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Lactic Acid Bacteria Production From Whey

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Abstract

The main purpose of this work was to isolate and characterize lactic acid bacteria (LAB) strains to be used for biomass production using a whey-based medium supplemented with an ammonium salt and with very low levels of yeast extract (0.25 g/L). Five strains of LAB were isolated from naturally soured milk after enrichment in whey-based medium. One bacterial isolate, designated MNM2, exhibited a remarkable capability to utilize whey lactose and give a high biomass yield on lactose. This strain was identified as *Lactobacillus casei* by its 16S rDNA sequence. A kinetic study of cell growth, lactose consumption, and titratable acidity production of this bacterial strain was performed in a bioreactor. The biomass yield on lactose, the percentage of lactose consumption, and the maximum increase in cell mass obtained in the bioreactor were 0.165 g of biomass/g of lactose, 100%, and 2.0 g/L, respectively, which were 1.44, 1.11, and 2.35 times higher than those found in flask cultures. The results suggest that it is possible to produce LAB biomass from a whey-based medium supplemented with minimal amounts of yeast extract.

Index Entries: Ammonium salts; biomass; lactic acid bacteria; whey; yeast extract.

Introduction

Whey is a byproduct of the cheese- and casein-manufacturing industry *(1)*, with an annual worldwide production of about 115 million t *(2)*.

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There is continuing interest in utilizing this byproduct as a fermentation substrate for the production of value-added products *(3,4)*. Lactic acid bacteria (LAB) biomass is one such product that has numerous applications in the pharmaceutical and food industries *(5)*. Nevertheless, the growth of LAB in whey is frequently a problem because these organisms are very fastidious concerning their nutritional requirements, especially nitrogen supplementation *(6)*.

Yeast extract is recognized as being among the best nitrogen sources for LAB, and it is the ingredient of choice in many media *(7,8)*. The highest production rates of LAB biomass have been obtained when 5–15 g/L of yeast extract were added to whey *(9)*. However, the addition of such amounts of yeast extract during large-scale fermentation of whey to produce biomass or other chemicals represents a significant cost; hence, the partial replacement of yeast extract by the far less expensive ammonium salts is of great commercial interest.

Whey supplementation with ammonium salts might have a positive effect on biomass and lactic acid production by LAB; it has been postulated that the presence of ammonium ions with amino acids in the medium influences the metabolism of certain amino acids even though these ions cannot be used as the sole nitrogen source *(10)*. It is possible that this is the case, particularly when the yeast extract concentration is low. The coaddition of such low-cost nitrogen sources would reduce the required amount of higher-cost sources.

Based on the aforementioned facts, we focused the present study on isolating LAB strains to be used for biomass production using a whey-based medium supplemented with mineral salts and with very low amounts of yeast extract. Such isolation was made using enrichment culture techniques, which have been extensively used for the isolation of organisms to be used for biomass production *(11)*. Afterward, we selected the bacterial strain capable of giving high values of biomass yield and lactose consumption efficiency. Furthermore, we conducted a kinetic study of cell growth, lactose consumption, and titratable acidity production of the selected bacterium in a stirred-tank bioreactor.

Materials and Methods

Culture Medium

The culture medium was prepared as follows: sweet whey powder (Land O'Lakes) was rehydrated with distilled water to obtain a lactose concentration similar to that obtained in the manufacture of cheese (approx 45–48 g/L). The whey proteins were removed by filtration after precipitation by thermocoagulation at 92°C for 10 min. The protein content of the whey was thus reduced from 6.6 to 2.45 g/L, as measured according to the method of Lowry et al. *(12)*, using bovine serum albumin as standard. Deproteinized whey was diluted with distilled water to a lactose concentration of about 12 g/L, in order to reduce growth inhibition by acidifica-

tion of the medium (in the experiments carried out without pH control) or by accumulation of lactate (fermentations performed under pH control). The resulting solution was supplemented with 2.5 g/L of $(NH_4)_2HPO_4$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.035 g/L of $MnSO_4$, and 0.25 g/L of yeast extract. The culture medium was sterilized by autoclaving (120°C for 20 min). The initial pH of the medium was 6.9 ± 0.1. Samples of the culture medium prepared in this way (whey-based medium) were used as the enrichment medium and for the fermentation studies. All experiments were carried out under anoxic conditions.

Isolation of LAB

A homemade fermented milk was used as inoculum to obtain isolates of LAB capable of growing in whey-based medium containing very low levels of nitrogen supplementation in the form of yeast extract. For the enrichment cultures, a small volume of the soured milk was added to 500-mL Erlenmeyer flasks with screw caps containing 100 mL of whey-based medium. Enrichment was made by cultivating the sample at 30°C for 48 h and subculturing 15 times over 1 mo. All culture transfers were conducted aseptically around a Bunsen burner to minimize contamination.

After the enrichment period, observations made at the microscope showed that the enrichment culture was formed by yeasts and bacteria. A portion of the enrichment culture was diluted to 10^{-7}-fold with sterile distilled water and spread onto a plate of MRS (deMan, Rogosa, and Sharpe) agar. After cultivation at 30°C for 72 h, single bacterial colonies with uniform morphology were picked up from the plate and cultured in MRS broth. By repeating the liquid and plate cultures alternatively several times, seven bacterial strains were isolated. Five of them showed the general characteristics of LAB (designated as MNM1, MNM2, MNM3, MNM4, and MNM5) and were identified on the basis of specific characteristics such as colonial and cellular morphology, Gram reaction, biochemical tests, and by the API (bioMérieux, Inc.) system.

Development of Inoculants

All LAB isolates were cultivated separately in 500-mL Erlenmeyer flasks with screw caps containing 100 mL of whey-based medium and were incubated in a shaker at 54 cycles/min at 30°C for 48 h. The cells obtained were separated aseptically by centrifuging at 1500*g* at 4°C for 20 min and washed twice with distilled water to eliminate medium components and cell debris. The resulting pellets were resuspended in a small amount of sterile culture medium. A sample of the cell suspensions was used as inoculum for the batch experiments.

Selection of Pure Bacterial Culture

Five-hundred-milliliter Erlenmeyer flasks with screw caps containing 100 mL of culture medium were sterilized in an autoclave at 120°C for

20 min and were inoculated with the cell suspensions of the isolated LAB. Equivalent initial biomass concentrations were used in all the bacterial cultures tested (0.475 mg/mL). Incubation took place with a constant shaking of 54 cycles/min at 30°C for 95 h. All the experiments were repeated three times, and the mean values are presented herein.

The most suitable bacterial strain for biomass production was selected on the basis of four criteria: biomass yield (Y) on lactose, maximum specific growth rate (μ_{max}) of the bacterial culture, percentage of lactose utilization, and maximum increase in cell mass (ΔX_{max} = maximum biomass concentration – initial biomass concentration). The maximum specific growth rate of every bacterial culture was estimated at the early stage of exponential growth.

16S rDNA Identification of Selected Bacterial Strain

The genomic DNA from the selected bacterial strain was isolated using a GenomicPrep™ cells and tissue DNA isolation kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions except that 20 µL of lysozyme (12.5 mg/mL) solution was added to the samples in the cell lysis step. The DNA preparations so obtained were applied directly in polymerase chain reactions (PCRs).

Universal bacterial 16S rDNA primers 8FPL (8–27) 5'-GCGGATCCG CGGCCGCTGCAGAGTTTGATCCTGGCTCAG-3' and 1492RPL (1510-1492) 5'-GGCTCGAGCGGCCGCCCGGGTTACCTTGTTACGACTT-3' were used to amplify the approx 1.5-kb bacterial 16S rDNA fragment *(13)*. PCR amplification was performed in a total volume of 25 µL in a DNA thermocycler (Applied Biosystems 2400). Each PCR mixture contained 1 µL of template DNA (45 µg/mL), 2.5 µL of 10X *Taq* DNA polymerase buffer (Bioline), 0.75 µL of 50 m*M* $MgCl_2$ solution, 0.5 µL of each deoxynucleoside triphosphate (10 m*M*), 0.125 µL of *Taq* DNA polymerase (5 U/mL), 18.125 µL of deionized water, and 1 µL of each primer (10 µ*M*).

Amplification was performed for 35 cycles under the following conditions: after 5 min of initial denaturation at 94°C, each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 2.5 min, followed by a 10-min final extension step at 72°C in the last cycle. PCR products were purified using a QIAquick spin column (Qiagen) according to the manufacturer's instructions.

Purified PCR products were subjected to electrophoresis in 1% (w/v) agarose gels and visualized after ethidium bromide staining with a TAE (Tris-acetate-EDTA) buffer system to verify that the DNA had not been degraded. Automated DNA sequencing was performed using an ABI Prism DNA sequencer and the 16S rDNA sequences were compared with known sequences in the NCBI database using Advanced Blast to identify the most similar sequence.

Batch Culture of Selected Strain in Stirred-Tank Bioreactor

To increase the biomass yield and the efficiency of whey lactose consumption, a study was performed on batch cultures of the selected bacterial strain in a 5-L Bioflo IIC bioreactor (New Brunswick Scientific) with a working volume of 3.5 L. In the three independent experiments conducted in the bioreactor, deproteinized whey with a lactose concentration of 12 g/L was used (whey-based medium). Because the specific growth rate of LAB is very low, a high initial biomass concentration (1.6 mg/mL) was used in all batch cultures, in order to shorten the incubation time and to minimize microbial contamination. None of the cultures were contaminated.

The fermentations were run with an agitation rate of 80 rpm at 30°C. During the first hours of incubation, the pH was not controlled (initial pH of 6.9 ± 0.1); as soon as the pH reached about 5.7 ± 0.1 (which is within the optimum range for growing *Lactobacillus* species), it was maintained thereafter by the automatic addition of 10% Na_2CO_3. Aeration was not provided. The batch cultures were carried out until no noticeable change could be detected in the cell concentration.

The data reported represent the mean value of three independent experiments.

Analytical Methods

Cell Concentration

The cell concentration was determined by optical density (OD) and dry cell measurements. OD measurement was carried out at a wavelength of 600 nm using a Bausch and Lomb spectrophotometer. The dry weight of cells was determined by filtering the culture samples through a preweighed 0.7-μm filter (Whatman GF/F), which was washed twice with sterile distilled water and dried subsequently at 95°C to a constant weight. The filtrate was used to determine the residual lactose concentration and the titratable acidity.

Lactose Concentration

The lactose concentration of the culture samples was estimated enzymatically using β-galactosidase and a glucose oxidase–peroxidase mixture *(14)*.

Titratable Acidity

The acidity of the culture samples was determined quantitatively by a titrimetric method. These analyses were performed according to the procedures described in *Official Methods of Analysis,* by the Association of Official Analytical Chemists *(15)*. Acidity values were expressed as lactic acid concentration.

The concentration of acids of the culture samples taken from the bioreactor throughout the cultivation period in which the pH was kept constant at 5.7 ± 0.1 was estimated by using the instantaneous volume of Na_2CO_3 delivered by the pH control peristaltic pump and correcting for the molarity of the neutralizing agent *(16)*.

Results and Discussion

Isolation and Biochemical Identification of LAB Strains

A total of seven bacterial strains were isolated from a homemade fermented milk under the selective pressure of carbon and nitrogen sources in the enrichment medium (whey-based medium). Of these strains, five showed the general characteristics of LAB (designated MNM1, MNM2, MNM3, MNM4, and MNM5), such as being Gram-positive, catalase-negative, oxidase-negative, non-spore-forming, facultative rods or cocci, which grow on MRS agar and carry out a lactic acid fermentation of sugars. On MRS agar, colonies were cream colored, smooth, 1 to 2 mm in diameter, circular, convex, with an entire edge, shiny, and moist.

Prior to biochemical identification, it was experimentally confirmed that the isolates MNM1–MNM5 were capable of growing in whey-based medium, which contained very low amounts of a nitrogenous growth factor (0.25 g/L of yeast extract). The biochemical tests corroborated that these isolates belonged to the LAB group. For these tests, a commercial kit (API Rapid CH-50), which has been approved for the identification of the genus *Lactobacillus* and related organisms by the Compendium of Microbiological Methods *(5)*, was used.

Four isolates belonged to the *Lactobacillus* genus (MNM1, MNM2, MNM3, and MNM4) and the remaining one to the *Leuconostoc* genus (MNM5). The *Lactobacillus* strains are the LAB that have been most widely used as probiotics in the production of dairy-based foods, because they belong to the indigenous human microflora, they have a long history of safe use, and there is a general body of evidence that supports their positive role in health *(17–19).*

The aforementioned results clearly show that the selective pressure applied during the batch-enrichment process allowed the isolation of LAB strains possessing the desirable feature of being capable of growing in a culture medium that is less rich in nitrogenous growth factors (yeast extract) than those commonly used for the cultivation of LAB. This would therefore result in a cheaper commercial process for bacterial biomass production and would minimize microbial contamination during the fermentation process.

Selection of Pure Bacterial Culture

Batch experiments were conducted in order to select the bacterial strain more suitable for biomass production. Table 1 shows that the highest values of maximum specific growth rate, overall biomass yield, lactose consumption, and maximum increase in cell mass were obtained in fermentation with *Lactobacillus* strains, and the lowest values were found in fermentation with *Leuconostoc* strain MNM5. Starting from a pH of 6.9 ± 0.1, the pH reached values as low as 3.6–3.8 for all the bacterial cultures tested, which might have affected the bacterial growth. Thus, it would be desirable to

Table 1
Kinetic Parameters of Isolated Bacterial Strains

Strain	μ_{max} (h^{-1})	Y (g of biomass/ g of lactose)	Lactose consumption (%)	ΔX_{max} (g of cells/L)
MNM1	0.033	0.097	72.34	0.68
MNM2	0.037	0.115	90.24	0.85
MNM3	0.031	0.105	65.58	0.58
MNM4	0.035	0.108	78.20	0.65
MNM5	0.013	0.063	42.28	0.25

control the pH of the medium in order to increase the biomass yield and lactose consumption. Furthermore, the different *Lactobacillus* strains showed different growth kinetic behaviors. Compared with the other isolated *Lactobacillus* strains, strain MNM2 gave the highest values of specific growth rate, overall biomass yield, percentage of lactose utilization, and maximum increase in cell mass (Table 1). For these reasons, strain MNM2 was used for all subsequent studies.

16S rDNA Identification of Selected Bacterium

Analysis of the 16S rDNA from the selected bacterium (strain MNM2) revealed that it was a species of *Lactobacilli*, with a high similarity (98%) to *Lactobacillus casei* (NCBI Sequence Viewer, accession no. AF526388); this affiliation was consistent with the morphologic and biochemical characteristics.

L. casei strains have been widely used to prevent or reduce the severity of infantile diarrhea, prevent antibiotic-associated diarrhea, enhance innate immunity, treat allergic disorders, modulate intestinal flora, and lower fecal enzyme activities. It has also been postulated that they have positive effects on superficial bladder cancer and cervical cancer *(19–22)*.

Batch Culture of L. casei *in Bioreactor*

Figure 1 shows the kinetic patterns of growth, lactose utilization, and titratable acidity production in batch culture of *L. casei* in a bioreactor. The biomass concentration curve followed the classic pattern for a microbial growth curve, and the maximum specific growth rate was found to be 0.025 h^{-1}, slightly lower than that found in agitated flasks; this difference could be owing to the different operational conditions (agitation rate, pH) used in both systems. Maximal production of biomass was observed at 44 h of incubation, and afterwards the biomass concentration remained almost constant at 3.6 g/L.

The concentration of residual lactose fell rapidly during the first 44 h of fermentation, and thereafter it decreased slowly until no measurable concentration of lactose could be detected in the medium at 65.6 h of incu-

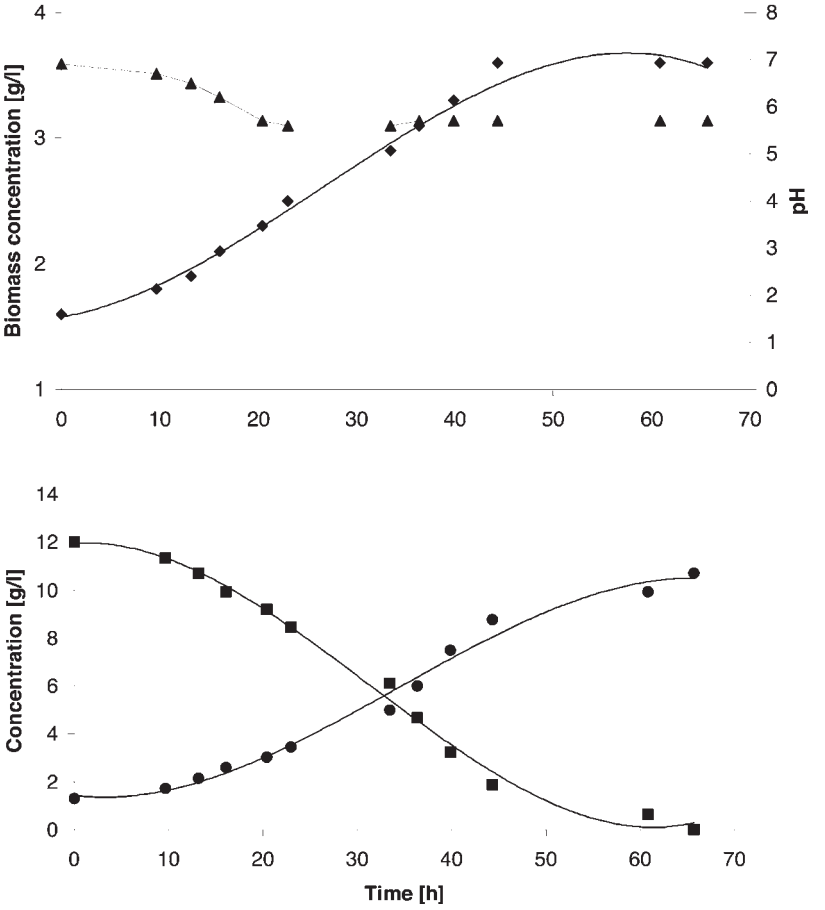

Fig. 1. Changes in biomass concentration, residual lactose concentration, titratable acidity (expressed as lactic acid concentration), and pH during batch culture of *L. casei* in bioreactor: (◆) biomass concentration; (■) lactose concentration; (●) titratable acidity; (▲) pH.

bation; thus, the percentage of disaccharide consumption was 100%, which was higher than that obtained in flasks (90% utilization at 95 h of incubation). On the other hand, titratable acidity increased rapidly during the first 44 h of fermentation and kept increasing at a slower rate, reaching a maximum at the end of the experiment (65.6 h), which coincided with lactose exhaustion. By thin-layer chromatography, it was determined that the major product of the fermentation was the lactic acid; some other compounds were also detected (data not shown), but they were neither identified nor quantified.

The lactose consumption pattern was consistent with the shape of the titratable acidity production curve, but not with the growth curve. At 44 h of cultivation, cell growth had completely ceased; however, titratable acidity production increased in the period from 44 to 65.6 h of cultivation.

The growth of *L. casei* resulted in the consumption of nutrients and the excretion of microbial products, events that influenced the growth of the organism. Thus, after a certain time, the growth rate of the culture decreased until growth ceased (after 44 h of incubation). The cessation of growth might be owing to the depletion of some essential nutrients in the medium, the accumulation of some toxic products of the organism in the medium, or a combination of both.

The maximum increase in cell mass obtained in the bioreactor was 2.0 g/L, which was 2.35 times higher than that found in the flask cultures. On the other hand, the maximum cell mass increases of *L. casei* ssp. *rhamnosus* ATCC 7469 *(10)* and *L. casei* strain 01 *(3)* were 2.6 and 3.2 g/L, respectively, and they were attained at a fermentation time of approx 30 h when whey was supplemented with 5 and 20 g/L of yeast extract. Furthermore, when *L. casei* ssp. *rhamnosus* was grown in batch cultures on 50 g/L of glucose medium supplemented with varying amounts of yeast extract and tryptone, it was found that the final biomass concentration increased from 1.4 to 4 g/L and the fermentation time decreased from 50 to 26 h as the nutrient supplementation increased from 2.5 g/L of yeast extract and 5 g/L of tryptone to 20 g/L of yeast extract and 40 g/L of tryptone *(23)*.

It is considered that the fermentation time (44 h) and the maximum increase in cell mass (2 g/L) obtained in the present work are satisfactory if one takes into account that the addition of yeast extract (0.25 g/L) to the culture medium was minimal.

The overall biomass yield on lactose obtained in our work for the culture of *L. casei* was 0.165 g of biomass/g of lactose, and it was higher than the yields found for *L. casei* ssp. *rhamnosus* ATCC 7469 (0.064 g of biomass/g of lactose) *(10)*, *L. casei* strain 01 (0.066 g of biomass/g of lactose) *(3)*, and *L. casei* ssp. *rhamnosus* (0.03–0.08 g of biomass/g of glucose) *(23)*.

These results clearly show that the *L. casei* strain isolated in the present work is well adapted to grow in whey-based medium containing an ammonium salt and very low levels of yeast extract.

Conclusion

LAB biomass could be produced from whey, which is a cheap and abundant crude feedstock. It was demonstrated that it is possible to produce LAB biomass using a whey-based medium supplemented with minimal amounts of yeast extract and to substitute most of the expensive yeast extract with the far less expensive ammonium salts without a significant decrease in the final biomass concentration and in the biomass yield.

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