Media Evaluation of Lactic Acid Repeated-Batch Fermentation with *Lactobacillus plantarum* and *Lactobacillus casei* Subsp. *rhamnosus*[†]

Ali Demirci,[‡] Anthony L. Pometto III,*[,‡] Byungtae Lee,[‡] and Paul N. Hinz[§]

Department of Food Science and Human Nutrition and Department of Statistics, Iowa State University, Ames, Iowa 50011

This paper describes a methodology to evaluate different fermentation media without interruption for long-term repeated batch fermentation by *Lactobacillus casei* subsp. *rhamnosus* or *Lactobacillus plantarum*. A repeated-batch fermentor was designed to evaluate various food byproducts as nitrogen source to replace yeast extract in lactic acid fermentation. Seven types of livestock- or marine-based byproducts were used in addition to corn steep liquor and yeast extract. Also, further enrichment of vitamins and minerals in the fermentation media was performed by the addition of 0.1% cane molasses. Data from lactic acid fermentation in duplicate were analyzed by analysis of variance and least significant difference. Marine hydrolysate BAP5301 supplemented with cane molasses resulted in a higher lactic acid production rate than that of yeast extract medium for *L. plantarum*. On the other hand, marine hydrolysate BAP5300 supplemented with cane molasses was the best for *L. casei*, which produced lactic acid similar to yeast extract supplemented medium.

Keywords: *Lactic acid; medium; Lactobacillus casei; Lactobacillus plantarum*

INTRODUCTION

Lactic acid exists in two optically active anantiomers, L-(+) and D-(−) (VickRoy, 1985). Lactic acid has found applications in the food industry as well as in nonfood industries. The newest market for lactic acid is in the production of polylactic acid (PLA)-based degradable plastics (Kharas et al., 1994). Lactic acid can also be used as a "green" solvent and as a slow release carrier for agricultural and pharmaceutical chemical delivery (Datta et al., 1995).

Lactic acid can be produced chemically from acetaldehyde and hydrogen cyanide or via microbial fermentation (Holten et al., 1971). The advantages of microbial fermentation over chemical synthesis of lactic acid from petroleum-based products are utilization of renewable carbon sources such as corn and exclusive production of the L- or D-isomer of lactic acid, which is critical in PLA production. However, fermentation must be cost-competitive with chemical synthesis.

Numerous attempts have been made to improve fermentation to decrease the production cost of lactic acid. Accelerated microbial production rates and high concentrations of lactic acid were achieved by strain development (Demirci and Pometto, 1992). Because lactic acid production is both type I (growth associated) and type II (non-growth associated), production rates can be increased by increasing cell densities in the bioreactor. To achieve this goal, hollow fiber (Roy et al., 1982), cell-recycled (Ohleyer et al., 1985), and artificially immobilized cell (Boyaval and Goulet, 1988) reactors were developed. However, high start-up costs and short lifetimes were drawbacks. On the other hand, biofilm reactors with plastic composite support (Demirci and Pometto, 1995; Ho et al., 1997a−c) demonstrated increased productivity rates and long-term (>2 months) slow release of nutrients.

A high lactic acid concentration in the fermentation broth inhibits growth of lactic acid bacteria, which can stop lactic acid production. Some integrated separation/recovery techniques have been coupled with fermentation to remove lactic acid continuously from the fermentation broth. Methods employed have included dialysis (Friedman and Gaden, 1980), electrodialysis (Namura et al., 1991), ion-exchange resin (Vaccari et al., 1993), biparticle fluidized-bed bioreactor (Kaufman et al., 1994), reverse osmosis (Schlicher and Cheryan, 1990), and liquid−liquid extraction (Hano et al., 1993).

All of these developments play an important role in reducing the production cost of lactic acid. However, cost of the medium can represent >30% of the total cost of production. Therefore, new low-cost nutrients for lactic acid fermentation are vital. Yeast extract is used in most laboratory fermentation media as the main source of nitrogen and vitamins. However, it is too expensive for industrial use. Corn steep liquor is an inexpensive source of essential nutrients. However, some residual lactic acid in corn steep liquor can negatively affect product purity of D- or L-lactic acid, and vitamin supplements may still be required.

This study evaluates animal and marine byproducts as nutrient substitutes for yeast extract in lactic acid fermentation. We developed a repeated-batch fermentation system that allowed aseptic medium blending and operated continuously without contamination for ∼5 months.

* Address correspondence to this author at the Department of Food Science and Human Nutrition, 2312 Food Sciences Building, Iowa State University, Ames, IA 50011 [telephone (515) 294-9425; fax (515) 294-8181; e-mail apometto@iastate.edu].

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[‡] Department of Food Science and Human Nutrition.

[§] Department of Statistics.

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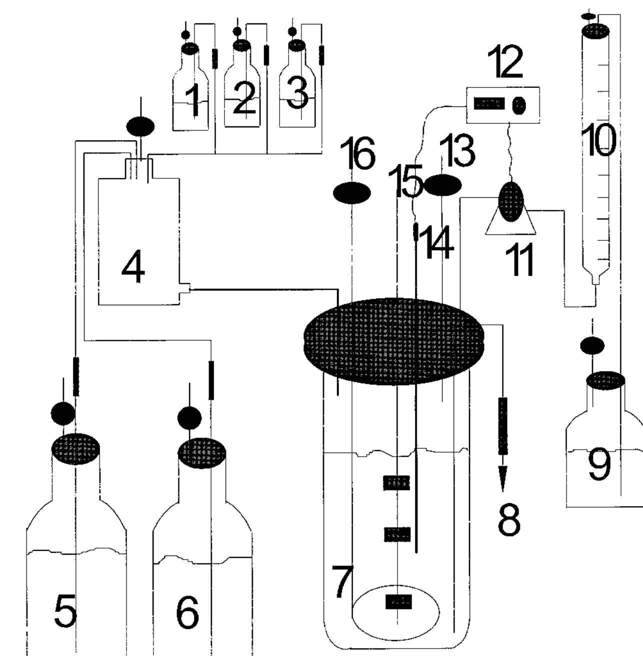

Figure 1. Schematic diagram of repeated-batch fermentor.

MATERIALS AND METHODS

Microorganism. *Lactobacillus casei* subsp. *rhamnosus* (ATTC 11443) and *Lactobacillus plantarum* (ATCC 14917) were maintained as freeze-dried cultures and as working cultures by monthly transfers in MRS broth (Difco Laboratories, Detroit, MI) and then stored at 4 °C. The culture medium used contained 20 g of glucose (Cerelose, International Ingredients, St. Louis, MO), 10 g of yeast extract (YE) (Ardamine-Z, Champlain Industries, Inc., Clifton, NJ), 6 g of $MgCl_2{\cdot}7H_2O$, 0.3 g of $MnSO_4{\cdot}H_2O$, 10 g of sodium acetate, 5 g of KH_2PO_4, and 5 g of K_2HPO_4 per liter of deionized water.

Reactor. Microferm benchtop fermentor (New Brunswick Scientific, Edison, NJ) equipped with pH, temperature, and agitation controls was employed. The 7.5 L vessel was equipped with filtered air in and out, alkali, medium addition, and broth removal ports (Figure 1). To control pH, alkali was added from a graduated buret, which was refilled aseptically from a reservoir. Before medium was pumped into the fermentation vessel, glucose, salt, and complex nutrients were transferred aseptically into a medium mixing carboy. The medium mixing carboy was equipped with 15 inlet ports and lines with quick connectors and an air port with filter. Once the desired blend was made up to volume, it was transferred from the bottom exit line to the fermentor vessel by pressuring the carboy with sterile air from the filter air port.

Fermentation Media. Media consisted of 80 g/L glucose (Cerelose), 6 g/L $MgCl_2{\cdot}7H_2O$, 0.3 g/L $MnSO_4{\cdot}H_2O$, 10 g/l sodium acetate, 5 g/L KH_2PO_4, 5 g/L K_2HPO_4, and 40 g/L complex nutrient; yeast extract (Champlain Industries, Clifton, NJ), corn steep liquor (Markor, Hackensack, NJ), and animal or marine byproducts (American Protein Corporation, Ames, IA). Fermentations were performed with or without 0.1% cane molasses (Westway Trading, Houston, TX). Table 1 lists the complex nutrients used.

Stock Media Preparation. Ninety-five liters of deionized water or 2× salt solution containing 12 g of $MgCl_2{\cdot}7H_2O$, 0.6 g of $MnSO_4{\cdot}H_2O$, 20 g of sodium acetate, 10 g of KH_2PO_4, and 10 g of K_2HPO_4 per liter of deionized water was sterilized in a B. B. Braun U-100 fermentor (Allentown, PA) with constant agitation (220 rpm) at 121 °C for 30 min. Sterile medium was then aseptically dispensed into sterile 50-L carboys with medium outlet, medium filling, and sterile air-inlet ports.

Table 1. Summary of Complex Nutrient Blend Used in Fermentation Culture Media[a]

complex nutrient	cane molasses 0.1% (w/v)	code
yeast extract	−	YE
corn steep liquor	−	CSL
corn steep liquor	+	CSLC
chicken broth	−	AP1020
chicken broth	+	AP1020C
hydrolyzed pork protein	−	AP5020
hydrolyzed pork protein	+	AP5020C
hydrolyzed beef protein	−	AP5030
hydrolyzed beef protein	+	AP5030C
hydrolyzed beef serum concentrate	−	AP5135
hydrolyzed beef serum concentrate	+	AP5135C
marine hydrolysate	−	BAP5300
marine hydrolysate	+	BAP5300C
marine hydrolysate	−	BAP5301
marine hydrolysate	+	BAP5301C

[a] All products were supplied as dry powders.

For each medium blend, a concentrated solution of each nutrient was prepared in a separate bottle. (1) The 500 mL of nitrogenous medium containing 40 g of yeast extract, corn steep liquor (CSL), or animal and marine byproducts; (2) the 500 mL of glucose solution containing 350 g of glucose (cerulose); or (3) the 500 mL of cane molasses medium containing 4 g of cane molasses was sterilized in a 1-L bottle equipped with a filtered air port and a medium exit port connected to one line with stainless steel liquid connector. All three or two bottles were autoclaved at 121 °C for 1 h. A 7.4 N NH_4OH solution was prepared for pH control by adding 2.5 L of concentrated NH_4OH (14.8 N) to 2.5 L of sterile deionized water in a 10-L carboy equipped with a filtered air port and a medium exit port and line which was connected to the alkali addition buret.

Repeated-Batch Fermentation. Five-hundred milliliters of glucose solution, 500 mL of nitrogenous medium (animal, marine byproducts, YE or CSL), and 2 L of salt solution were transferred to a medium mixing carboy, mixed, and then transferred into the fermentation vessel by pressuring the carboy with filter-sterilized air. One liter of deionized water was transferred into the carboy to wash the chamber and feed line and then transferred into the fermentation vessel, which made the final working volume (4 L). After a fresh 40-mL stock culture was added, the vessel was flushed with carbon dioxide for 10 min. Medium pH was maintained at 6 and 5 for *L. plantarum* and *L. casei*, respectively, with 7.4 N NH_4OH solution addition. Temperatures were maintained at 30 and 37 °C for *L. plantarum* and *L. casei*, respectively. Agitation was set at 200 rpm. Samples were taken three or four times a day for 5 days. Alkali consumption was also recorded at each sampling time. At the end of fermentation, medium was drained from the vessel by pressurizing the fermentation vessel, and new medium was aseptically transferred into the empty vessel to begin the next repeated-batch fermentation.

Analysis. Samples were analyzed for cell density at 620 nm by using a Spectronic 20 and for glucose and lactic acid concentrations by using a Waters high-pressure liquid chromatograph (Waters Corp., Milford, MA) equipped with a Waters model 401 refractive index detector, column heater, autosampler, and computer controller. Lactic acid and glucose were separated on a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using 0.012 N sulfuric acid 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.

Statistical Analysis. The order of each medium blend addition to the fermentation was randomized, and the duplicate fermentations were initiated only after the first fermenta-

tions of all the medium compositions were completed. The data were analyzed using an analysis of variance for a completely randomized design using Statistical Analysis System package (version 6.09) (SAS Institute, Cary, NC). The 5% least significant differences (LSD) was used to determine significance between treatment means. Overall significance level (p value) among culture media from the analysis of variance is presented in each figure legend.

RESULTS AND DISCUSSION

Repeated-batch fermentations with *L. plantarum* and *L. casei* were performed in media containing various animal and marine byproducts (AP1020, AP5020, AP5030, BAP5300, and BAP5301) and CSL at the concentration of 10 g/L with or without 1 g/L cane molasses to compare with the 10 g/L YE medium. The fermentation design used here was unique in its capacity to evaluate different media efficiently. Between each batch fermentation, reactor cleaning and reautoclaving were not needed as long as the working microorganism was not changed. The mixing carboy and lines were washed with sterile deionized water, which was then transferred into the fermentation vessel to make the final volume of 4 L. For each culture medium inoculation, 40 mL of fresh culture was injected into the fermentation vessel in addition to the residual culture medium from the previous batch fermentation. Fresh culture inoculation was essential to ensure repeatable fermentation. Because *L. plantarum* and *L. casei* are facultative anaerobes, media were flushed with CO_2 for 10 min at the beginning of each fermentation to decrease dissolved oxygen. To maintain pH at 6 and 5 for *L. plantarum* and *L. casei*, respectively, 7.4 N NH_4-OH solution in a graduated buret was used, which allowed continuous and rapid estimation of the production rate and the lactic acid concentration based on alkali consumption.

The order of each medium blend addition to the fermentation was randomized and performed in replicates of two. There were high correlations, 0.88 and 0.93, between lactic acid concentrations and production rates for both *L. plantarum* and *L. casei*, respectively, as expected. However, correlations between production rates and growth rates were poor for both cultures because of the inaccurate absorbance readings due to the suspended particles in the media.

L. plantarum. The LSD suggested that YE was not different from BAP5301C, BAP5301, CSLC, and CSL in terms of lactic acid concentration. Also, YE was not different from BAP5301C in terms of production rate (Figure 2). Therefore, BAP5301C could be used to replace with YE for lactic acid fermentation using *L. plantarum.*

When the fermentation media were supplemented with 1 g/L cane molasses, all measured parameters were only slightly improved. For example, averages of lactic acid concentrations were 45.7 and 51.9 g/L without and with cane molasses, respectively. AP5020 and AP5030 produced 35.7 and 34.8 g/L lactic acid without cane molasses but 45.9 and 48.9 g/L lactic acid with cane molasses, respectively (Figure 2). These higher concentrations were achieved by slightly higher lactic acid production rates for both.

BAP5300 benefited significantly by cane molasses supplement, because lactic acid concentration, production rate, and yield increased from 39.9 to 52.4 g/L, from 0.46 to 0.77 g L^{-1} h^{-1}, and from 82.6 to 85.1% with cane molasses, respectively. Cane molasses seemed not to increase lactic acid concentration with BAP5301, be-

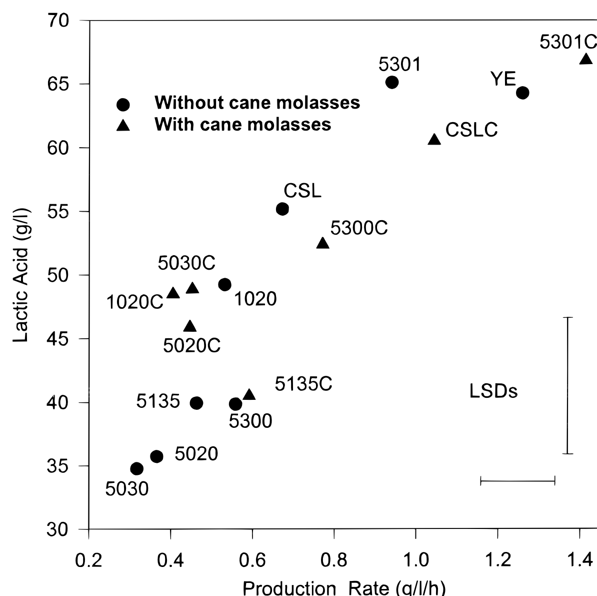

Figure 2. Final lactic acid concentrations and production rates in repeated-batch fermentation with *L. plantarum* in various media. Each point is the average of two replicates. Correlation coefficient (r) is 0.88. Significance levels from the analysis of variance are $p = 0.0001$ for both lactic acid concentration and production rate.

cause possible maximum lactic acid concentration (~60 g/L) due to product inhibition had been reached. However, cane molasses benefited BAP5301 in terms of a faster lactic acid production rate. Therefore, YE, CSL, and animal and marine byproducts can be grouped in terms of final lactic acid concentration by using LSD (7.7 g/L) to determine averages of fermentation with or without cane molasses as BAP5301, YE > CSL > AP1020, BAP5300 > AP5030, AP5020, AP5135 (Figure 2).

L. casei. *L. casei* in YE medium produced 67.6 g/L lactic acid, whereas BAP5300 with cane molasses produced 61.9 g/L lactic acid, which was not significantly different within the LSD interval for lactic acid (Figure 3). Also, production rates (1.17 and 0.95 g L^{-1} h^{-1}) and yields (83.9 and 81.7%) of YE and BAP5300C, respectively, were not significantly different (Figure 3).

The data showed that lactic acid fermentation with *L. casei* was improved when animal and marine byproducts were supplemented with 0.1% (w/v) cane molasses. Overall, the animal and marine byproducts with cane molasses did not have a significant effect on yield ($p = 0.62$), whereas they had a significant effect on lactic acid concentration ($p = 0.01$) and production rate ($p = 0.02$). The overall averages of lactic acid concentrations were 30.4 and 36.4 g/L without and with cane molasses, respectively (Figure 3).

Surprisingly, cane molasses did not benefit CSL lactic acid concentration, which dropped from 46.3 to 40.6 g/L lactic acid in the presence of cane molasses. This was not significantly different within the LSD of 11.5 g/L lactic acid, whereas the production rate decreased slightly (Figure 3). BAP5300 and BAP5301, respectively, produced 49.3 and 36.1 g/L lactic without cane molasses and 61.9 and 54.0 g/L lactic acid with cane molasses under the same fermentation conditions.

AP1020 produced 30.1 g/L without and 35.8 g/L lactic acid with cane molasses, which also increased production rate. Addition of cane molasses to CSL, AP5020, AP5030, and AP5135 demonstrated no benefit, which

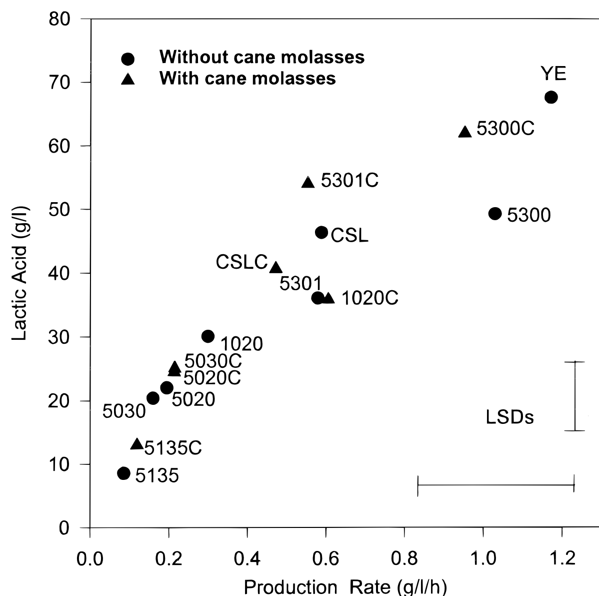

Figure 3. Final lactic acid concentrations and production rates in repeated-batch fermentation with *L. casei* in various media. Each point is the average of two replicates. Correlation coefficient (r) is 0.93. Significance levels from the analysis of variance are $p = 0.0001$ and 0.0005 for lactic acid concentration and production rate, respectively.

suggests some important nutrient(s) for *L. casei* was (were) still missing. Therefore, YE, CSL, and animal and marine byproducts can be grouped in terms of final lactic acid concentration by using LSD (8.1 g/L lactic acid) for averages of both fermentation with or without cane molasses as YE > BAP5300 > BAP5301, CSL > AP1020 > AP5020, AP5030 > AP5135 (Figure 3).

Conclusions. Long-term repeated-batch lactic acid fermentations with *L. plantarum* and *L. casei* were successfully performed for >5 months to evaluate different fermentation media. *L. plantarum* demonstrated higher lactic acid production than *L. casei* for all evaluated media except in YE. Marine hydrolysates BAP5301 and 5300 were best among animal and marine byproducts evaluated for *Lactobacillus* fermentation and could be further enhanced with cane molasses addition. Chicken broth (AP1020) was not as good as marine hydrolysates. Hydrolyzed pork and beef proteins (AP5020 and 5030) cause the production of lactic acid to slow, especially for *L. casei*. However, supplementation with cane molasses slightly improved the lactiv acid fermentation, whereas BAP5301 supplemented with 0.1% cane molasses was the best among the animal and marine byproducts for *L. plantarum* with lactic acid concentration as high as YE and with higher production rate. Also, lactic acid production rate and yield for BAP5301 were not significantly different from those of YE. BAP5300 supplemented with 0.1% (w/v) cane molasses was optimal for *L. casei*, which produced lactic acid as high as YE with a similar production rate. The fastidious nature of *Lactobacillus* clearly illustrated that these inexpensive nutrients obtained from animal and marine sources represent a new family of complex nutrients for an expanding industrial fermentation industry.

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LITERATURE CITED

Boyaval, P.; Goulet, J. Optimal conditions for production of lactic acid from cheese whey permeate by Ca-alginate-entrapped *Lactobacillus helveticus*. *Enzyme Microb. Technol.* **1988**, *10*, 725–728.

Datta, R.; Tsai, S. P.; Bonsignore, P.; Moon, S. H.; Frank, J. R. Technological; economic potential of poly(lactic acid) and lactic acid derivatives. *FEMS Microbiol. Rev.* **1995**, *16*, 221–231.

Demirci, A.; Pometto III, A. L. Enhanced production of D-(−)-lactic acid by mutants of *Lactobacillus delbrueckii* ATCC 9649. *J. Ind. Microbiol.* **1992**, *11*, 23–28.

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

Friedman, M. L.; Gaden, E. L. Growth and acid production by *Lactobacillus delbrueckii* in a dialysis culture system. *Biotechnol. Bioeng.* **1980**, *12*, 961–974.

Hano, T.; Matsumoto, M.; Uenoyama, S.; Ohtake, T.; Kawano, Y.; Miura, S. Separation of lactic acid from fermented broth by solvent extraction. *Bioseparation* **1993**, *3*, 321–326.

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

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

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

Holten, C. H.; Muller, A.; Rehbinder, D. *Properties and Chemistry of Lactic Acid and Derivatives*; Verlag Chemie: Weinheim. Germany, 1971.

Kaufman, E. N.; Cooper, S. P.; Davison, B. H. Screeining of resins for use in a biparticle fluidized-bed bioreactor for the continuous fermentation and separation of lactic acid. *Appl. Biochem. Biotechnol.* **1994**, *45/46*, 545–554.

Kharas, G. B.; Sanchez-Riera, F.; Severson, D. K. Polymers of lactic acid. In *Plastics from Microbes*; Mobley, D. P., Ed.; Hanser Publishers: Munich, Germany, 1994.

Namura, Y.; Yamamoto, K.; Ishizaki, A. Factors affecting lactic acid production rate in the built-in electrodialysis fermentation, an approach to high-speed batch culture. *J. Ferm. Bioeng.* **1991**, *71*, 450–452.

Ohleyer, E.; Wilke, C. R.; Blanch, H. W. Continuous production of lactic acid from glucose and lactose in a cell-recycled reactor. *Appl. Biochem. Biotechnol.* **1985**, *11*, 457–463.

Roy, T. B. V.; Blanch, H. W.; Wilke, C. R. Lactic acid production by *Lactobacillus delbrueckii* in a hollow fiber fermenter. *Biotechnol. Lett.* **1982**, *8*, 483–488.

Schlicher, L.; Cheryan, M. Reverse osmosis of lactic acid fermentation broths. *J. Chem. Technol. Biotechnol.* **1990**, *49*, 129–140.

Vaccari, G.; Gonzalez-Vara, A.; Campi, A. L.; Dosi, E.; Brigidi, P. Fermentative production of L-lactic acid by *Lactobacillus casei* DSM 20011 and product recovery using ion-exchange resins. *Appl. Microbiol. Biotechnol.* **1993**, *40*, 23–27.

VickRoy, T. B. Lactic acid. In *Comprehensive Biotechnology: The Principles, Applications and Regulations of Biotechnology in Industry*; Moo-Young, M., Ed.; Pergamon Press: New York, 1985.

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