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Bacillus stearothermophilus for Thermophilic Production of L-Lactic Acid

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ABSTRACT

A process for the continuous production of high purity L-lactic acid in a membrane bioreactor at 65°C has been developed. Two different *Bacillus stearothermophilus* strains have been tested in batch experiments. Lactic acid yields are between 60 and more than 95% of theoretical yields. The amounts of ethanol, acetate, and formate formed varied between 0 and 0.4, 0 and 0.1, and 0 and 0.5, respectively (mol/mol glucose). All byproducts are valuable and may be separated easily by rectification of the fermentation broth. Complete cell retention enables high volumetric productivity (5 g/Lh), and a minimum of growth supplements. The high temperature of 65°C allows the autoselective fermentation without problems with contamination.

Index Entries: *Bacillus stearothermophilus*; L-lactic acid; thermophilic; continuous fermentation.

INTRODUCTION

Lactic acid may be easily fermented from organic substances such as molasses, whey, or potato sap *(1)*. Although it has been called a "commodity chemical sleeping giant" *(2)*, it still has a relatively small world market of 54,500-59,000 t/yr *(1)*. At present, the global market is estimated to be growing at about 3–5% annually *(3)*, but, to gain access to large markets, conversion of lactic acid to other chemicals or polymers is required. At the end of 1996, market prices for both food and technical grade 88% lactic acid were about 1.8 US $/kg *(4)*.

According to Kharas et al. *(5)* physical and biological properties of lactic acid polymers are related to the enantiomeric purity of lactic acid stereocopolymers. The homopolymers have very regular structures and develop a crystalline phase. When copolymerized with D- or L-lactides or

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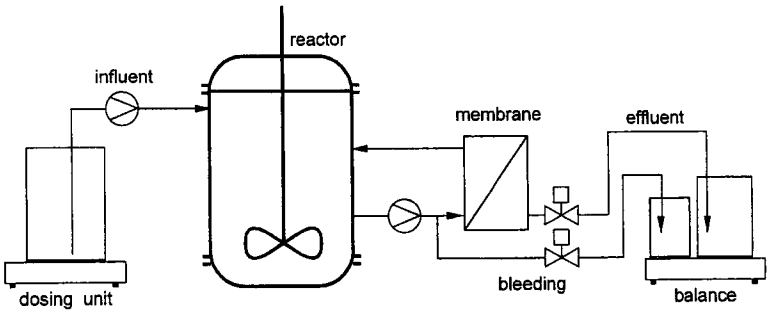

Fig. 1. Schemata of the membrane bioreactor system with cross-flow filtration unit.

lactic acid, their regular structures are interrupted, producing amorphous materials. Therefore, it is desirable to produce high-purity D- or L-lactic acid monomers.

In principle, various methods for the production of lactic acid enantiomers are described in the literature, including chemical synthesis, biotechnological fermentation processes, and enzymatic conversion of pyruvate or mixtures of D- and L-lactic acid. From an economic point of view, direct fermentation of sugars to lactic acid seems the most promising production method. Various microorganisms, including bacteria like lactobacilli, streptococci, or bacilli, and molds like *Rhizopus*, are known to produce either D- or L-lactic acid *(1)*. But fermentation processes suffer from the main disadvantage of possible contaminations. Although *Lactobacillus* fermentations can be performed at quite autoselective conditions at pH 5.0–6.0 and high temperatures of 45°C *(6)*, contamination, of other lactobacilli, which may produce both enantiomers, are possible.

To overcome the problem of contamination, *Bacillus stearothermophilus* was applied in this study. *B. stearothermophilus* is a gram-positive, endosporeforming microorganism, which is capable of growth up to 65°C. The organism may be grown anaerobically where L-lactic acid is the main product. It also grows aerobically when organic acids are further converted to CO_2. Unfortunately, *B. stearothermophilus* requires complex media constituents like yeast extract and peptone for cell growth *(7)*, which raise the production costs. The application of a membrane bioreactor (Fig. 1) should help to overcome this problem.

Membrane bioreactor systems are widely applied in the fermentation industry. Several applications of membranes in combination with bioreactors have been described recently. In all cases an increase of cell density within the bioreactor is the main purpose *(8–11)*. As a result of the high cell concentration, the volumetric productivity may be raised from less than 10 g/Lh lactic acid to more than 85 g/Lh *(12)*.

Because microbes can be recycled completely in membrane bioreactors, cell growth is not a significant parameter, the addition of cost-inten-

sive supplements, such as yeast extract or peptone for growth, can be minimized.

MATERIAL AND METHODS

B. stearothermophilus strain IFA6 was obtained from the German strain collection; strain IFA9 is an isolate which was made available from the high school of chemistry Rosensteingasse in Vienna. Conservation of the strains was performed according to Jones et al. *(13)*, using glass beads at −70°C.

Inocula preparation, strain collection, and batch cultivation experiments were done on the standard synthetic media, consisting of the following substances: 3 g/L meat extract 5 g/L meat peptone, 4 g/L yeast extract, and 30 g/L glucose.

For continuous fermentation, two solutions were applied: solution (a) was pure sucrose from a local sugar company (50 g/L), which was prepared in 1000 L scale. Solution (a) was sterile-filtrated (Durapore CVGL71TP3 from Millipore (Bedford, MA) with pore size of 0.22 μm), and could be stored over weeks. Solution (b) was the supplement solution consisting of (40 g/L casein peptone and 32 g/L yeast extract). This solution was prepared in 10 L scale and sterilized at 120°C. All chemicals were obtained from Sigma. Membranes for cell retention were obtained by Millipore (Ceraflow MSDN 40U50, pore size 50,000 da, 3 modules, membrane surface 0.378 m^2). All the equipment was steam-sterilized at 120°C prior to use.

Analysis of lactose, glucose, galactose, ethanol, formate, and lactic acid were done with HPLC (HP 1050C) using Bio-Rad (Hercules, CA) HPX-87H column and RI (HP1047 A) detectors. The mobile phase was 0.01 *N* H_2SO_4 (flow 0.6 mL/min, temperature 35°C). Samples were diluted 1:5 with 0.01 *N* H_2SO_4 and centrifuged (Beckmann GS-15, 10 min). Five μL of the supernatant were injected into the HPLC. Determination of L- and D-lactic acid was done enzymatically according to Gawehn *(14)*.

Cell density was determined by absorbance using a Perkin-Elmer (Norwalk, CT) UV/VIS spectrometer Lambda 2S at 600 nm wavelength. On-line determinations were done using the same spectrometer, with a Perkin-Elmer flow-through cell (model 175, 10 mm light path). Cell dry wt was determined after centrifugation of 5 mL cell suspension, washing the pellet with bidistilled water, and drying at 100°C till weight was constant.

RESULTS AND DISCUSSION

Batch Experiments

Supplement Requirement

Figure 2 shows the results of a batch experiment, which was done in 20 mL flasks with varying substrate constituents. This experiment indicates

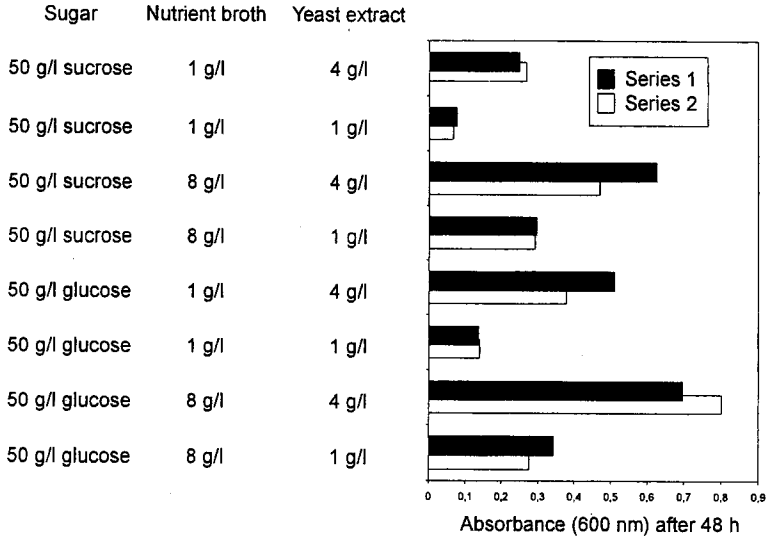

Fig. 2. Influence of media constituents on the growth of *B. stearothermophilus*.

that the amount of yeast extract in the media is the most important factor in the growth of *B. stearothermophilus* IFA9, the influence of nutrient broth, which consists of meat extract and peptone, is significant, but less effective. It also indicates that sucrose from sugarbeet is a suitable substrate, although the optical density of the fermentation broth is lower. This is probably a result of preculturing the inocula in glucose.

Growth Kinetics

Experiments for the estimation of growth parameters, such as maximum growth rate, maximum production rate, and cell or product yields, were done in 5-L reactors (Biostat ED), which were obtained from B Braun Biotech International (Germany). Absorbance (600 nm) and base consumption were detected on-line; sugar, ethanol, and organic acids were determined in intervals of 1–12 h by HPLC.

Figures 3 and 4 show typical fermentation curves of *B. stearothermophilus* IFA6 and IFA9. Prior to inoculation, the reactor was sparged with nitrogen to eliminate oxygen.

B. stearothermophilus IFA6 produces considerable amounts of byproducts such as formic acid, acetic acid, and ethanol (Table 1). The sum of produced ethanol and acetate more or less equals the amount of formate formed; the formation of ethanol is about 1.2–3.5 times more than the amount of detectable acetate. The authors assume that these variations are dependent on dissolved oxygen presented during fermentation. The higher the oxygen level, the more acetate will be formed, instead of ethanol. The formation of CO_2 under anaerobic conditions is negligible. This also confirms the proposed pathway for *B. stearothermophilus (15)*.

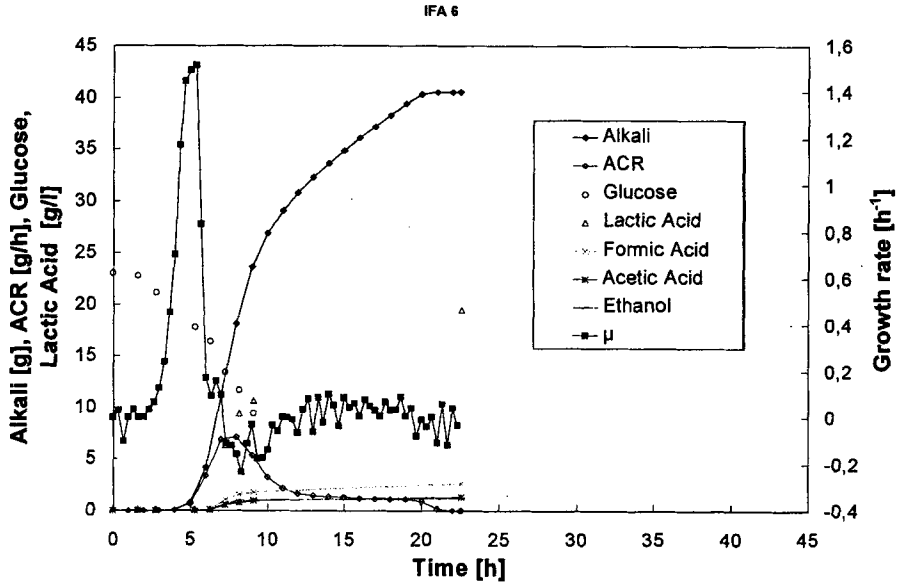

Fig. 3. Growth of *B. stearothermophilus* IFA6 on standard synthetic media. pH was maintained at 7.2 by adding 4 *N* NaOH (alkali). ACR means alkali consumption rate. The calculation of μ is based on the measurements of the optical density (not shown in figure).

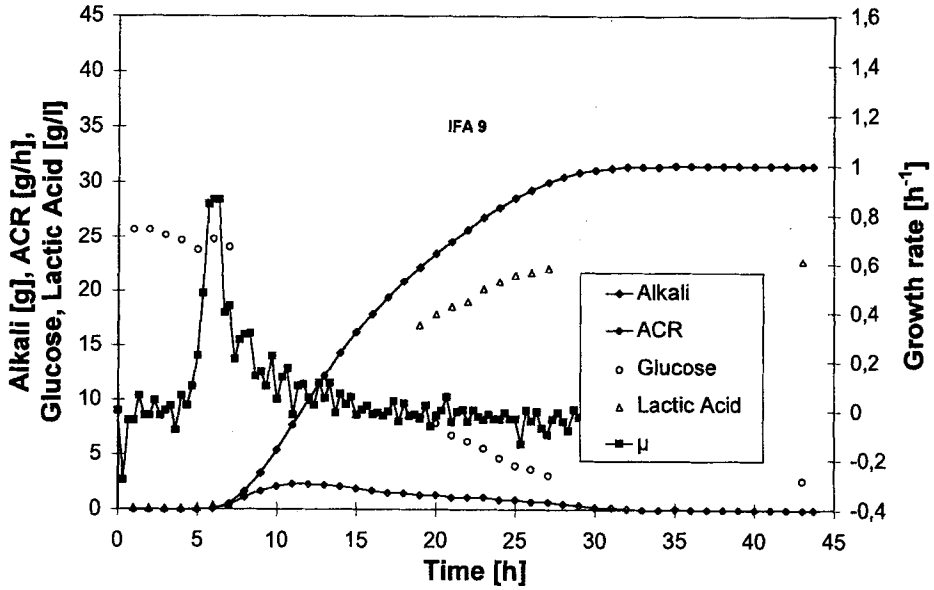

Fig. 4. Growth of *B. stearothermophilus* IFA9 on standard synthetic media. pH was maintained at 7.2 by adding 4 *N* NaOH (alkali). ACR means alkali consumption rate. The calculation of μ is based on the measurements of the absorbance at 600 nm (not shown in figure).

Table 1
Observed Yields of Growth and Metabolism of *B. stearothermophilus* Strain IFA6 and IFA9 Under Comparable Conditions

	IFA6	IFA9
Maximum growth rate μ_{max} (h^{-1})	1.36–1.54	0.863
Cell yield Y_x (g DW/g glucose)	0.023–0.025	0.030
Lactic acid produced Y_{la} (mol/mol glucose)	1.24–1.68	1.974
$Y_{la\ observed}$ /$Y_{theoretical}$ (%)	60.5–84.0	98.7
Optical purity (% L-lactic acid/lactic acid)	99.22–99.85	99.4
Ethanol produced Y_{EtOH} (mol/mol glucose)	0.12–0.39	–
Acetate produced $Y_{acetate}$ (mol/mol glucose)	0.086–0.127	–
Formate produced $Y_{formate}$ (mol/mol glucose)	0.27–0.51	–
Maximum productivity (g lactic acid/lh)	1.2–6.24	1.20
Remaining sugar (g/L)	–	3.67

Given values are the minimum and maximum observed values of various batch experiments. DW, cell dry wt.

B. stearothermophilus IFA9 converts glucose mainly to lactic acid (98.7% of theoretical maximum yield of 2 mol lactic acid/mol glucose). Almost no formation of byproducts could be observed. Unfortunately, not all sugar is converted. The remaining sugar concentration varies between 2.5 and 4 g/L.

Strain Selection for Continuous Fermentation

As already mentioned above, in membrane bioreactor systems, growth of cells is not the key parameter, because cells are kept in the system. According to Boyaval et al. *(16)* and Ferras et al. *(8)*, total cell recycling does not seem suitable: They suggest to install a so-called bleeding of the reactor. This prevents the accumulation of dead cells and a significant decrease in specific productivity. On the other hand continuous growth of biomass is required. Hence, the demands for a suitable strain for continuous conversion of sugar to lactic acid in a membrane reactor are cell growth at high lactic acid concentrations, complete conversion of sugar, and high productivities.

To evaluate the most suitable strain, the lactic acid concentrations (alkali consumed) vs the actual productivity (alkali consumption rate) is shown in Fig. 5. From this, it follows, that IFA6 has higher productivities at higher product concentrations.

Continuous Fermentation in a Membrane Bioreactor

The results of the continuous fermentation (in a membrane bioreactor [Fig. 6]) are shown in Fig. 7. The substrate contains pure sucrose from sugar beets at a concentration of 40 g/L. After a 20 h batch phase, continuous feed

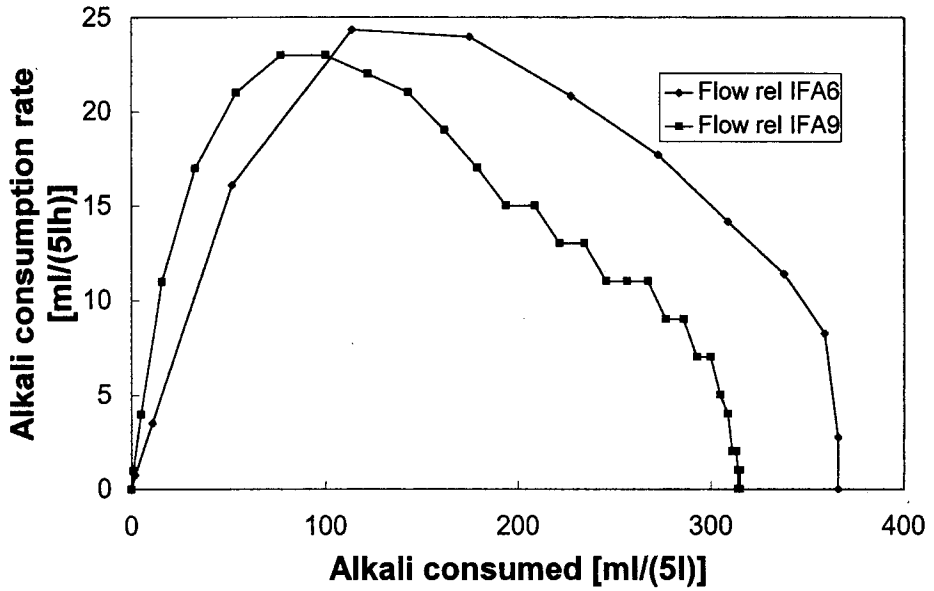

Fig. 5. Alkali (4 *N* NaOH) consumed vs alkali consumption rate (mL 4 *N* NaOH/h and 5 L reactor volume). Strain IFA6 has higher productivities at higher product concentrations.

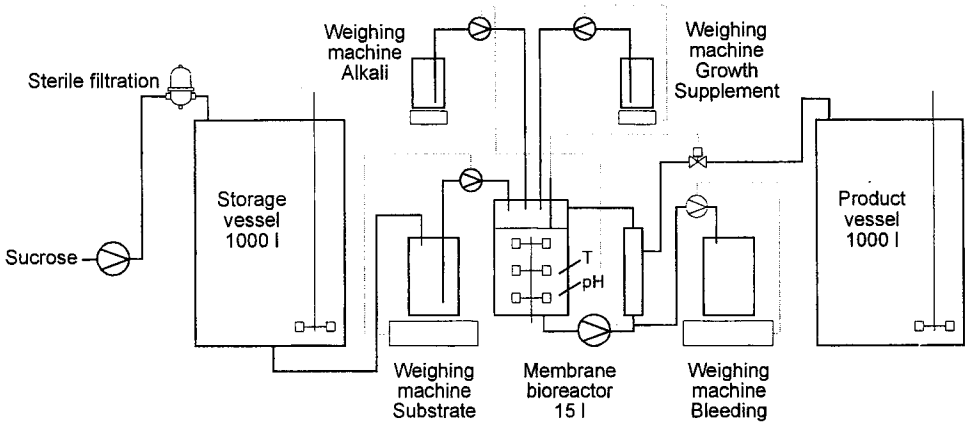

Fig. 6. Flow scheme of the continuous fermentation in membrane bioreactor. The amounts of alkali consumption, growth supplements, substrate, and bleeding were determined by weighing machines. The reactor volume (15 L) was kept constant with a level control sensor, which was connected with the permeate valve.

of 1500 mL sucrose solution/h was started. The feeding rate of sucrose was kept constant over the experiment. Supplement addition (solution [b], consisting of 40 g/L casein peptone and 32 g/L yeast extract) was varied between 10 g/h and 175 g/h. From 20 to 90 h, supplement addition was kept at 10 g/h; from 90 to 138 h, the rate was doubled (20 g/h); and from

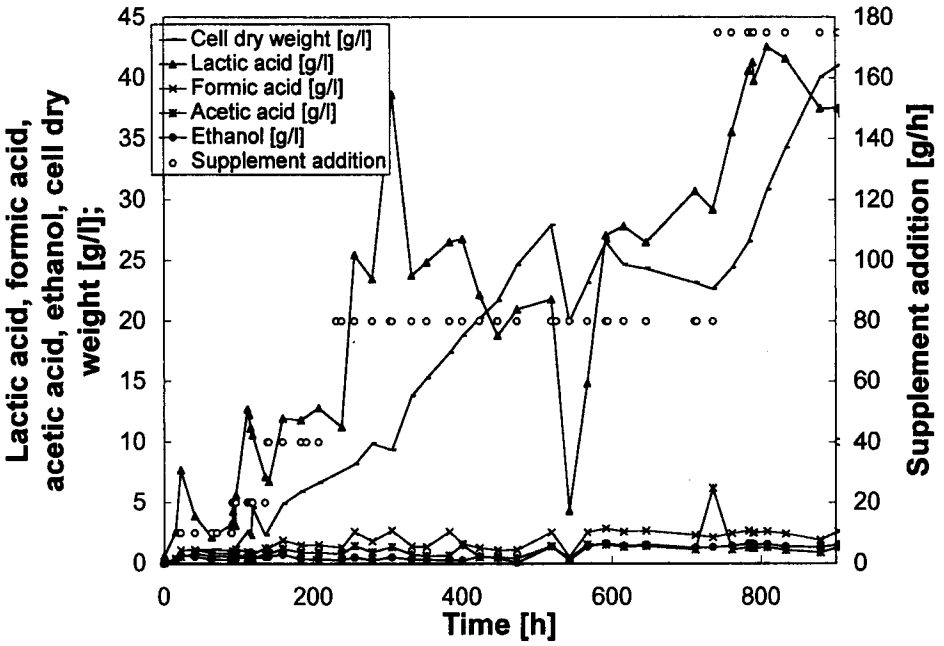

Fig. 7. Course of product formation and cell growth during continuous fermentation in a membrane bioreactor.

138 to 231 h, supplement addition was increased to 40 g/h. Complete conversion of sugar was achieved after raising the supplement addition to 80 g/h and stopping the sucrose feeding for 12 hs (big spike at 300 hs). Complete conversion in continuous fermentation was observed only when supplement addition was set to 175 g/h. The membrane cleaning procedure is responsible for the big dip at 550 h, when losses of biomass are inevitable.

Volumetric productivity was 5 g/Lh. This value is comparable with technical fermentation productivity in batch processes.

Byproduct formation was more or less constant over the whole fermentation process. It is not proportional to the formed lactic acid. The observed concentrations of about 40 g/L lactic acid prove that most of the sucrose is converted to lactic acid. This indicates that no contamination of thermophilic, nonlactic acid producers occurred, and that therefore fermentation under nonsterile conditions is possible.

No problems caused by sporulation were observed.

For the technical realization of the process, the involvement of the product recovery process is required. The application of electrodialysis for separation of lactic acid enables the partial reuse of growth supplements, and therefore will reduce the amounts of cost-intensive substances such as yeast extract and peptone. Bipolar electrodialysis may also lower the costs for alkali, which play a major role in the economics of the process. Further experiments are currently in progress at the institute.

CONCLUSION

The experiments described above have shown that complete conversion of sucrose to L-lactic acid is possible. The chief advantage of this process is the opportunity for continuous production of optically pure lactic acid under nonsterile conditions. The experiments in batch mode and in membrane bioreactor have proved, that growth supplements are necessary for complete conversion of sugar.

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