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Strain Screening and Development for Industrial Lactic Acid Fermentation†

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ABSTRACT

Thirty-four lactic acid bacterial strains have been screened for their potential in industrial lactic acid fermentations. The screening criteria included substrate utilization, product yield, resistance to product inhibition, product stereospecificity, growth temperature, fermentation rate, and ease of cell-broth separation. Acclimation techniques were also used to increase growth temperature and improve product tolerance. This effort has resulted in the identification and improvement of several promising strains, and a superior mixed culture. The mixed culture exhibited a >98% product stereospecificity, cell viability at 106.6 g/L lactic acid, and a productivity of 5.3 g/(L·h) in batch fermentation.

Index Entries: Lactic acid; fermentation; strain development; stereospecific; product inhibition.

INTRODUCTION

Lactic acid fermentation has recently been receiving considerable interest from industry, especially for making degradable polylactic acid (PLA) plastics and coatings (*1*). Commercial lactic acid fermentation was practiced as early as 1881, and the characteristics of the conventional lactic acid bacteria are well known (*2*). These bacteria are facultative

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anaerobes that can be homo- or heterofermentative, are nutritionally fastidious, and often are able to grow at temperatures above 40°C and at a pH between 5 and 7. Most strains do not have saccharolytic enzymes and cannot metabolize starch effectively. The lactic acid produced can be in the D-, L-, or DL-isomeric form.

This article discusses our method of screening bacterial strains and our work in developing promising strains for use in the transformation of high-carbohydrate food byproducts into degradable PLA plastics and coatings. The specific objective was to screen and develop lactic acid bacterial strains that would be suitable for industrial lactic acid fermentation processes using glucose-containing materials as the carbon source. The selected or developed strains are intended for batch fermentation in a stirred-tank bioreactor or continuous fermentation in a cell-recycle bioreactor. No *in situ* product removal in the fermenter is intended.

Although lactose in cheese whey or cheese-whey permeate is also promising as an inexpensive carbon source, screening of lactose-utilizing strains is beyond the scope of this work. Also, this work is concerned with conventional lactic acid bacteria only. The potential use of unconventional strains, such as *Rhizopus oryzae* (3) and *Bacillus laevolacticus* (4), is not discussed in this work.

Considerable research work is ongoing for the use of lactic acid bacterial strains as starter cultures in dairy products and fermented foods (5,6). However, criteria used for such purposes are not necessarily applicable for industrial lactic acid production processes. The screening criteria used in this work include the following:

- The strains should be able to grow on glucose
- The strains should be homofermentative to obtain high product yield and simplify product purification
- The strains should have appropriate product stereospecificity; i.e., they should produce the desired optical isomer(s) of lactic acid
- The strains should be resistant to product inhibition, so that a high product concentration can be obtained
- A growth temperature at 42°C or higher is desired to minimize microbial contamination
- A high batch fermentation rate is desired
- Strains that have easy cell-broth separation characteristics are preferred

The product stereospecificity desired is influenced by the intended application of lactic acid. The synthesis of degradable PLA is expected to be the most significant market growth for lactic acid. Physical properties of PLA, such as crystallinity and degradability, can be adjusted by manipulating the D/L isomeric ratio of monomeric lactic acid (7). Some of the potential applications of PLA are in such agricultural areas as mulch

films and controlled release of fertilizers and pesticides, where the stimulatory effect of lactic acid oligomers on plant growth is considered a potentially important benefit. However, this effect was found to exist only with the oligomers (2–10 lactic acid molecules) of L- and DL-lactic acid, but not with D-lactic acid (*8*). Therefore, it is desirable to have both L-specific and racemic (D/L = 50/50) strains available. This work focused on the L-specific strains.

MATERIALS AND METHODS

Microorganisms

Thirty-four homofermentative and heterofermentative lactic acid bacterial strains were screened. These strains were either obtained from various collections or isolated in our laboratory from food-waste streams. All the homofermentative strains were of the genus *Lactobacillus*. Because of the proprietary nature of this work, strains are identified by their genus and species names, but coded with our strain numbers.

Media

Three types of media, *Lactobacilli* MRS (Difco, Detroit, MI), STLM-B, and PM-C, were used for this work. The STLM-B medium, at a final pH of 6.3, contained 120 g/L glucose (or as specified), 3 g/L KH_2PO_4, 3 g/L K_2HPO_4, 1 g/L sodium acetate, 10 g/L trypticase peptone, 5 g/L yeast extract, 3 g/L tryptose, 1 mL/L Tween 80, 229 mg/L L-cystein $HCl \cdot H_2O$, 573 mg/L $MgSO_4 \cdot 7H_2O$, 34 mg/L $FeSO_4 \cdot 7H_2O$, and 120 mg/L $MnSO_4$; "saccharification tank liquor," obtained from a corn wet-milling plant, was used as a source of glucose. The PM-C medium, at a final pH of 6.3, contained 60–120 g/L glucose obtained from hydrolysis of starch in potato byproducts, 2 g/L KH_2PO_4, 5 g/L sodium acetate, 10 g/L trypticase peptone, 5 g/L yeast extract, 3 g/L tryptose, 1 mL/L Tween 80, 573 mg/L $MgSO_4 \cdot 7H_2O$, 34 mg/L $FeSO_4 \cdot 7H_2O$, and 120 mg/L $MnSO_4$. In some experiments, lactate was added to the medium, either as lactic acid, which was subsequently neutralized by addition of NaOH, or as sodium lactate. The concentration of lactate is expressed as percent sodium lactate (i.e., mol wt = 112) for the Phase I sodium lactate tolerance test and as percent lactic acid (i.e., mol wt = 90) for subsequent experiments.

The Screening Program

The overall strain screening and development program consisted of two phases (I and II), followed by additional characterization of a promising culture. The flow charts for Phase I and Phase II programs are shown in Figs. 1 and 2, respectively. In the first step, the strains received or

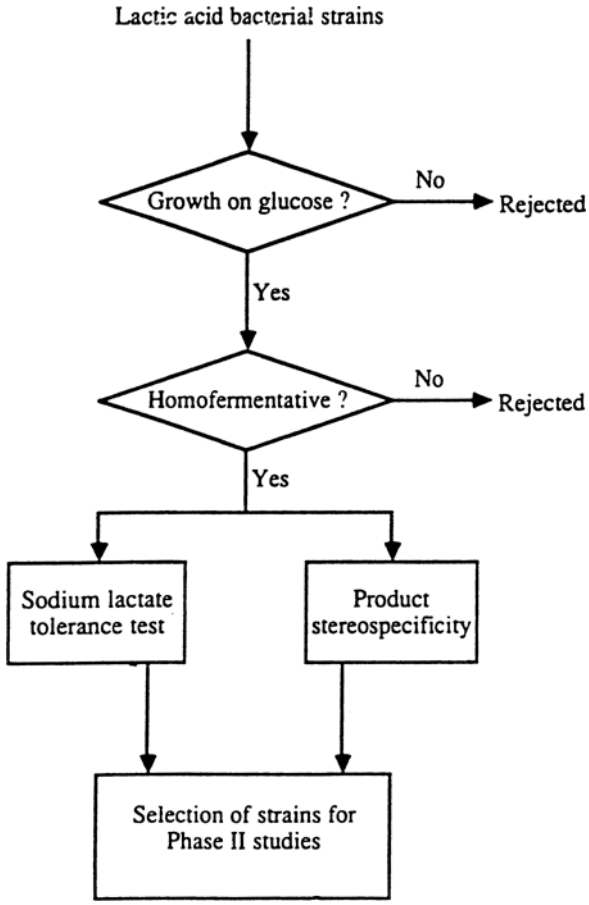

Fig. 1. Flow chart of the Phase I screening program.

isolated were cultivated on MRS medium at 37°C to test their ability to grow on glucose. Strains that grew well were subsequently tested by the methods described below. It should be noted that our screening of some strains started several years ago, and Phase I screening of these strains was carried out using slightly different procedures.

Homofermentative Test

The homofermentative test was carried out using an inverted-tube technique. The strains were cultivated in a 15-mL screw cap culture tube in which a small inverted tube had been immersed in the medium. Heterofermentative lactic acid strains produced CO_2 as a byproduct, which was trapped inside the inverted tube.

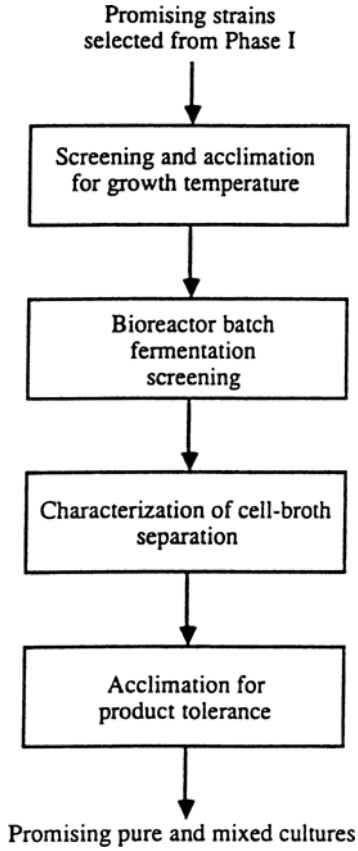

Fig. 2. Flow chart of the Phase II screening program.

Sodium Lactate Tolerance Test

Lactic acid strains were cultivated at 37°C in tubes containing 5 mL of PM-C medium with 7–10% added sodium lactate (i.e., 5.6–8.0% lactic acid equivalent), at pH 6.3. Cell growth, as indicated by turbidity, was observed at 24, 96, and 144 h after inoculation.

Product Stereospecificity Test

Strains were cultivated at 37°C for 16–24 h in 5 mL of PM-C or MRS medium. The culture broth was assayed for D- and L-lactic acid concentrations, as described later, to calculate the percent L-isomer of the total lactic acid.

Growth Temperature Screening and Acclimation

Strains were first cultivated at 37°C in MRS or STLM-B medium. After good growth (usually in 2 d), the culture was transferred to fresh medium at a temperature that was 2–3°C higher. This serial transfer was continued until a final culture temperature of 45°C was reached. If the initial growth at a given temperature was poor, the culture was subcultured at the same temperature for acclimation before being transferred to the next higher temperature.

Bioreactor Batch Fermentation Screening

Several strains were grown in a 1- or 3-L fermenter to screen their batch fermentation performance. These strains were grown in STLM-B medium at 42°C. The pH of the medium was 6.3 initially and was allowed to drop to 5.5 as lactic acid was produced. Further decrease of pH was prevented by addition of 10*N* NaOH. An inoculum grown in the shake-flask for about 12 h was used at 10% (v/v) inoculum size.

Characterization of Cell-Broth Separation

Centrifugation and filtration were selected as the separation methods for this screening, because they are both processes likely to be used in commercial production. Cell broth was prepared from the shake-flask or bioreactor culture, and diluted, when necessary, to an optical density (OD) of 6 at 560 nm for centrifugation and 1.0 for filtration. For the centrifugation process, 10 mL of the broth sample were centrifuged at RCF = 35.5 G for 3 min. the ease of separation for each sample was measured by taking the ratio of the supernatant OD to the original OD. For the filtration method, 100 mL of sample were flitered through a 47-mm diameter membrane filter (0.45 μm) under vacuum (pressure = 130 mm Hg absolute). Filtration was carried out for 10 min or until all 100 mL were filtered, whichever came first. The ease of separation was determined by measuring the average filtration rate over the period.

Product Tolerance at 42 °C

The lactic acid tolerance of five strains was tested at 42°C in shake-flasks using the STLM-B medium containing 8 and 10% L-lactic acid (4 and 2% glucose, respectively). Cell growth was observed at 24 h and at 7 d. The effect of optical isomeric forms on product tolerance was also tested at concentrations from 2 to 10% for DL-lactic acid for *L. casei* RC 1040 and at 8 to 12% for *L. lactis* ST 1510. For acclimation, broth cultures were transferred to media containing higher concentrations of lactic acid (0, 2, 4, 6, 8, 10%).

Batch Bioreactor Characterization of the Mixed Culture LBM5

The five most promising L-specific strains, generated via Phases I and II screening and acclimation, were mixed in equal parts to produce a combined culture designated LBM5. Initially, the batch fermentation performance of LBM5 was determined. These experiments were carried out in a 3-L fermenter under the same conditions as the bioreactor was run for the pure culture screening. Two trials were carried out; one used an inoculum grown for 11 h in a shake-flask, and the inoculum for the other run had been grown in a 0.5-L fermenter at a controlled pH of 5.5.

Chemostat Characterization of the Mixed Culture LBM5

To determine the effect of lactic acid inhibition on cell growth, the mixed culture LBM5 was tested in a chemostat at a moderate dilution rate (0.05/h) and at the lactic acid concentrations (80–110 g/L) anticipated for continuous fermentation in a cell-recycle bioreactor. With the conventional chemostat operation, high product concentrations cannot be achieved at a moderate dilution rate because of continuous withdrawal of cell mass and, thus, low volumetric productivity. Thus, in this experiment, lactic acid, the fermentation product, was added in the feed medium to obtain a high product concentration in the bioreactor. One medium tank (Medium A) contained STLM-B medium with 105 g/L of glucose. The second medium tank (Medium B) contained STLM-B medium that was devoid of glucose, but contained 105 g/L of lactic acid. Initially, the bioreactor containing the regular STLM-B medium was operated in the batch mode for 28 h to accumulate cell mass. In the continuous mode, the total feed rate was maintained at 25 mL/h, whereas the individual feed rates of Medium A and Medium B were varied. Consequently, the effective lactic acid concentration in the feed varied in the range of 0 to 67.2 g/L. The bioreactor agitation speed was 80 rpm, the working vol was 550 mL, and the pH was controlled at 5.5 with 10*N* NaOH solution. The effective dilution rate, taking into account the rate that NaOH was added, varied between 0.045 and 0.05/h, depending on the lactic acid production rate.

Preservation of Cultures

The stock cultures were prepared in 20% glycerol by placing 2 mL of a fully grown culture into a sterile tube containing 2 mL of 40% (v/v) glycerol solution. The 20% glycerol stock cultures were then stored at −70°C.

Assays

The concentrations of lactic acid and glucose were measured by HPLC, using a Bio-Rad (Richmond, CA) Aminex HPX-87H column. An enzymatic method was used as a stereospecific assay to determine D- and L-lactic acids, thereby allowing calculation of the percent L-isomer of the total lactic acid. The method was adapted from the SIGMA Diagnostics Procedure No 826-UV (Sigma, St. Louis, MO) for L-lactic acid assay. For D-lactic acid assay, the D-lactate dehydrogenase was substituted for L-lactate dehydrogenase. An enzymatic method (Glucose [Trinder], SIGMA Diagnostics Procedure No. 315; Sigma) was also used for glucose assay. In the chemostat experiment, the total cell concentration was measured as optical density at 560 nm (OD_{560}), and the viable cell concentration was determined by the plate count procedure using MRS agar to cultivate viable bacteria in the broth.

RESULTS AND DISCUSSION

Phase I Screening

Of the 34 strains tested, two were found to be heterofermentative, and these were rejected for further screening. Seven strains had been previously cultivated at 40°C or higher and were not screened for sodium lactate tolerance at 37°C. Twenty-five homofermentative strains were screened for sodium lactate tolerance at 37°C, with the results shown in Fig. 3. Note that for the 8% sodium lactate 24-h cultures, the inocula were previously grown in the 7% sodium lactate medium. In all other cultures, the inocula were not acclimated. Sodium lactate significantly inhibited cell growth, and the inhibitory effect increased with the sodium lactate concentration. None of the strains showed good 24-h growth at 7% sodium lactate concentration, although some strains were able to grow well after a long lag phase. However, more than 50% of the strains tested did not grow at 7% sodium lactate even after 96 h. Acclimation of the inocula at 7% sodium lactate resulted in improved 24-h cell growth at 8% sodium lactate, compared with the 24-h growth at 7%.

Results of the product stereospecificity screening are summarized in Fig. 4. Product stereospecificity varied broadly, but the distribution seemed to be skewed to favor production of the L-lactic acid isomer. Several strains were found to be highly L-specific (>90 or 95%), but none were found to be D-specific (>90%) from this screening.

Phase II Screening

Based on results of Phase I screening, eight strains were selected for Phase II screening. They were *L. lactis* ST 1510, *L. casei* RC 1040, *L. casei* RC 1020, *L. casei* RC 1010, *L. delbrueckii* RC 1230, *L. helveticus* RC 1260, *L.*

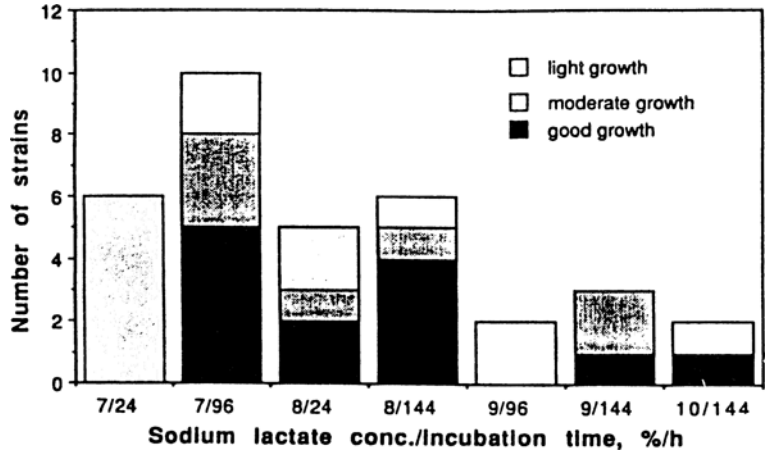

Fig. 3. Lactic acid bacterial growth in the sodium-lactate-tolerance screening experiment. Numbers in the X-axis correspond to the culture conditions. For example , 7/24 is for cultures grown at 7% sodium lactate concentration for an incubation time of 24 h.

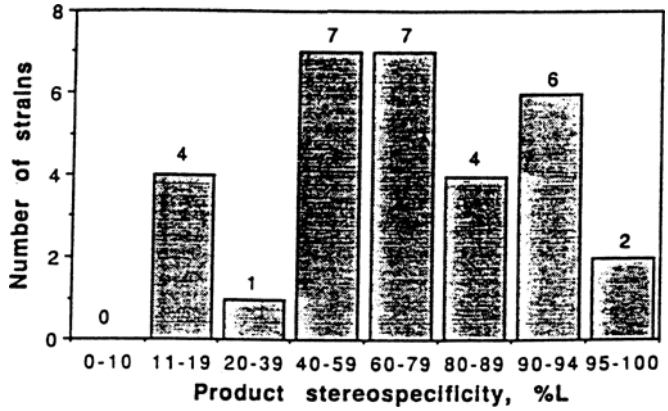

Fig. 4. Results of the product stereospecific screening.

salivarius RC 1190, and *L. bulgaricus* ST 1530. They were first screened and acclimated for growth temperature. Only *L. casei* RC 1010 was unable to grow at 42°C. Of the remaining strains, *L. casei* RC 1020, *L. casei* RC 1230, and *L. helveticus* RC 1260 grew well at 45°C, but *L. lactis* ST 1510 showed only light growth. Therefore, seven strains were tested in the bioreactor for batch fermentation.

Bioreactor Batch Fermentation Screening

From the bioreactor batch fermentation screening, good L-specific lactic-acid-producing strains were found to be (in decreasing order of fermentation rate) *L. lactis* ST 1510 > *L. delbrueckii* RC 1230 > *L. helveticus* RC 1260 > *L. casei* RC 1020 > *L. casei* RC 1040. These strains fermented 70

g/L or more glucose in a 48-h period and had a product stereospecificity of at least 95% L-lactic acid. The fermentation using *L. lactis* ST 1510, which had the highest fermentation rate, was completed in 35 h, resulting in a final fermentation broth composition of 108 g/L lactic acid and 0.3 g/L glucose, and a batch productivity of 3.1 g/(L·h). There were two good nonstereospecific strains, *L. salivarius* RC 1190 and *L. bulgaricus* ST 1530, which consumed more than 100 g/L of glucose in 48 h with a product stereospecificity of 90 and 78% L-lactic acid, respectively.

Characterization of Cell-Broth Separation

Five strains, *L. lactis* ST 1510, *L. casei* RC 1020, *L. delbrueckii* RC 1230, *L. helveticus* RC 1260, and *L. casei* RC 1040, were screened for ease of cell-broth separation. Both the centrifugation and filtration methods gave the same ranking of separation difficulty, which was, in the order of increasing difficulty, *L. helveticus* RC 1260 < *L. casei* RC 1020 < *L. casei* RC 1040 < *L. lactis* ST 1510 < *L. delbrueckii* RC 1230. It is interesting to note that, except for *L. helveticus* RC 1260, strains that had faster fermentation rates appeared to be more difficult to separate.

Product Tolerance at 42 °C

Five strains, *L. lactis* ST 1510, *L. casei* RC 1020, *L. delbrueckii* RC 1230, *L. helveticus* RC 1260, and *L. casei* RC 1040, were screened for lactic acid tolerance. Among them, only *L. lactis* ST 1510 showed good 24-h growth in the presence of either 8 or 10% L-lactic acid. The strains *L. casei* RC 1020 and *L. delbrueckii* RC 1230 showed good growth in the presence of 8% L-lactic acid in 7 d, but not at 10%. *L. helveticus* RC 1260 and *L. casei* RC 1040 did not grow well in concentrations of either 8 or 10% L-lactic acid, even after 7 d. After acclimation in tube cultures, all strains were able to grow well at a concentration of 6% L-lactic acid.

DL-lactic acid and L-lactic acid at the same concentration showed statistically similar inhibitory effects for both *L. lactis* ST 1510 and *L. casei* RC 1040. This implies that when addition of lactate to the medium is desired for screening or acclimation of product tolerance, it is appropriate to use the DL-lactate, if so desired, instead of the exact optical isomer(s) that the culture produces. Organic acids are known to cause growth inhibition by acidification of the cytoplasm and collapse of the proton motive force (*9*). The lack of a stereospecific effect on inhibition suggests that the disturbance of the proton concentration, instead of a possible feedback inhibition of the stereospecific lactate dehydrogenase, would be the primary mechanism of inhibition.

Batch Bioreactor Characterization of the Mixed Culture LBM5

The five most promising strains, *L. lactis* ST 1510, *L. casei* RC 1020, *L. delbrueckii* RC 1230, *L. helveticus* RC 1260, and *L. casei* RC 1040, were mixed in equal parts to form a mixed culture designated LBM5. In an experiment using the shake-flask culture as the inoculum, the batch fermentation, with an initial glucose concentration of 110 g/L, was completed (i.e., glucose was consumed to < 0.1 g/L) in 24.5 h, resulting in a lactic acid concentration of 99.2 g/L. This is a batch productivity of 4.0 g/(L·h), which is significantly higher than that obtained for the best constituent pure culture, *L. lactis* ST 1510 (3.1 g/[L·h]), alone. Symbiotic interactions seem to exist in the mixed culture. When the active combination inoculum was grown under controlled pH and used, the batch fermentation was completed in 18.6 h, resulting in further improved batch productivity of 5.3 g/(L·h).

Lactic fermentations by mixed bacterial cultures were reviewed in the literature (*10*). As an example, the synergistic effects of the yogurt starter consisting of *L. bulgaricus* and *Streptococcus thermophilus* have been studied intensively (*11,12*). In this culture, exchange of nutrients leads to improved growth and acid production; i.e., the proteolytic strain *L. bulgaricus* supplies *S. thermophilus* with amino acids, whereas *S. thermophilus* provides *L. bulgaricus* with essential formate. It was also observed by several investigators that the nutrient requirements of mixed cultures of lactic bacteria were considerably less fastidious than those of pure cultures (*10*). However, it is not possible to elucidate the mechanism of interactions of the mixed culture LBM5 in this study. To the best of our knowledge, there has been no reported stereospecific *Lactobacilli* mixed culture to date.

Chemostat Characterization of the Mixed Culture LBM5

The steady-state chemostat results are summarized in Table 1. The first steady state of the chemostat occurred at 17.2 g/L of glucose and 82.9 g/L of lactic acid. The subsequent increases in the feed lactic acid concentration caused the bioreactor lactic acid concentration to increase and the lactic acid production rate to decrease, as anticipated. A considerable number of viable cells were alive at 106.6 g/L of lactic acid at 42°C. The cells were growing continuously under this condition and never washed out from the bioreactor. The cell viability index, defined as a value proportional to the ratio of viable cell density (CFU/mL) to the total cell concentration (as measured by OD_{560}) and set to be 100 for the end of the

Table 1
Steady-State Results of Chemostat Characterization of the Mixed Culture LBM5

Feed		Bioreactor				
Lactic acid, g/L	Glucose, g/L	Lactic acid, g/L	Glucose, g/L	OD_{560}	Viable cells, 10^9 CFU/mL	Viability index
0.0	105.0	82.9	17.2	14.2	8.0	57
33.6	71.4	90.2	6.1	12.8	7.5	59
54.6	50.4	93.9	7.7	9.2	5.0	55
67.2	37.8	106.6	1.3	5.9	3.4	58

batch period, did not change significantly during the change in lactic acid concentration from 82.9 to 106.6 g/L. The results imply that cell activities (cell growth and lactic acid production) were not strongly affected by the lactic acid concentration in this concentration range.

CONCLUSIONS

The characteristics of lactic acid strains vary widely, and only some selected strains are suitable for industrial lactic acid production processes. Acclimation methods can effectively improve the product tolerance and growth temperature characteristics of the cultures. An effective strain screening and development program has been developed and used successfully, resulting in the identification of pure and mixed cultures suitable for industrial lactic acid fermentation. The superior performance of the mixed culture seemed to be owing to the symbiotic interactions among its constituent strains.

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