to increase production rates and maximize down-stream processing. Presently, lactic acid is produced by batch fermentation because it exhibits both Type I (growth associated) and Type II (non-growth associated) fermentation (8).

To increase cell density in bioreactors, fermentation using cell immobilization by attachment and entrapment has been researched. PCS biofilm reactors have demonstrated increased lactic acid production rates, minimal lag phase, tolerance to high concentration of glucose, reduced requirement of micronutrients, and increased cell density (24, 25, 28).

Continuous fermentations allow a constant flow of fresh sterile media into a stirred tank bioreactor to eliminate the nutrient limitations that are present in batch fermentations. Microbial growth rate equals bioreactor dilution rate. Higher productivities can be achieved, but contamination and cell wash out at high dilution rates for suspended cell bioreactors remain problems.

Down stream recovery processes still remain the significant expense for microbial production of lactic acid. Liquid-liquid extraction has recently been studied as method to minimize recovery costs. Liquid-liquid extraction employs organic solvents to extract organic acids and metal ions from aqueous waste streams and fermentation broth. The process involves two steps: extraction of lactic acid from the aqueous fermentation broth into an organic phase, then back extracting the lactic acid from the organic phase with stripping solution into an aqueous solution. The organic phase consists of organic diluents and extractants (carrier compounds) with high selectivity for the organic acid being extracted. Besides the chemical nature of the carrier compound, extraction is affected by several factors: extractant concentration, diluent, pH of aqueous solution, and temperature.

Extraction by solvents and stripping processes may be combined and performed simultaneously in a process known as emulsion liquid membrane extraction. The organic phase serves as a hydrophobic liquid membrane between the external aqueous phase (fermentation broth) and internal aqueous phase (stripping solution). The organic phase contains a surfactant for emulsion stabilization.

Toxicity of solvents and pH requirements for extractive fermentation remain the major disadvantages of liquid-liquid and emulsion liquid membrane extraction. Optimal recovery is achieved at a pH below or near the pKa of the organic acid being extracted. Most solvents used in these recovery processes demonstrate a toxic effect toward microorganisms (4, 13, 14, 52). Toxicity can be reduced with cell immobilization via biofilm reactors (14) or by using a physical barrier, such as hollow fibers, to reduce direct contact between organic and aqueous phases.

The first paper demonstrates the effectiveness of continuous lactic acid fermentation using PCS tubes fixed to the agitator shaft for cell immobilization via biofilm reactor. The second paper studies the effect of carrier concentration, surfactant concentration, and stripping solution concentration on lactic acid recovery to optimize the emulsion composition using selected solvents and carriers with a minimal and high toxic effect on *Lactobacillus casei*. The effect of external aqueous phase pH on emulsion liquid membrane extraction is reported. Lactic acid recovery via emulsion liquid extraction in a hollow fiber contactor with a toxic solvent combination is also evaluated.

Thesis Organization

This thesis follows an alternative format and is divided into two papers. Each paper contains an abstract, introduction, materials and methods, results and discussion, conclusions, acknowledgments, and references with tables and figures included in the text. The papers are written to conform to the specifications of Applied and Environmental Microbiology, the journal to which the papers will be submitted. A general introduction chapter including a literature review and general conclusion chapter have been included. All experiments, data collection, and data analysis were performed by the candidate.

Literature Review

Lactic Acid

Lactic acid (2-hydroxypropionic acid) is found widely throughout nature in two isomeric forms. In mammalian systems, lactic acid occurs in the L(+) isomer. Both L(+) and D(-) enantiomers exist in bacterial systems. Lactic acid can be made by fermentation or by chemical synthesis. It is also a major metabolite intermediate in many living organisms (56).

Applications of Lactic Acid

Lactic acid has two major application classes. The first class being food, medicine and cosmetic industries. Secondly, lactic acid is used as a chemical for chemistry and technology. Lactic acid that is commercially purchased is one of three grades: technical, food, and pharmaceutical. Lactate salts, such as calcium lactate, are also available. Technical grade lactic acid was traditionally used by the tanning industry for deliming hides. It is also used in the textile industry as mordant for color prints (32).

The food and food-related applications constitute 85% (wt/vol) of the demand for lactic acid in the United States (10, 40). Lactic acid and calcium lactate as food additives are considered "generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA). Lactic acid is also a "natural" ingredient in many foods. Lactate salts are soluble, and lactic acid is easily applicable as a liquid (32). As a preservative, lactic acid displays bacteriostatic properties (3). Food grade lactic acid, also referred to as edible grade, is used as an acidulent and preservative. It has many advantages over other acids used in food systems.

Lactic acid has a mild acid taste and can therefore be used in combination with vinegar to reduce the stringency of vinegar. In fruit flavored beverages, lactic acid will not interact with aromatic flavors and, therefore, does not mask the strong fruit flavors. In practicality, it often enhances these fruit flavors. Lactic acid is used as a pickling agent for sauerkraut, olives, and pickled vegetables. In the beer and wine industry, lactic acid can be added to prevent butyric acid bacteria from producing butyric acid. Lactic acid is also used in the dairy industry in the production of cheeses for adjustment of acidity, flavor and texture. Lactic acid can be used as a preservative in unsalted butter. Processed meats have lactic acid added for flavor or as a preservative. Lactic acid can also be used for pH adjustment of juices and jellies (32). More than 50% of the lactic acid produced for the food industry is used in the production of emulsifying agents for bakery products. Esters of lactate salts are combined with long chain fatty acids to produce the emulsifying agents (10).

A new use of lactic acid and its salts is the disinfection and packaging of carcasses. The fish and poultry industry use aqueous lactic acid solution and salts to increase the shelf life and reduce the growth of anaerobic spoilage organisms, such as *Clostridium botulinum* (10).

The pharmaceutical and cosmetic industries have used lactic acid for many years. Lactic acid and ethyl lactate are used in topical ointments and lotions. Sodium lactate is used for dialysis applications. Calcium lactate is used widely for calcium-deficiency therapy. Degradable lactate polymers also have medical applications. These thermoplastics have potential markets in prosthetic devices and the production of surgical sutures. In cosmetics, ethyl lactate acts as a humectant, and it is the active ingredient of

many anti-acne products (11).

Polymers of lactic acid have the potential to be used in a wide variety of consumer products, such as paper coatings, films, molded articles, foamed articles and fibers. Polylactic acid (PLA) films are a new emerging industry of huge environmental importance. Conventional plastics are difficult to degrade because of their high molecular weight and hydrophobic character. Polyesters of lactic acid are of low molecular weight and have been found to degrade with temperature and humidity (11, 25, 26, 27, 29, 31). Ho et al. (26, 27, 29, 31) confirmed PLA degradation in laboratory and field studies.

Stimulation of plant growth of various crops and fruits has been demonstrated by low molecular weight oligamers (degree of polymerization 2-10) of L-lactic acid. The incorporation of polylactic acid into the formulation of controlled release or degradable mulch films for large scale agricultural applications (10).

Lactic acid esters and its derivatives have the potential of becoming 'green' chemicals/solvents for plasticizers. Since these compounds are non-volatile, non-toxic, and degradable, they could have substantial benefit to food processing and packaging (10).

Economic Importance

The annual world-wide production of lactic acid exceeds 50,000 tons (10). The consumption is growing by 3 to 5% per year. The potential markets are increasing the demand for an inexpensive source of L- lactic acid. The current complex production of lactic acid drives the market price of this intermediate-volume specialty chemical. In

2000, the market prices for food grade and technical grade lactic acid are $0.70 to 0.80 and $0.72 to 0.85 per pound, respectively (2). This high cost of monomers for polymerization is the limiting factor to the development and growth of the PLA industry. In 1998, PLA prices ranged from $1.30 to $3.00 per pound (44). The degradable plastics alone have an estimated value as high as $1.6 billion per year. The expanding potential markets including biodegradable polymers, oxygenated chemicals, 'green' solvents, and plant-growth regulators could use 6.4 to 8.4 billion pounds of lactic acid per year. The markets would be worth an estimated $4.3 to 6.2 billion (10).

Chemical Production of Lactic Acid

Chemical synthesis of lactic acid can be achieved using coal, petroleum and natural gas. Another process of producing lactic acid uses acetaldehyde and hydrogen cyanide as starting materials. The chemical methods produce a racemic mixture of L(+) and D(-) lactic acid. Chemical synthesis produces a high purity, heat-stable lactic acid which is required for polylactic acid synthesis (56).

Microbial Production of Lactic Acid

The microbial production of lactic acid offers two advantages: utilization of renewable carbon sources such as corn and exclusive production of L(+) or D(-) isomer. Table 1.1 lists some lactic acid bacteria that produce one isomer or a racemic mixture. The exclusive production of the L(+) isomer is necessary for PLA production. The disadvantage of microbial production is the cost of production, recovery and purification processes.

Table 1.1. Homo- and heterofermentative lactic acid bacteria and configurations of lactic acid produced (17).

Bacterium	Homofermentative	Heterofermentative	Lactic Acid Configuration
Lactobacillus			
L. delbrueckii	+		D(-)
L. lactis	+		D(-)
L. bulgaricus	+		D(-)
L. casei	+		L(+)
L. curvatus	+		D/L
L. plantarum	+		D/L
L. brevis		+	D/L
L. fermentum		+	D/L
Streptococcus			
S. faecalis	+		L(+)
S. cremoris	+		L(+)
S. lactis	+		L(+)
Pediococcus			
P. damnosus	+		D/L
Leuconostoc			
L. mesenteroides		+	D(-)
L. dextranicum		+	D(-)
Bifidobacterium			
B. bifidum		+	L(+)

Microbial fermentation producing lactic acid are of two types: homofermentative and heterofermentative. *Lactobacillus delbrueckii, L. bulgaricus, L. leichmanii,* and *L. casei* are bacteria that exhibit homolactic fermentations producing two molecules of lactic acid from one glucose molecule. The bacteria degrade glucose to pyruvate via Embden-Meyerhoff pathway, and the pyruvate is converted to lactic acid by lactate dehydrogenase. Heterolactic fermentations produce one lactic acid molecule and other by-products such as ethanol, glycerol, carbon dioxide or acetic acid from one glucose. Heterolactic bacteria are plentiful, however, only homofermentative lactic acid bacteria are of industrial importance due to higher production yields of 80 to 99% (56).

Lactic acid bacteria have complex nutritional requirements for vitamins, amino acids, nucleotides and inorganic salts. These nutritional requirements can be addressed by using complex medium components, such as yeast extract, corn steep liquor, cottonseed flour, and soy flour.

The selection of bacterium is highly based upon the carbohydrate to be fermented. The factors driving the selection of carbohydrates in commercial scale processes are cost, availability, purity and ease of product recovery. In general, glucose and sucrose are used as substrates for microbial fermentation. Other sources include maltose, polysaccharide hydrolysates, cane and beet sugar and lactose.

Fermentation Systems

Lactic acid production in batch fermentation (closed system) is considered to be a Type I (growth associated) and Type II (non-growth associated) fermentation. In the initial phases of cell growth, lactic acid production is proportional to cell growth. However, as the cells enter the stationary phase, lactic acid concentration continues to increase. Cell growth in stirred tank reactors at a specific agitation rate, temperature and pH is limited by complex nutrient requirements and accumulation of end-product. Since productivities of batch fermentations are lower than desired by industry, there has been extensive research on continuous fermentations (8).

Fed-batch fermentation takes advantage of the Type II fermentations by building up a high biomass concentration, then by converting residual substrate to lactic acid (55). To feed the batch, the media is "spiked" with substrate. It is assumed the bacteria (biocatalyst) do not die and the limiting factor is nutrients. Eventually, production rates

decrease due to cells death and increased end-product accumulation. At this point the batch is harvested.

Continuous fermentations (open system) allow for a constant flow of fresh sterile media into the steady state bioreactor while, at the same time, an equal amount of media is removed from the system. Agitation, temperature and pH are controlled. Continuous fermentations have the advantage of having higher productivity rates (g/l/h) than batch, but contamination and cell wash out at high dilution rates ($\geq 1\ h^{-1}$) remain problems. Microbial growth rate equals bioreactor dilution rate, thus cell wash out occurs when dilution rate exceeds microbial growth rate.

Cell Immobilization

Cell immobilization is used to increase productivity rates and yields over suspended-cell fermentations (20, 24, 28, 30, 35, 36). The two types of cell immobilization are attachment and entrapment. Entrapment is the process in which organisms are trapped in the inner portions of a fibrous or porous matrix, such as a stabilized gel or membrane. Entrapment does not depend on cellular properties. The most widely used cell-entrapment methods involve alginate or κ-carrageenan beads. Industrial scale application of cell entrapment in fermentation has been limited by bead swelling, cell leakage, limited mass transfer across the beads, high cost of carrier and poor operational stability.

When cells adhere to surfaces by self adhesion or chemical bonding, the type of immobilization is attachment. This simple method of immobilization involves reversible

surface interactions between cells and support materials. Biofilms are naturally formed when microorganisms attach to surfaces, grow, and produce extracellular components (biological glue). Biofilm accumulation results from the combination of the following processes (Figure 1.1): transport of cells to the substratum, adsorption of cells to the substratum, growth and other metabolic processes within the biofilm, and detachment of portions of the biofilm (6). Advantages of this system include: the ability to process large volumes of nutrient flow for long times, better mass transfer, the freedom to innoculate with mixed cultures to regulate multiple bioconversions, and the use of inexpensive support material.

Support material must be sterilizable, easy to handle, inexpensive, and have a long term benefit. Research has been conducted using the following support materials: wood chips, porous bricks, glass (18, 20, 36), foam (15), cotton cloth, ceramics (18, 21), and plastic composite supports (24, 28, 30, 35).

Organic Acid Recovery

For lactic acid and other organic acids produced by fermentation to be suitable for the various food and non-food applications discussed, it must be free of impurities such as residual sugars. Recovery and purification processes contribute largely, as much as 50%, to the final cost of lactic acid. The purification of synthetically made lactic acid requires less effort and is, therefore, traditionally preferred (56).

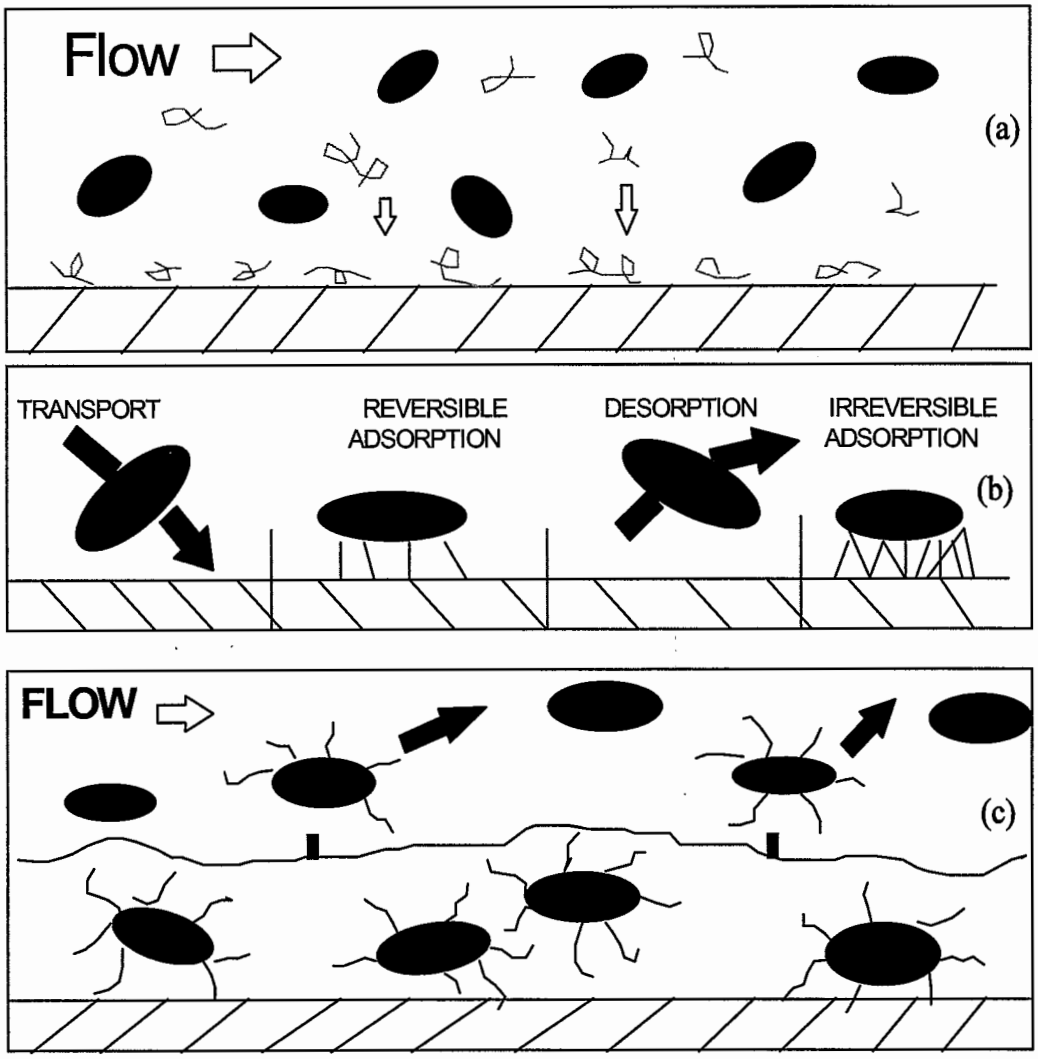

Figure 1.1. (a) Transport and adsorption of organic molecules on a clean substratum, forming a conditioning film. (b) Transport of microbial cells to the conditioned substratum. (c) Attachment and detachment of cells and particles to and from the biofilm (6).

Calcium Precipitation

Traditional fermentations have an excess of calcium carbonate added to maintain the pH. Upon production of lactic acid, calcium lactate is formed. Recovery processes using this system require pretreatment of fermentation broth. The first step is to increase

the temperature to 80 to 100°C and adjusted the pH to 10 to 11. This step will kill organisms, coagulate the proteins, solubilize calcium lactate and degrade some residual sugars. Filtration removes additional cells and coagulated proteins. The crude extract can then be purified with one of the following methods (56).

Filtration, Carbon Treatment, and Evaporation

This simple recovery process is suitable for fermentations of pure sugars and minimal amounts of nitrogenous nutrients. The process by which the American Maize-Products Company (Hammond, IN) commercially produces lactic acid is an example of this type of fermentation. The starting material consists of 15% (wt/vol) corn sugar, 10% (wt/vol) $CaCO_3$, 0.375% (wt/vol) malt sprouts, 0.25% (wt/vol) $(NH_4)_2HPO_4$. After filtration, a carbon treatment is performed using activated carbon to bleach calcium lactate for edible grade lactic acid. The lactate is acidified with sulfuric acid and filtered to remove calcium sulfate (gypsin). Additional activated carbon treatments and evaporation result in 52 and 82% lactic acid, respectively. If necessary, heavy metals are then removed by sodium sulfite or an ion exchange column. Food grade lactic acid requires additional bleaching steps. The disadvantages to this recovery process include the calcium sulfate waste disposal and the large amounts of energy required to carry out the process (56).

Calcium Lactate Crystallization

For fermentation media composed of crude raw materials (i.e. whey or molasses), the recovery process that is used is calcium lactate crystallization. Filtered broth

undergoes activated carbon treatment under alkaline conditions followed by acidic conditions. The broth is then subjected to evaporation. Subsequent evaporation, acidification with sulfuric acid, filtration of calcium sulfate, activated carbon treatment and precipitation of heavy metals are required for the manufacturing of technical grade lactic acid. The manufacturing of food grade and pharmaceutical grade lactic acid involve cooling the broth, crystallizing and washing. Disadvantages of this recovery process is the presence of ash from calcium sulfate and difficult washing when crystals form clusters. This process also requires large amounts of energy and labor (56).

Distillation of Lactate Esters

When fermentation broth exposed to an excess of low molecular weight alcohol, such as methanol, lactate esters will be formed. Distillation of lactate esters and hydrolysis produces a high quality lactic acid. The lactic acid is practically free of residual sugars, ash and other impurities. Processes have been developed using methyl lactate, alkyl lactate and ammonium lactate. Ammonium lactate is a fermentation product when ammonium salts or ammonia is used for pH control during the fermentation. Since this recovery process produces a heat stable lactic acid, the commercial feasibility has been explored and is currently in practice (56).

Electrodialysis

Electrodialysis or dialysis in the presence of an electric field utilizes an apparatus made up of anion and cation membranes arranged alternately between a cathode and an anode. The electric potential between anode and anions and cathode and cations is the

driving force for anions and cations to cross their respective membranes. Lactic acid, propionic acid and acetic acids have been successfully recovered using electrodialysis (16, 58). Lactic acid penetrates the anionic membrane into the concentration compartments. To remove other anionic compounds present in the lactic acid fermentation media other processes are employed. Electrodialysis can be implemented as a continuous or semi-continuous recovery process. Disadvantages of electrodialysis include high cost and membrane fouling. Cells from the fermentation broth adhere to the anion exchange membranes and cause fouling which impairs product recovery. Electrodialysis apparatuses are expensive to implement into commercial production (16).

Adsorption

Adsorption recovery is suitable for recovery of substances produced in a dilute concentrations in complex aqueous solutions. Polymeric absorbents can be classified as a weak or strong acid cation exchanger or weak or strong base anion exchanger. A basic anion exchanger is appropriate for the removal of lactic acid. The recovery of lactic acid using weak and moderate base sorbents was evaluated by Evangelista et al. (16). Lactic acid was recovered using three steps after cell removal. A weak-acid cation exchanger was used to acidify the broth. Extraction of lactic acid was conducted using the basic sorbents. After adsorption, the lactic acid was eluted with methanol or sodium hydroxide solution. The final step was concentration. Disadvantages include the need for pre- or post-treatment of organic acid. The amount of impurities can be large, and lactic acid must be pre-treated or treated following elution (16).

Liquid-Liquid Extraction

Liquid-liquid extraction employs organic solvents to extract organic acids and metal ions from waste streams and fermentation broth. The process involves two steps: extraction of lactic acid from the fermentation broth into an organic phase and removing the lactic acid from the organic phase with stripping solution into an aqueous solution. The organic phase consists of organic diluents and extractants (carrier compounds) with high selectivity for the organic acid being extracted. The criteria for solvents used in extraction of organic acids produced by fermentation are summarized in Table 1.2. Batch extraction, extraction apart from fermentation, and *in situ* extraction, also referred to as extractive fermentation, of organic acids including lactic acid has been studied (Table 1.3).

Table 1.2. Criteria for solvents used in liquid-liquid and emulsion liquid membrane extraction.

Biocompatibility
Distribution Coefficient
Selectivity
Viscosity
Personnel Safety
Solubility
Interfacial tension, density, boiling point, melting point, polarity
Chemical Stability
Cost
Environmental Risks
Availability

Table 1.3. Liquid-liquid extraction of carboxylic acids other than lactic acid.

Carboxylic Acid	References	Extractant:Diluent
Propionic	Yang et al. (60)	Alamine 336:Kerosene
		Alamine 336:2-octanol
		Aliquat 336:Kerosene
		Aliquat 336:2-octanol
	Lewis and Yang (39)	Alamine 336:2-octanol
	Matsumoto et al. (41)	Tri-octylphosphine oxide:hexane
	Gu et al. (19)	Alamine 304:2-octanol
		Alamine 304:1-dodecanol
Malic	Prochazka et al. (46)	Trialkylamine:1-octanol
		Trialkylamine:1-heptane
	Matsumoto et al. (41)	Tri-octylphosphine oxide:hexane
Citric	Prochazka et al. (46)	Trialkylamine:1-octanol
		Trialkylamine:1-heptane
	Matsumoto et al. (41)	Tri-octylphosphine oxide:hexane
Acetic	Tamada et al. (53)	Alamine 336:Nitrobenzene
	Yang et al. (60)	Alamine 336:Kerosene
		Alamine 336:2-octanol
		Aliquat 336:Kerosene
		Aliquat 336:2-octanol
		Alamine 336:2-octanol
	Matsumoto et al. (41)	Tri-octylphosphine oxide:hexane
Succinic	Tamada et al. (53)	Alamine 336:Methylisobutyl ketone
	Poole and King (45)	Alamine 336:Methylisobutyl ketone
Fumaric	Poole and King (45)	Alamine 336:Methylisobutyl ketone
Butyric	Yang et al. (60)	Alamine 336:Kerosene
		Alamine 336:2-octanol
		Aliquat 336:Kerosene
		Aliquat 336:2-octanol
		Alamine 336:2-octanol
	Hatzinikolaou and Wang (23)	Dodecanol:oleyl alcohol

Besides the chemical nature of the carrier compound, extraction is affected by several factors: extractant concentration, diluent, pH of aqueous solution, and temperature. Table 1.4 summarizes research evaluating different factors for liquid-liquid extraction of lactic acid.

Table 1.4. Survey of published research of liquid-liquid extraction of lactic acid.

Reference	Solvent Selection	Effect of pH	Solvent Toxicity	Extractant Selectivity	Effect of Extractant Concentration	Use of Hollow Fiber	Effect of Stripping Solution	In Situ Extraction	Effect of Temperature	Effect of Diluents
Choundhury et al. (4)	x	x	x	x	x					x
San-Martin et al. (49)					x				x	
Lazarova and Peeva (38)	x	x			x		x			
Scheler et al. (50)						x				
Yabannavar and Wang (59)							x	x		
Achour et al. (1)										x
Honda et al. (33)	x		x		x			x		
Hano et al. (22)	x	x			x		x	x		x
Chen and Lee (7)	x	x				x		x	x	x
Han and Hong (21)					x				x	x
Dai and King (9)	x	x		x						x
Ye et al. (61)		x	x			x				
Prochazka et al. (46)									x	x
Wang et al. (57)	x			x					x	x
San-Martin et al. (48)				x	x				x	x
Tamada et a. (15)	x								x	x
Juang and Huang (34)					x				x	
Lazarova and Peeva (37)				x	x		x			
Yang et al. (60)	x	x			x					x
Seevaratham et al. (52)	x	x	x					x		

Of the solvents and carrier compounds studied, long chain aliphatic amines (i.e., Alamine 336, Alamine 304, and tri-octyl amine) and phosphorous containing compounds (i.e., tri-butyl phosphate and trioctylphosphine oxide) have been shown to be suitable extractants due to the polar nature and weak acid characteristics of lactic acid. Tri-octylphosphineoxide and tri-butyl phosphate in hexane have been reported to extract 23 and 0.73% lactic acid, respectively (22). Several diluents mixed with tri-butyl phosphate were evaluated by Achour et al. (1).

The extractability of carrier compounds under a variety of extraction conditions with different amounts of extractant and in various diluents is clearly reported in literature. Table 1.5 gives a summary of the results obtained from previous research using tertiary and quaternary amines. The results are reported as percent recovery or distribution coefficients.

$$\text{Percent Recovery} = \frac{[\text{lactic acid}]_{aq_{initial}} - [\text{lactic acid}]_{aq_{final}}}{[\text{lactic acid}]_{aq_{initial}}} * 100$$

$$\text{Distribution Coefficient } (K_D) = \frac{\left([\text{lactic acid}]_{aq_{initial}} - [\text{lactic acid}]_{aq_{final}}\right)}{[\text{lactic acid}]_{aq_{initial}} * \left(\frac{vol_{org}}{vol_{aq}}\right)}$$

Table 1.5. Summary of liquid-liquid extraction results from previous research using tertiary and quaternary amines.

Extractant	Diluent	pH	Initial Lactic Acid Concentration (g/L)	Result	Reference
Aliquat 336 (70%)	Methylisobutyl ketone (30%)	2.8	86.9	$K_D = 0.71$ 41% recovery	4
Aliquat 336 (5%)	Nonane (75%)	5-6	3.0	$K_D = 0.36$	38
Aliquat 336 (5%)	N-Octane (95%)	6.5	9.4	31.9% recovery	37
Aliquat 336 (50%)	Paraffin Oil (50%)	6.0	50.0	$K_D = 0.50$	52
Alamine 336 (10%)	Nonane (90%)	5-6	3.2	$K_D = 0.01$	38
Alamine 336 (50%)	Toluene (50%)	-	40.0	$K_D = 2.06$ 60% recovery	48
Alamine 336 (20%)	Oleyl alcohol (20%) Kerosene (40%)	2.3	15.1	$K_D = 0.28$	7
Alamine 336 (50%)	Oleyl alcohol (50%)	5-6	12.0	$K_D = 2.62$	33
Di-octylamine	Hexane	2.2	16.0	69% recovery	22
Tri-octylamine (10%)	Nonane (90%)	5-6	3.8	$K_D = 0.03$	38
Tri-octylamine (50%)	Paraffin Oil (50%)	6.0	50.0	$K_D = 0.27$	52
Tri-octylamine (50%)	Methylisobutyl ketone (50%)	2.8	86.9	$K_D = 3.75$ 79% recovery	4

Tertiary and quaternary amines are slightly more effective for liquid-liquid extraction and less expensive than phosphorous containing compounds (60). Primary and secondary amines are not effective due to the high solubility of primary amines and the ability of secondary amines to regenerate amide structure after distillation (60). Quaternary amines, such as Aliquat 336, extract disassociated and undissociated acid, but stripping is difficult (60). Amines, including Aliquat 336 (4, 37, 38, 52, 60), tri-n-octylamine (19, 34, 52, 57), di-n-octylamine (22) and Alamine 336 (7, 9, 33, 48, 49, 53, 57, 59, 60, 61) have been evaluated for lactic acid recovery.

The utilization of a solution 40% (vol/vol) Alamine 336 in oleyl alcohol for extractive fermentation with a hollow fiber contactor reported a maximum 48% recovery and an increase in productivity from 0.93 to 2.91 g/l per hour (61). Honda et al. (33) also reported a productivity of 1.4 times the control for an *in situ* extraction system using 50/50 (vol/vol) Alamine 336/oleyl alcohol. Extractive fermentation conducted with κ-carrageenan immobilized bacteria and a solution of 15% (vol/vol) Alamine 336 in oleyl alcohol reported a productivity of 12 g/l per hour, compared to the control with a productivity of 7 g/l per hour without in situ extraction (59).

Continuous removal of lactic acid by liquid-liquid extraction decreases the concentration of end product in the fermentation system which reduces the effects of end product inhibition. However, pH requirements and the biocompatibility of solvents to microorganisms is a major concern for integrated production and recovery fermentation. Toxicity is of two levels, molecular and phase level. Molecular toxicity is caused by the soluble portions of solvents. The presence of two phases (organic and aqueous) causes

phase level toxicity. The use of a physical barrier , such as a hollow fibers, can eliminate molecular toxicity (61). Although the ability to recover organic acids by many solvents and carrier compounds has been extensively studied, the toxicity information is relatively new. The toxicity of a variety of solvents and extractants to lactic acid bacteria has been studied (33, 60, 61, 52). Demirci et al. (13) screened many solvent and carrier compound combinations for toxicity to *Lactobacillus casei*. Research also conducted by Demirci et al. (14) indicates that immobilization may be able to reduce the effect of toxic solvents.

Maximal extraction of organic acids is obtained at a pH near or below the pK_a of the acid (4, 7, 9, 22, 38, 52, 53, 57, 60). Table 1.6 gives pKa values for several organic acids. The pK_a of lactic acid is 3.96. However, the maximal physiological conditions for most lactic acid bacteria to grow is a pH of ≥ 5.

After extraction, the acid is removed from the organic phase with a stripping solution. The stripping solution is an aqueous phase that removes the organic acid bound to the carrier. Both type and concentration of stripping solution affect product recovery. Sodium carbonate, ammonium carbonate, and sodium hydroxide have been determined appropriate stripping reagents (22, 37, 38).

Emulsion Liquid Membrane Extraction

The process which combines extraction and stripping processes simultaneously in a process known as emulsion liquid membrane extraction. The organic phase serves as a hydrophobic liquid membrane between the external aqueous phase (fermentation broth) and internal aqueous phase (stripping solution) (Figure 1.2). The organic phase contains a surfactant for stabilization of the emulsion.

Table 1.6. The pKa values of several carboxylic acids (23).

Carboxylic Acid	pKa (25°C)
Lactic	3.86
Propionic	4.85
Malic	3.22, 4.70
Citric	3.13, 4.76, 6.40
Acetic	4.75
Succinic	4.20, 5.64
Fumaric	3.02, 4.38
Butyric	4.81

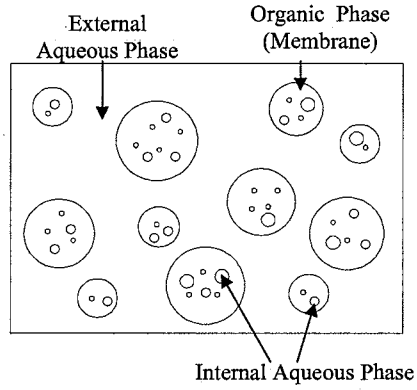

Figure 1.2. Schematic of emulsion liquid membrane.

The major disadvantage of emulsion liquid membrane extraction is emulsion swelling. Over extended contact with the aqueous phase, the emulsion begins to swell causing a dilution of the organic phase in the internal phase. Swelling can be controlled by choosing diluents with low polarity and adjusting the concentration of surfactant and stripping solution (51). Instability of the emulsion allows the solvent and carrier compounds to leach into the aqueous phase. For *in situ* extraction, biocompatibility of

solvents remains a problem for emulsion liquid membrane extraction. Demirci et al. (13, 14) evaluated the toxicity of a variety of solvents in an emulsion system to *Lactobacillus casei*. Solvent combinations that reported low toxicity, good recovery and minimal swelling were hexane:Alamine 304 and xylene:Alamine 304.

Solvent criteria for emulsion liquid membrane extraction is the same as those for liquid-liquid extraction (Table 1.2). The additional component to the emulsion system that is absent from liquid-liquid extraction is the surfactant. The type and concentration of surfactant plays a critical role in interfacial tension, emulsion stability, and extraction capability (42, 43, 54, 62). Previous research indicates the surfactant component depends on the components of the organic phase and the acid being recovered.

Emulsion liquid extraction, like liquid-liquid extraction, is affected by several factors: diluents, carrier concentration (5, 43, 54, 62, 47), surfactant concentration (5, 42, 43, 62), stripping solution (5, 51), pH of the external aqueous solution (51), and viscosity (42, 62). Like liquid-liquid extraction, optimum recovery is achieved at pH values lower than the pKa of the organic acid being recovered (51). However, Scholler et al. (51) report extraction rates are not affected by temperature.

To eliminate the leaching of organic compounds into the aqueous phase, a physical barrier, such as a hollow fiber unit which consists of a shell and tube configuration, is employed. The organic phase and aqueous phase flow in opposite directions on different sides of the membranes. The hollow fibers and casing materials must be compatible with the organic solvents employed. The major advantage of this method is no contact between the two phases; therefore, emulsion swelling and breakage are reduced and molecular toxicity is eliminated.

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CONTINUOUS LACTIC ACID FERMENTATION USING A PLASTIC COMPOSITE SUPPORT BIOFILM REACTOR

A paper to be submitted to Applied and Environmental Microbiology

Julie C. Cotton and Anthony L. Pometto III

Abstract

An immobilized-cell biofilm reactor was used for the continuous production of lactic acid by *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443). At Iowa State University, a unique plastic composite support (PCS) which stimulates biofilm formation has been developed. The optimized PCS blend for *Lactobacillus* contains 50% (wt/wt) agricultural products (35% (wt/wt) ground soy hulls, 5% (wt/wt) soy four, 5% (wt/wt) yeast extract, 5% (wt/wt) dried bovine albumin, and mineral salts) and 50% (wt/wt) polypropylene produced by high temperature extrusion. The PCS tubes have of wall thickness of 3.5 mm, outer diameter of 10.5 mm and were cut into 10 cm lengths. Six PCS tubes, three rows of two parallel tubes, were bound in a grid fashion to the agitator shaft of a 1.2 liter vessel for a New Brunswick Bioflo 3000 fermentor. PCS stimulates biofilm formation, supplies nutrients to attached and suspended cells, and increases lactic acid production. Biofilm thickness on the PCS tubes was controlled by the agitation speed. The PCS biofilm reactor and polypropylene control reactor achieved optimal average production rates of 9.0 and 5.8 g/l per hour, respectively at 0.4 h^{-1} dilution rate and 125 rpm agitation with yields of approximately 70%.

Introduction

Lactic acid (2-hydroxypropanoic acid) exists in two optically active enantiomers, L(+) and D(-), and it is widely used by the food, cosmetic, pharmaceutical and plastics industries (14). The organic acid is produced by chemical synthesis or microbial fermentation. Microbial fermentation is advantageous because it is able to produce optically pure lactic acid. However, for fermentation processes to be competitive, improvements are needed to increase production rates and to maximize down-stream processing. Presently, lactic acid is produced by batch fermentation because it exhibits both Type I (growth associated) and Type II (non-growth associated) fermentation (1). Accelerated production rates and high lactic acid concentrations were achieved by strain development (2).

To increase cell density in bioreactors, fermentation using cell immobilization by attachment and entrapment has been researched. The industrial application of cell entrapment with calcium alginate or κ-carrageenan beads are not feasible due to bead disintegration and mass transfer limitations. Attachment immobilization has been explored with several types of support materials: wood chips, porous bricks, glass (5, 7, 11), foam (4), cotton cloth, ceramics (5, 7) and plastic composite supports (PCS) (8, 9, 10). PCS biofilm reactors have demonstrated increased lactic acid production rates, minimal lag phase, tolerance to high concentration of glucose, reduced requirement of micronutrients, and increased cell density (8, 9, 10).

Continuous fermentations allow a constant flow of fresh sterile media into a stirred tank bioreactor to eliminate the nutrient limitations that are present in batch

fermentations. Microbial growth rate equals bioreactor dilution rate. Higher productivities can be achieved, but contamination and cell wash out at high dilution rates for suspended cell bioreactors remain problems. The purpose of this research was to demonstrate the effectiveness of continuous lactic acid fermentation using PCS tubes made of polypropylene and agriculture materials fixed to the agitator shaft for cell immobilization via biofilm reactor.

Materials and Methods

Bacterial Culture Preparation

Stock culture of *Lactobacillus casei* subsp. *rhamnosus* (ATTC 11443) from the American Type Culture Collection (Rockville, MD) was maintained at 4°C in *Lactobacillus* MRS broth (Difco Laboratories, Detroit, MI). To maintain viability, monthly transfers were made into fresh media. An active *L. casei* culture was prepared by adding 1-ml stock solution to 100-ml MRS and incubated as a static culture for 18 hours at 37°C.

Medium Preparation

Lactic acid fermentation medium (40 g of glucose, 5 g of yeast extract [Ardamine Z; Champlain Industries Inc., Clifton, NJ], 1 g of sodium acetate, 0.6 g of $MgSO_4 \cdot 7\ H_2O$, 0.03 g of $MnSO_4 \cdot 7H_2O$, 0.5 g of KH_2PO_4, and 0.5 g of K_2HPO_4 per liter deionized water) was used for all experiments. Glucose, yeast extract, sodium acetate, $MgSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 7H_2O$ were added to 86 liters water, sterilized in a B-Braun 100-D fermentor (Allentown, PA) with continuous agitation for 25 min at 121°C, and adjusted to pH 5.0

with 3 N hydrochloric acid. Phosphate buffer solution was autoclaved separately in a 4 liter carboy for 40 min at 121 °C and added aseptically to other medium components. The sterilized media was aseptically transferred into two sterilized 50 liter carboys equipped with a carboy filling port, a medium delivery line with a liquid break, and an air vent capped with a 0.45 μm air filter for storage.

Plastic Composite Supports (PCS)

PCS tubes composed of 50% (wt/wt) polypropylene (PP), 35% (wt/wt) ground soy hull (Cargill Soy Processing Plant, Iowa Falls, IA), 5% (wt/wt) soy flour (Archer Daniels Midland, Decatur, IL), 5% (wt/wt) yeast extract and 5% (wt/wt) dried bovine albumin (American Protein Corp., Ames, IA) and mineral salts were produced according to Ho et al. (10). These dry ingredients were mixed in separate container prior to being poured into extruder hopper. The twin screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, N.J.) was operated at a rate of 11 rpm, barrel temperatures of 200, 220, and 200 °C, and a die temperature of 167 °C to form a continuous tube. Composite supports with a wall thickness of 3.5 mm and an outer diameter of 10.5 mm were cut into 10 cm lengths. Polypropylene (PP) tubes (control) were cut identical to PCS tubes.

Continuous Fermentation Systems

A continuous stir tank bioreactor with PCS tubes fixed to the agitator shaft was compared to a control continuous reactor with PP tubes fixed to the agitator shaft (Figure

2.1). PP and PCS tube ends were cut at an angle to allow fermentation media to flow through the inside of tubes. Six PCS or PP tubes, 3 rows of 2 parallel tubes, were bound to the agitator shaft in a grid-like fashion. A schematic of the reactor design in a 1.2 L vessel (inside diameter of 12 cm) of a computer controlled New Brunswick Bioflo 3000 fermentor (Edison, NJ) is given in Figure 2.2. Fermentations were controlled at 37°C and pH 5.0 with concentrated ammonium hydroxide. To determine the working volume of each reactor, water was passed through the continuous system until steady state, at least 5 working volume exchanges, was reached. Agitation rates of 100 and 125 rpm did not affect working volume of reactors.

The series of steps for repeated continuous fermentation are summarized in Figure 2.3. The reactor was sterilized with water in the reactor for 1.25 h at 121°C. After sterilization, media was used to dilute out water at a dilution rate of 0.6 h^{-1} overnight. The batch fermentation (1% inoculum) prior to continuous flow, employed agitation of 100 rpm. Dilution rates varied from 0.1 to 1 h^{-1}. A dilution rate of 0.1 h^{-1} at 100 rpm for 5 days was needed to establish good biofilm formation on fresh PCS supports. Dilution rates were then increased. To repeat, reactors were washed with

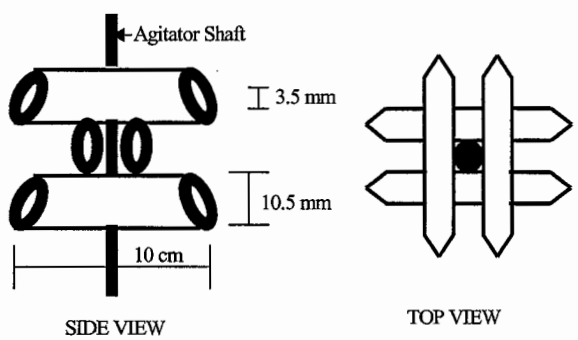

Figure 2.1. PCS and PP tubes bound to agitator shaft.

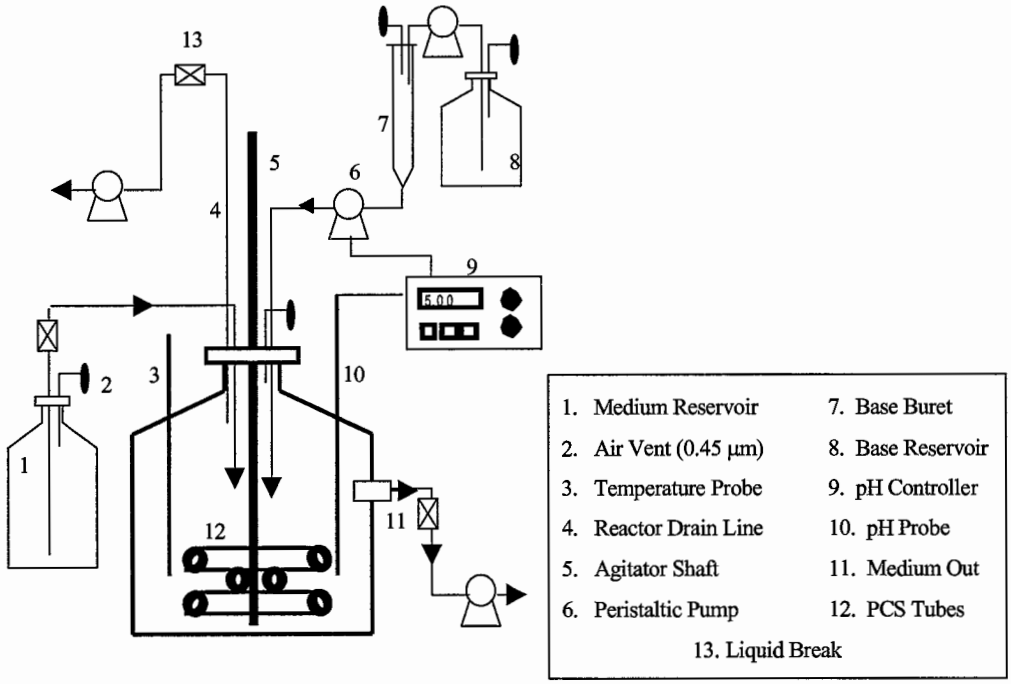

Figure 2.2. Bioreactor Design.

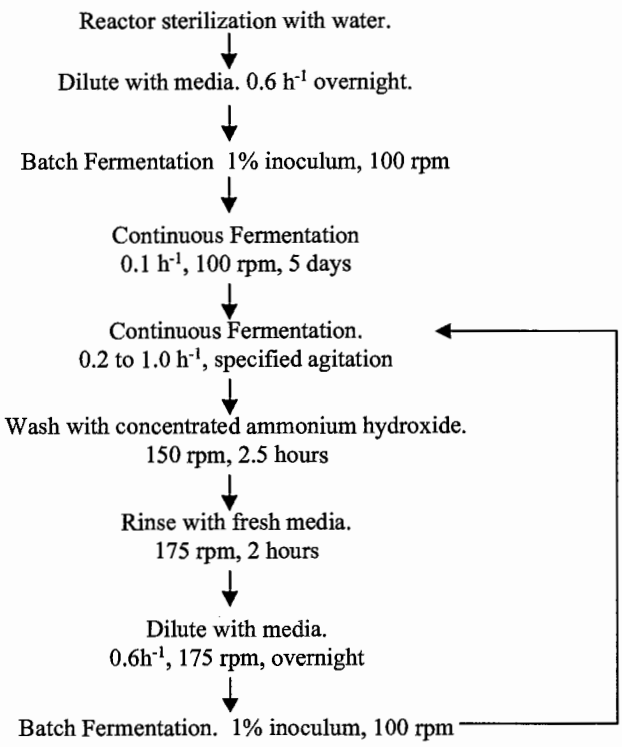

Figure 2.3. Series of steps for repeated continuous lactic acid fermentations for bioreactors protocol.

concentrated ammonium hydroxide for 2.5 h at an agitation of 150 rpm, rinsed with sterile media for 2 h at an agitation of 175 rpm, and diluted with fresh sterile media at a dilution rate of 0.6 h^{-1} overnight. Long exposure at a dilution rate of 0.1 h^{-1} was not necessary for repeat fermentations. The effect of agitation, 100 and 125 rpm, was also evaluated.

Sample Analysis

After reaching steady state, at least 5 working volume exchanges, samples were collected and analyzed for glucose consumption, lactic acid production, and the presence of exopolysaccharide. Fermentation samples were centrifuged, diluted 1:2 and filtered with a 0.45 μm filter prior to being analyzed for D-glucose and L(+)-lactic acid concentrations. Samples were analyzed using Water's high performance liquid chromatograph (HPLC) equipped with column heater, autosampler, computer controller and Water's model 2410 refractive index detector (Milford, MA). Components were separated on a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) with 0.12 N sulfuric acid used as the mobile phase at a flow rate of 0.8 ml/min with a 20 μl injection volume and a 65°C column temperature.

The amount of exopolysaccharides (EPS) (total complex carbohydrates) present in freeze-dried fermentation media was measured according to Ho et al. (10) by comparing the reducing sugar concentration determined by the Somogyi-Nelson to the phenol sulfuric assay (13). The phenol sulfuric assay hydrolzyes any polysaccharides that may be present into monosaccharides. The Somogyi-Nelson assay measures free

reducing sugars prior to hydrolysis. After steady state at each dilution rate was achieved, 500 ml samples were collected. Samples were centrifuged to collect biomass and EPS, washed with deionized water, centrifuged, and freeze dried for carbohydrate analysis.

Results and Discussion

Figure 2.4 and Table 2.1 demonstrate the benefit of PCS tubes fixed to the reactor agitator shaft by increased microbial production rates and yields compared to a reactor with PP tubes (control). Agitation rates also had an effect on production rates and exopolysaccharide production.

At a dilution rate of 0.4 h^{-1} the biofilm reactor demonstrated excellent productivities, 5.1 and 9.0 g/l per hour with acceptable yields of 68 and 70% at agitations of 100 and 125 rpm, respectively. Furthermore, at a dilution rate of 0.6 h^{-1} and agitation of 125 rpm, the biofilm reactor demonstrated excellent productivity and yield of 8.2 g/l per hour and 72%, respectively. Whereas, the PP control reactor demonstrated acceptable performance at a dilution rate of 0.4 h^{-1} and agitation of 125 rpm with a productivity rate and yield of 5.8 g/l per hour and 70%, respectively. The PP control reactor achieved a highest average productivity of 12.0 g/l per hour, at 1.0 h^{-1} dilution rate and 100 rpm agitation. The PCS bioreactor achieved a highest average productivity of 9.88 g/l per hour, at 0.8 h^{-1} dilution rate and 125 rpm agitation. However, the percentage yield for the control and biofilm reactors giving these productivities were unacceptable at 52 and 58%, respectively. The typical percentage yield for *L. casei* is 70 to 72% (8).

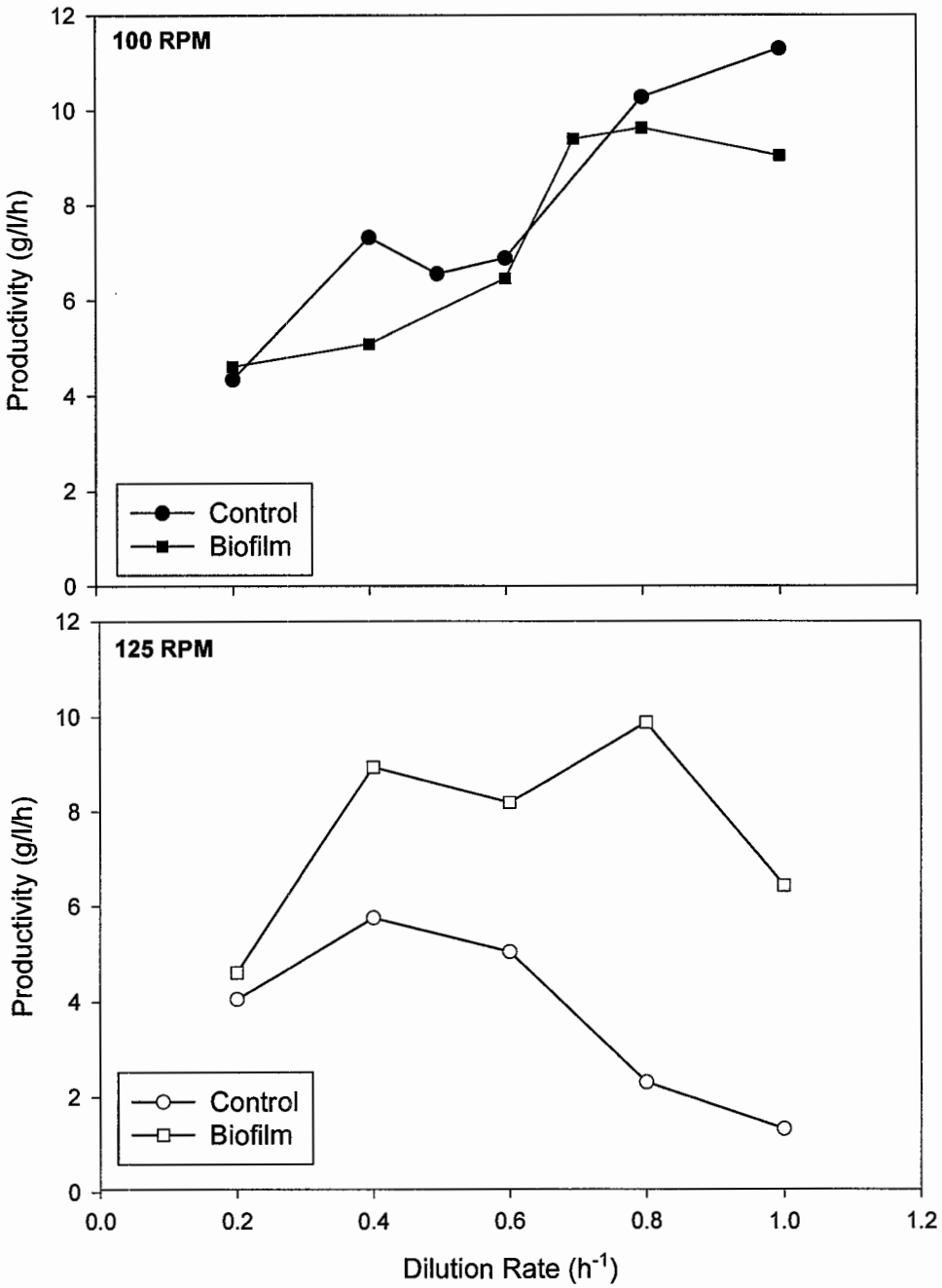

Figure 2.4. Lactic acid production rates for PCS and PP control reactors. Each value is an average of repeated continuous fermentations.

Table 2.1. Summary of results for control and biofilm reactors.[a]

	Dilution Rate h^{-1}				
	0.20	**0.40**	**0.60**	**0.80**	**1.00**
Lactic Acid Production (g/l/h)					
Control-100 rpm	4.34	7.32	6.88	10.27	11.29
Control-125 rpm	4.05	**5.75**	5.04	2.31	1.33
Biofilm-100 rpm	**4.61**	**5.08**	6.45	9.62	9.04
Biofilm-125 rpm	**4.61**	**8.95**	**8.19**	9.88	6.43
% Yield[b]					
Control-100 rpm	64.50	61.39	51.35	55.88	52.38
Control-125 rpm	64.83	**69.55**	48.28	28.78	11.68
Biofilm-100 rpm	**72.12**	**68.89**	52.82	29.63	48.07
Biofilm-125 rpm	**74.73**	**70.70**	**71.68**	58.61	46.95
Final Lactic Acid Concentration (g/l)					
Control-100 rpm	21.68	18.30	11.46	12.84	11.29
Control-125 rpm	20.23	**14.38**	8.39	2.88	1.33
Biofilm-100 rpm	**23.05**	**12.70**	10.75	12.03	9.04
Biofilm -125 rpm	**23.03**	**22.36**	**13.65**	12.34	6.43

[a] Each value is an average of repeated continuous fermentations, with the exception of biofilm reactor at 100 rpm.
[b] Bolded data illustrated acceptable percentage yields of 68 to 75% for *L. casei.*

Conversion yields below 69% represent a physiological shift in the bacterium to the over production of exopolysaccharide and less to lactic acid production. Biofilm formation by *Lactobacillus* to PCS corresponds to EPS production (8). Since EPS is generated by the PCS biofilm then randomly released into the liquid medium, the data presented in Table 2.2 appears inconsistent between repeated fermentations. At a higher agitation, more EPS is sloughed off and suspended in the culture media. Therefore, the amount of EPS reported in Table 2.2 is not the "absolute amount" of EPS present in the reactor. With the data being so inconsistent, no correlations can be made regarding dilution rate or agitation and EPS formation. However, there is a visible increase of suspended EPS at higher dilution rates and increased agitation. There were also visible

Table 2.2 Amount of exopolysaccharide (g/l) in suspended media.

	Dilution Rate h^{-1}				
	0.20	**0.40**	**0.60**	**0.80**	**1.00**
Control-100 rpm	387	252	1528	175	317
Control-100 rpm	x	17	122	745	464
Control-125 rpm	x	2955	221	297	x
Control-125 rpm	808	138	236	391	706
Biofilm-100 rpm	1141	844	1265	2516	2021
Biofilm-125 rpm	x	455	335	849	1993
Biofilm-125 rpm	269	273	465	1333	822

x Samples at this dilution rate were not collected.

changes in the characteristics of the freeze dried media. At higher dilution rates, a flaky, white material was produced. This change in carbon flow by *Lactobacillus* has been observed previously. Krischke et al. (11) reported production rates of 10 and 13.5 g/l per hour at dilution rates of 0.4 and 1.0 h^{-1}, respectively, using a fluidized bed reactor with glass fittings. However, they also reported low yields of 50% for the higher dilution rate. Whereas, a continuous packed bed reactor with foam glass achieved a production rate and yield of 6.16 g/l per hour and 60%, respectively (7). However, this packed bed reactor also made pH control difficult. Gonclaves et al. (5) achieved a production rate of 20.1 g/l per hour with a continuous packed bed made of sintered glass beads. However, the reactor could be operated for only 24 hours and percentage yields were not reported.

The PCS reactors in this research were operated continuously for 3 months. Repeated batch lactic acid PCS biofilm reactors have operated for > 2.5 years in our laboratory. A reactor with PCS tubes fixed to the agitator shaft provides an open continuous bioreactor design for an immobilized cell bioreactor. This design has advantages over packed and fluidized beds and cell recycling bioreactor, which have

problems with reduced flows and membrane fouling. Thus, this bioreactor can increase cell density, productivity and yield for continuous lactic acid fermentations over a longer period of time. Other benefits to PCS biofilm reactors include control of biofilm thickness with agitation and excellent pH control.

Conclusions

The use of PCS tubes fixed to the agitator shaft of a bioreactor demonstrated a positive effect on production rates and yields. A dilution rate of 0.4 h-1 and agitation of 125 rpm demonstrated the highest productivities (9.0 g/l per hour) and yields (71%). Agitation had a significant effect on cell density, biofilm formation, and yields. Further research is needed to optimize and increase the final concentration of lactic acid in the fermentation media to $\geq$120 g/l.

Acknowledgments

This research was supported by the National Corn Grower Association, the Iowa Corn Promotion Board, the Center for Crops Utilization Research of Iowa State University, and the Iowa Agriculture and Home Economics Experiment Station. We thank Jelena Jeremic and Dr. John Strohl for their technical support and the use of equipment in the Iowa State University Fermentation Facility.

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EMULSION LIQUID MEMBRANE EXTRACTION OF LACTIC ACID SOLUTION WITH AND WITHOUT A HOLLOW FIBER CONTACTOR

A paper to be submitted to Applied and Environmental Microbiology

Julie C. Cotton, Anthony L. Pometto III, and Ali Demirci

Abstract

Lactic acid extraction using emulsion-liquid extraction was evaluated. The optimum composition of the organic phase of the emulsion contains 91% (vol/vol) solvent, 5% (vol/vol) carrier, and 4% (vol/vol) surfactant. The most effective concentration of sodium carbonate (stripping solution) for iso-octane:tri-butyl phosphate and hexane:Alamine 304 was 1.2 and 0.6 M, respectively. The initial pH of aqueous lactic acid solution affected product recovery, with low pH (2.0) being optimum. Optimal lactic acid recovery of 53 and 47% for iso-octane:tri-butyl phosphate and hexane: Alamine 304, respectively, were achieved when extraction was carried out in an orbital shaker at 37°C and 125 rpm for 15 minutes. Lactic acid recovery using emulsion liquid extraction in a hollow fiber contactor was also evaluated. Using the hollow fiber contactor to prevent intimate interaction between emulsion and lactic acid solutions minimizes swelling and increases stability of the emulsion. The hydrophobic nature of the polypropylene fibers allowed the lactic acid solution to be pumped through shell side and emulsion phase through the lumen. Since pH of the lactic acid solution affects recovery, lactic acid solution was adjusted to pH 5.0 to simulate fermentation conditions of *Lactobacillus casei*. Recovery by emulsion liquid extraction in the hollow fiber was

minimal due to the controlled pH at 5.0 of aqueous solution and incompatibility of solvents and hollow fiber material.

Introduction

Microbial production of lactic acid used for food and non-food applications offers many advantages over chemical synthesis. As a food additive, it qualifies as natural. The newest non-food market for lactic acid is the production of polylactic acid (PLA) degradable plastic. Long chain polymerization requires one specific form, thermally stable L(+) or D(-)-lactic acid, which can only be produced by microorganisms. However, for fermentation to be competitive with chemical synthesis, improvements are needed to increase microbial production rates and recovery processes. Increased cell density and high productivities have been achieved by strain development (4), hollow fiber fermentors (14), cell-recycle reactors (13), and biofilm reactors (5, 7). However, down stream recovery processes still remain the significant expense for lactic acid production.

Liquid-liquid extraction employs organic solvents to extract organic acids and metal ions from aqueous waste streams and fermentation broth. The process involves two steps: extraction of lactic acid from the aqueous fermentation broth into an organic phase, then back extracting the lactic acid from the organic phase with stripping solution into an aqueous solution. The organic phase consists of organic diluents and extractants (carrier compounds) with high selectivity for the organic acid being extracted. Besides the chemical nature of the carrier compound, extraction is affected by several factors: extractant concentration, diluent, pH of aqueous solution, and temperature.

The criteria for solvents used in extraction of organic acids include: biocompatibility, high distribution coefficient, solubility, viscosity, selectivity, cost, and environmental risks. Of the extractants studied for lactic acid extraction, long chain aliphatic amines (i.e., Alamine 336, Alamine 304, Aliquat 336, and tri-octyl amine) and phosphorous containing compounds (i.e., tri-butyl phosphate and tri-octylphosphine oxide) are suitable (2, 9, 10, 16, 17, 18, 19).

Extraction by solvents and stripping processes may be combined and performed simultaneously in a process known as emulsion liquid membrane extraction (11). The organic phase serves as a hydrophobic liquid membrane between the external aqueous phase (fermentation broth) and internal aqueous phase (stripping solution) (Figure 3.1). The organic phase contains a surfactant for emulsion stabilization. Scholler et al. (15) and Demirci et al. (6) have evaluated several solvent/carrier combinations for emulsion liquid extraction of lactic acid.

However, toxicity of solvents and pH requirements for extractive fermentation remain the major disadvantages of liquid-liquid and emulsion liquid membrane extraction. Optimal recovery is achieved at a pH below or near the pKa of the

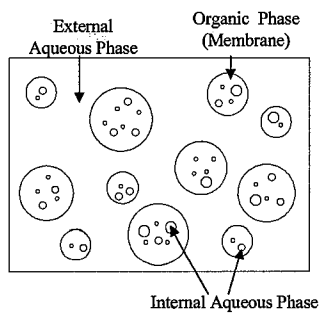

Figure 3.1 Schematic of emulsion liquid membrane (3).

organic acid being extracted. Most solvents used in these recovery processes demonstrate a toxic effect toward microorganisms (2, 6, 7, 16, 20). Toxicity is of two levels, molecular and phase level. Molecular toxicity is caused by the soluble portions of solvents. Whereas, phase level toxicity results from the presence of two phases (organic and aqueous). For *in situ* extraction, toxicity is the major concern. Toxicity can be reduced with cell immobilization via biofilm reactors (7) or by using a physical barrier, such as hollow fibers, to reduce direct contact between organic and aqueous phases (19).

This research evaluated the effect of carrier, surfactant, and stripping solution concentrations on lactic acid recovery to optimize the emulsion composition using selected solvents and carriers with minimal and high toxic effect on *Lactobacillus casei.* The effect of pH on emulsion liquid membrane extraction is investigated. Lactic acid recovery via emulsion liquid extraction in a hollow fiber contactor with a toxic solvent combination was evaluated.

Materials and Methods

Preparation of Aqueous Lactic Acid Solution:

Fresh concentrated lactic acid solution (85%) (Sigma Chemical Compnay, St. Louis, MO) was diluted to 100 g/L and heated to 95°C to eliminate the presence of lactic anhydride. Deionized water was added during heating to maintain the volume. To study the effect of the initial pH of lactic acid solution on extraction, lactic acid solution was adjusted with 4 N sodium hydroxide.

Preparation of Emulsion Phase

Demirci et al. (6) screened organic solvents and carrier compounds for optimal lactic acid extraction and degree of toxicity to *L. casei*. Solvent combinations hexane:Alamine 304 and xylene:Alamine 304 have good recovery and minimal toxicity to *L. casei* immobilized by biofilm (7). Iso-octane:tri-butyl phosphate and N-dodecane:tri-butyl phosphate have good recovery, but have a high toxic effect. These solvents were chosen to be further evaluated and optimized (Table 3.1). All combinations contained sorbitan monooleate (Span 80; Sigma Chemical Company, St. Louis, MO) as a surfactant and sodium carbonate solution as the stripping solution.

The emulsion phase was prepared according to Demirci et al. (6) using a Sorvall Omni-Mixer (Norwalk, Connecticut). Solvent, carrier and surfactant were mixed for one minute at 1000 rpm to prepare the organic phase. Sodium carbonate solution (stripping solution) was slowly added at 5700 rpm until the volume ratio of organic phase to stripping solution reached 1:1. The mixture was further emulsified at 12,700 rpm for five minutes in an ice bath.

Emulsion Liquid Membrane Extraction

After preparation of emulsion phase, the emulsion was transferred to a vial and an equal amount of lactic acid solution was added. The vials were placed in a 37°C orbital shaker in a tilted position and shaken for 15 min at 125 rpm. After an hour at ambient temperature for phase separation, aqueous phase was collected and the residual lactic acid concentration was determined by HPLC.

Table 3.1. Solvent:carrier combinations used for emulsion liquid membrane extraction.

Solvent	Carrier	Toxicity
Hexane[a]	Alamine 304[b]	Low
Xylene[a]	Alamine 304	Low
Iso-octane[a]	Tri-butyl Phosphate[c]	High
N-dodecane[a]	Tri-butyl Phosphate	High

[a]Fisher Scientific, Fair Lawn, NJ.
[b]Henkel Inc., Kankakee, IL.
[c]Sigma Chemical Company, St. Louis, MO.

Emulsion Liquid Extraction in a Hollow Fiber Contactor

Flow rates and pressures of aqueous and emulsion streams were varied to determine optimal extraction conditions using a hollow fiber contactor (Liqui-Cel Model G21, Hoechst Celenase, Charlotte, NC). The hydrophobic nature of the polypropylene fibers allows the emulsion phase to be pumped on the shell side of the contactor.

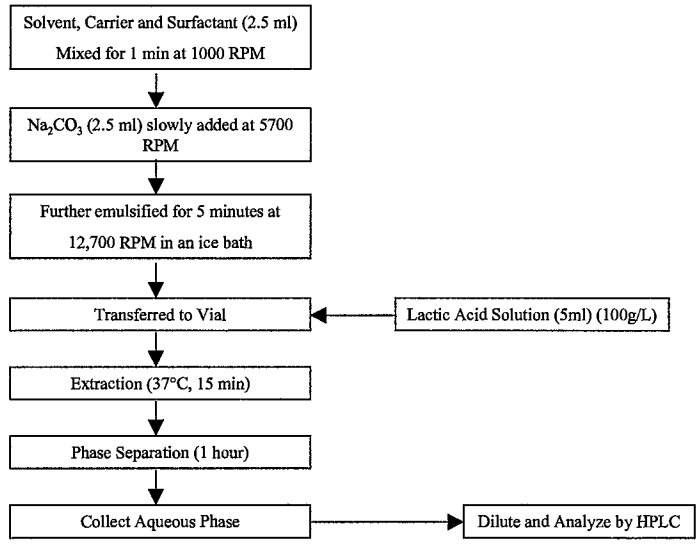

Figure 3.2. Steps for emulsion preparation and emulsion liquid membrane extraction.

Figure 3.3 shows the experimental setup for emulsion liquid extraction in the hollow fiber contactor. The organic phase was comprised of 91% (vol/vol) iso-octane, 5% (vol/vol) tri-butyl phosphate, 4% (vol/vol) Span-80, and 1.2 M Na_2CO_3 was used as the internal aqueous phase. Both reservoirs were maintained at room temperature and agitated slowly. To simulate fermentation conditions, the lactic acid solution was initially adjusted to pH 5.0 with 4 N NaOH and adjusted during extraction with 4 N HCl to maintain a pH of 5.0. Samples collected over time from aqueous reservoir were diluted 1:3 prior to analysis. To recover the internal aqueous phase, the 5 ml samples in 15 x 2.5 cm culture tubes were heated (125°C) on a heating block for 4 to 6 hours capped with a glass stopper. The length of tube exposed to air acted as a condensing unit and allowed the aqueous phase to be collected. The aqueous phase was diluted 1:2 and analyzed by HPLC.

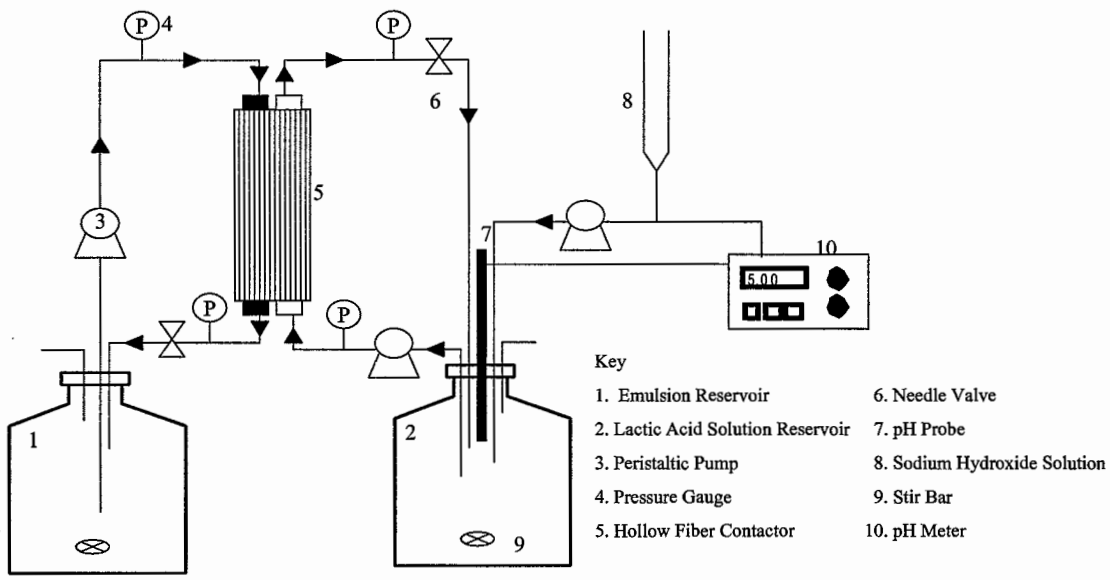

Figure 3.3. Schematic representation of emulsion liquid extraction with a hollow fiber unit.

Analysis

Lactic acid concentrations were analyzed using Hewlett Packard's and Water's high performance liquid chromatograph (HPLC) each equipped with column heater, autosampler, and Water's model 2410 refractive index detector (Milford, MA). Lactic acid was separated on a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) with 0.12 N sulfuric acid used as the mobile phase at a flow rate of 0.8 ml/min with a 20 µl injection volume and a 65 °C column temperature.

Results and Discussion

Effect of Carrier Concentration

To evaluate the effect of carrier concentration on lactic acid recovery, the amount of carrier in the organic phase was varied from 2.5 to 10%. The organic phase also contained 4% (vol/vol) Span-80, and 0.6 M Na_2CO_3 was used as the internal aqueous phase. The use of 2.5% (vol/vol) carrier did not form a stable emulsion. Extraction with 5% (vol/vol) carrier gave the highest recovery for all solvent combinations (Table 3.2). Maximal extraction by liquid-liquid extraction has been reported with up to 50% (vol/vol) extractant (2, 9, 16). An increase in carrier concentration in emulsion liquid membrane extraction has been reported to increase recovery for emulsion liquid membrane extraction as well (12, 1). However, Chaundhuri and Pyle (1) indicate in emulsion liquid membrane extraction, a carrier concentration > 10% (vol/vol) can act antagonistically to emulsion stabilizing surfactant.

Table 3.2. Summary of results from emulsion liquid membrane extraction at pH 2.

	Iso-Octane: Tri-butyl Phosphate		Hexane: Alamine 304		Xylene: Alamine 304		N-Dodecane: Tributyl Phosphate	
Experiment	% Recovery	Volume Loss	% Recovery	Volume Loss	% Recovery	Volume Loss	% Recovery	Volume Loss
Carrier Concentration[a]								
2.5	42.75	13	28.32	17	35.27	-11	29.84	22
5.0[d]	39.48	-10	40.16	17	35.30	-16	36.19	-14
7.5	37.16	-25	40.06	20	33.63	-18	34.77	-32
10.0	34.75	-31	33.83	19	34.53	-20	33.40	-33
Surfactant Concentration[b]								
4.0	38.37	-5	47.38	23				
6.0[e]	43.74	13	50.29	29				
8.0[e]	39.15	17	50.00	36				
Stripping Solution Concentration[c]								
0.3[d]	41.86	1	41.48	17				
0.6	40.42	-17	46.63	12				
1.2	52.27	-7	46.86	16				
1.8[d]	55.89	8	57.25	31				

[a] 4% Span-80, 0.6 M Na_2CO_3
[b] 5% carrier, 0.6 M Na_2CO_3
[c] 4% Span-80, 5% carrier
[d] Did not form a stable emulsion.
[e] Unable to break emulsion.

Effect of Surfactant Concentration

Surfactant concentrations of 4, 6, and 8% (vol/vol) of the organic phase were evaluated using 5% (vol/vol) carrier in the organic phase and an internal phase of 0.6 M Na_2CO_3. Besides the ability to extract, emulsion stability and the ability to break the emulsion to recover the aqueous phase are important factors in the selecting solvents and carriers for emulsion liquid membrane extraction. Surfactant concentration has a large effect on these factors. Under 4 % of surfactant, emulsions were not stable. Xylene:Alamine 304 with 4% (vol/vol) Span-80 could never be broken. Although recovery increased with surfactant concentration (Table 3.2), iso-octane:tri-butyl phosphate and hexane:Alamine 304 combinations with 6 and 8% (vol/vol) surfactant could not be broken. The increase of surfactant concentration also increased swelling, as noted by the volume loss of aqueous phase (Table 3.2). These combinations that increase swelling and cannot be broken are not suitable for emulsion liquid membrane extraction. Previous research also suggests an increase of surfactant benefited lactic acid recovery, but also increases swelling of the emulsion which represents migration of external aqueous phase into the emulsion (12, 1).

Effect of Stripping Solution Concentration

The concentration of sodium carbonate solution had an effect on lactic acid recovery and emulsion stability (Table 3.2). Solvent combinations with 0.3 and 1.8 M Na_2CO_3 did not form stable emulsions. The optimum lactic acid recovery (53%) from aqueous solution (100 g/l) for an organic phase of 91% (vol/vol) iso-octane, 5% (vol/vol) tri-butyl phosphate, and 4% (vol/vol) Span-80 was achieved with 1.2 M Na_2CO_3. When

91% (vol/vol) hexane, 5% (vol/vol) Alamine 304, and 4% (vol/vol) Span-80 was used for the organic phase, lactic acid recovery was not increased by increasing the stripping solution concentration from 0.6 to 1.2 M (Table 3.2). The concentration of stripping solution also affected emulsion swelling (Table 3.2). Scholler et al. (15) indicate an increase in concentration of stripping solution increased the initial extraction rate, but the overall recovery for 0.57 and 1.13 M stripping solutions remained constant.

Effect of Lactic Acid Solution Initial pH

The initial pH of lactic acid solution had a significant effect on lactic acid recovery (Figure 3.4). Greatest recovery was achieved at pH 2.0, well below the pKa of lactic acid. The pH requirements of extractive fermentation still remain a large hurdle. Liquid-liquid extraction by Choundhury et al. (2) and Seevaratham et al. (16), and emulsion liquid membrane extraction by Scholler et al. (15) report similar results.

Emulsion Liquid Extraction with a Hollow Fiber Contactor

Recovery using a hollow fiber unit with the model system (organic phase of 91% (vol/vol) iso-octane, 5% (vol/vol) tri-butyl phosphate, 4% (vol/vol) Span 80 and internal aqueous phase of 1.2 M Na_2CO_3) was not greatly affected by pressure and flow rates (Table 3.3). A maximum recovery rate of 1.34 g per hour were achieved. The recovery rate measured by the aqueous phase was higher than the recovery rate measured by the emulsion phase. This could be caused by the small amounts that were recovered, but could also indicate that not all the lactic acid can be recovered after breaking the

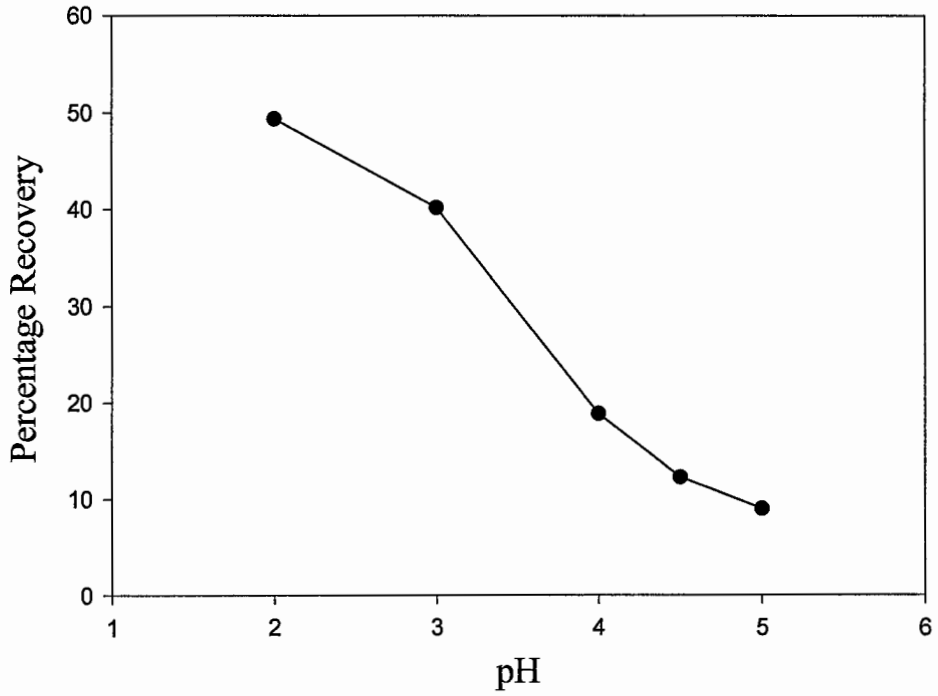

Figure 3.4. Effect of initial lactic acid solution pH on lactic acid recovery. Organic phase employed contained 91% (vol/vol) iso-octane, 5% (vol/vol) tri-butyl phosphate, and 4% (vol/vol) Span-80, and 1.2 M Na_2CO_3 used as the internal stripping solution. Each point is an average of two replicates.

emulsion. Recovery was limited due to the controlled pH of the aqueous lactic acid solution at 5.0. The organic phase was not compatible with the material of the hollow fibers, and thus gave membrane wet-out (crossing over of aqueous phase into emulsion phase) and minimal trials. Therefore, this solvent combination is not recommended for long-term recovery with hollow fiber units.

Table 3.3. Summary of emulsion liquid extraction in a hollow fiber contactor.[a]

Aqueous Flow Rate (ml/min)	Emulsion Flow Rate (ml/min)	Aqueous Pressure (psi)	Emulsion Pressure (psi)	Lactic Acid Recovery (g/h)[b]	Lactic Acid Recovery (g/h)[c]
12	10	10-15	1-5	1.34[d]	0.84[d]
12	10	25-30	1-5	1.19[d]	0.93[d]
12	10	25-30	5-10	0.80	0.56
36	30	10-15	1-5	0.59	0.40
36	30	25-30	5-10	0.71	0.60

[a] Lactic acid solution contained 85 g/l lactic acid with pH controlled at 5.0.
[b] Recovery rate measured by aqueous lactic acid solution samples.
[c] Recovery rate measured by aqueous phase of emulsion samples broken with heat.
[d] Average of two replicates

Conclusions

The optimum emulsion composition for lactic acid recovery is an organic phase consisting of an organic phase of 91% (vol/vol) solvent, 5% (vol/vol) carrier, and 4% (vol/vol) Span-80 and an internal phase of 0.6 and 1.2 M Na_2CO_3 for hexane:Alamine 304 and iso-octane:tri-butyl phosphate, respectively. Lactic acid recovery using emulsion liquid extraction in a hollow fiber contactor offers many challenges, with solvent and fiber compatibility being the most important.

Acknowledgments

This research was supported by the National Corn Grower Association, the Iowa Corn Promotion Board, the Center for Crops Utilization Research of Iowa State University, and the Iowa Agriculture and Home Economics Experiment Station.

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GENERAL CONCLUSIONS

The use of PCS tubes fixed to the agitator shaft of a bioreactor demonstrated a positive effect on production rates and yields. Agitation had a significant effect on biofilm formation which was controlled by agitation. A PCS biofilm continuous reactor has the potential to operate as a truly open system for long periods of time. Other benefits to this PCS biofilm reactor design includes control of biofilm thickness with agitation and excellent pH control. Further research must be conducted to optimize fermentation conditions and to increase the final concentration of lactic acid in the fermentation media.

Emulsion liquid membrane extraction was evaluated to determine the optimum lactic acid recovery conditions. For lactic acid recovery, an organic phase of 91% (vol/vol) solvent, 5% (vol/vol) carrier, and 4% (vol/vol) Span-80 and an internal phase of 0.6 and 1.2 M Na_2CO_3 for solvent combinations hexane:Alamine 304 and iso-octane:tri-butyl phosphate, respectively, were optimal. The large effect of initial pH of external aqueous phase on lactic acid recovery is a hurdle yet to be overcome with emulsion liquid membrane extraction. Furthermore, lactic acid recovery using emulsion liquid extraction in a hollow fiber contactor offers many challenges, with solvent and fiber compatibility being the most important.

ACKNOWLEDGMENTS

I would like to extend my greatest appreciation and sincere thanks to my major professor, Dr. Anthony L. Pometto III, for his support, advice, guidance, and encouragement. A large thank you goes to Drs. Ali Demirci and John Strohl for their support and technical guidance throughout my research. I would also like to thank my lab-mates for their willingness to take samples and creating a great working environment: Dr. Kai-Lai Grace Ho, Mohammad Khiyami, Jelena Jeremic, Ben Voss, and Susan Urbance. I would also like to thank my dear friends Pat Flannery, Adrianna Heywood, Marcy Billings, and Kerry Rees for their kind words and support. Finally, I would like to extend my love and thank to my parents and family for their constant support, encouragement, patience, and love.