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Comparison of Lactic Acid Production by *L. casei* in Batch, Fed-batch and Continuous Cultivation, Testing the use of Feather Hydrolysate as a Complex Nitrogen Source

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HIGHLIGHTS

- Batch, fed-batch and continuous lactic acid production by *Lactobacillus casei*
- Feather hydrolysate as complex nitrogen source substitute

Abstract: A comprehensive comparison of the main fermentation parameters, productivity, yield and final L-lactic acid concentration, obtained through batch, fed-batch and continuous cultivations using *Lactobacillus casei* CCDM 198 and a model cultivation medium was carried out. Using this data, a pulse-feed fed-batch process was established for testing chicken feather hydrolysate as a replacement for all complex nitrogen sources (yeast and beef extracts and peptone) in the medium. As comparably high values of productivity (about 4.0 g/L/h) and yield (about 98 %) were reached under all cultivation conditions, the maximum final L-lactic acid concentration (116.5 g/L), as achieved through pulse-feed fed-batch fermentation, was chosen as the most important criterion for process selection. Fed-batch cultivation with chicken feather hydrolysate as both a complex nitrogen source and a neutralizing agent for maintaining constant culture pH yielded half the concentration of L-lactic acid compared to the model medium. We demonstrate here that chicken feather hydrolysate has potential for use in the production of L-lactic acid but its utilization requires further optimization

Keywords: Lactic acid; homofermentative fermentation, process design, *Lactobacillus casei*, chicken feather hydrolysate

INTRODUCTION

Beside the traditional wide range of applications of lactic acid in food and beverages, personal care, cosmetics, and the pharmaceutical, chemical, leather and textile industries, there is an increasing demand for lactic acid as a precursor for biodegradable polylactic acid polymers that may be used as eco-friendly packaging [1]. Advantages of fermentative lactic acid over industrial chemical production include the use of renewable resources and the possibility of achieving the formation of a specific lactic acid isomer if the appropriate microorganism, such as *Lactobacillus casei*, is selected. Using this species, an L-lactic acid concentration of up to 180 g/L was reached [2].

Process design influences all the main bioengineering variables i.e., yield, productivity and final product concentration³ and for this reason, it is of crucial importance. Maintaining a yield close to its maximal theoretical value (100% in the case of lactic acid and homofermentative lactic acid fermentation) decreases the costs of raw materials, while an increase in productivity and a reduction in non-productive periods can contribute to efficient use of manufacturing equipment. A high product concentration also reduces the cost of downstream processing. Many industrial processes are carried out in batch mode due to its simplicity and low risk of contamination, but the maximum potential of the process is often not achieved. Substrate and product inhibition, low productivity and the non-effective use of time between batches are major problems in this process arrangement. Substrate inhibition is avoided in a fed-batch process, which leads to a shortening of the lag phase, while correctly designed feeding of concentrated nutrients can increase the product concentration and process productivity. The highest productivity is achieved under a continuous arrangement but at the expense of the concentration of final product. Although it is possible to control specific growth rate and product formation in a chemostat by setting a strain- and condition-specific dilution rate, the high volumes of low concentration broth coming from the system, together with the increased risk of contamination and low concentration of product are problematic.

Most of the described cultivation modes have already been employed for lactic acid fermentation, but comprehensive comparisons of all cultivation conditions using the same cultivation medium and the same strain are scarce. An exception is the work of Liu et al.⁴ carried out using the fungus *Rhizopus* sp., but otherwise, authors working with lactic acid bacteria have each focused on only one type of fermentation condition [5–8].

In addition to general bioprocess features, it is necessary to consider the specificity of lactic acid production by homofermentative lactic acid bacteria. Maintaining the pH at a strain-specific value, usually within the range of 5.0-7.0, is a necessity, because otherwise fermentation stops due to low pH [9]. Various hydroxides [10,11] or calcium carbonate [12] have frequently been added as neutralizing agents for lactic acid. Furthermore, it must be considered that homofermentative lactic acid bacteria are usually fastidious and require supplementation with complex nitrogen sources for non-inhibited growth and lactic acid production [13].

Frequently used methods to optimize lactic acid production have focussed on culture medium optimization, inoculum size, temperature and pH using a selected producer and one cultivation mode [14,15]. In this paper, the homofermentative L-lactic acid producer *Lactobacillus casei*, selected in previous screening experiments [16], was tested in batch, fed-batch and continuous systems using free and immobilized lactic acid bacteria in a complex cultivation medium with glucose as a model substrate. The reason for this work was to enable a precise comparison of the influence of fermentation mode on bioprocess parameters, such as yield, productivity, and final product concentration, obtained under conditions where influences of other variables (e.g. cultivation medium composition, lactic acid producer) were minimal. Using the highest lactic acid concentration as a criterion for further process improvement, fed-batch fermentation with pulse feeding was selected as the optimal variant. To decrease operational costs of the process, this process was then repeated using chicken feather hydrolysate as an alternate to other complex and expensive components of the culture medium (yeast extract, peptone and beef extract) and also as an agent for maintaining a constant pH during fermentation.

MATERIAL AND METHODS

Microbial strain and storage

Lactobacillus casei CCDM 198 obtained from the Culture Collection of Dairy Microorganisms Czech Republic was used in all experiments.

Lactobacillus casei CCDM 198 bacteria (culture volume 1 mL) was inoculated into 100 mL of sterile MRS medium and cultured statically in an Erlenmeyer flask for 24 h at 37 °C. Subsequently, 1 mL of culture was transferred into a sterile Eppendorf tube and centrifuged for 5 min at 10000 g (Hettich Mikro 220, Germany). The supernatant was decanted aseptically and 1 mL of a sterile 30% glycerol solution was added to the cell pellet. The microorganism was stored at -70 °C.

Preparation of inoculum

One mL of *Lactobacillus casei* CCDM 198 culture was inoculated into 100 mL of sterile MRS [5] medium and cultured statically in an Erlenmeyer flask for 12 h at 37 °C. For inoculation of a bioreactor, 50 mL of inoculum were used.

Culture medium

MRS broth of the following composition was used in all experiments: 20 g/L glucose (or higher as specified in each experiment), 10 g/L peptone (Carl Roth, Germany), 10 g/L beef extract (Merck KGaA, Germany), 5 g/L yeast extract (Merck KGaA, Germany), 1 mL Tween 80, 2 g/L, dipotassium hydrogen phosphate, 5 g/L, sodium acetate, 2 g/L ammonium citrate, 0.2 g/L magnesium sulphate heptahydrate, 0.2 g/L manganese sulphate monohydrate.

To vary the complex nitrogen source in MRS medium in pulse-feed fed batch experiments, a chicken feather hydrolysate was prepared. Degreased chicken feather (20 g) was mixed with 1 L of 2% NaOH solution and the mixture was hydrolysed for 24 h at 70°C and 130 rpm using a rotary shaker. A volume (450 mL) of hydrolysate was then supplemented with all components of MRS medium with the exception of peptone, beef and yeast extracts, the volume was adjusted to 1 L with tap water and the pH was adjusted to 6.5 with phosphoric acid. The volume of hydrolysate was chosen based on its composition [17] relative to alternate nitrogen sources in MRS medium.

The chicken feather hydrolysate used for neutralizing lactic acid was prepared in 20% NaOH, at a feather concentration of 20 g/L.

Cultivation

Batch cultivation

Batch cultivations were carried out in parallel 1 litre Multifors laboratory bioreactors (Infors AG, Switzerland) with 500 mL of initial working volume, and the inoculation ratio was always 10%. Temperature and pH were controlled at 37 °C and 6.5, respectively. Stirring was set at 300 rpm, and the pH was controlled by automatic addition of 20% NaOH or 10% H₂SO₄. Initial glucose concentrations of MRS medium in batch experiments were 20-70 g/L.

Fed-batch cultivation

Fed-batch cultivations were initiated as a batch, where the initial glucose concentration in MRS medium was 40 or 50 g/L. The type of bioreactor, temperature, pH and stirring were the same as in batch cultivation. For pH control, 20% NaOH or 10% H₂SO₄ were used. Feeding of a concentrated glucose solution in fed-batch was started as soon as glucose was consumed (as indicated by cessation of feeding of solutions used for pH control) and was achieved using a programmable pump. Fed-batch cultivation was performed using three strategies: pulse feeding, constant feeding and linear feeding. Pulse feeding was calculated to deliver 9.5 g of glucose in each pulse, and feeding was carried out at 4-hourly intervals. The constant rate of feed was calculated to deliver 3.5 g of glucose per hour (85% of the maximum glucose consumption rate calculated from batch to prevent glucose accumulation). Linear feeding was carried out according to equation 1. The amount of substrate was measured gravimetrically and feeding was carried out using bioreactor control software (Iris).

Linear feed was calculated as:

$$F = F_0 \cdot t \quad [\text{L/h}] \quad (1)$$

where F is a feeding rate, F_0 is a constant calculated as follows and at this time.

$$F_0 = \frac{\mu \cdot X_0}{c_s} \cdot Y_{X/S} \cdot V_0 \quad [\text{L/h}] \quad (2)$$

where μ is required specific growth rate, X_0 and V_0 are concentrations of biomass and volume in batch and $Y_{X/S}$ is the yield of biomass from substrate consumed.

A strategy of pulse feeding fed-batch cultivation was also tested using feather hydrolysate medium containing (in 1 L): 20 g of glucose, 450 mL of chicken feather hydrolysate, 1 mL of Tween 80, 2 g of dipotassium hydrogen phosphate, 5 g of sodium acetate, 2 g of ammonium citrate, 0.2 g of magnesium sulphate heptahydrate, and 0.2 g of manganese sulphate monohydrate. Preparation of the chicken feather hydrolysate is shown above (see Culture medium part). The type of bioreactor, temperature, pH and stirring were the same as in batch cultivation. For pH control, 20% feather hydrolysate or 10% H_2SO_4 , were used.

Continuous cultivation

Submerged continuous cultivations with free cells were carried out in a Biostat MCU-200 bioreactor (B. Braun, Germany), and pH, temperature, and stirring rate were controlled at the same values as specified for batch and fed-batch experiments. For pH control, 20% NaOH or 10% H_2SO_4 , were used. Inlet and outlet of MRS medium (20 g/L of glucose) were controlled gravimetrically to keep the dilution rate at the required level (0.05-0.25 1/h).

Continuous cultivation with immobilized cells was carried out in an in-house built double walled packed bed bioreactor (described in [18,19]). The cells were immobilized by adsorption using cut corncob residues (particle diameter 10 mm). This type of surface (adsorption) immobilization was used for the first time for *L. casei* cells, but was previously utilized for butanol production by *Clostridium beijerinckii* [18] and *Clostridium pasteurianum* [19] in the same bioreactor. The bioreactor was filled with 115 g of corncob particles and 200 mL of the MRS culture medium (40 g/L glucose), and was sterilised in an autoclave at 121 °C for 20 min. After cooling, the bioreactor was placed in a sterile box and inoculated with an exponentially grown culture of *L. casei* CCDM 198. After a batch lasting for 24 h, gravimetric feeding of MRS broth (20 g/L glucose) was initiated at a rate to achieve the required dilution rate (0.05-0.25 1/h). Temperature was controlled at 37 °C by thermostatically controlled water flow in the double walled bioreactor, and pH was measured in a flow-through cell placed in the outlet of the bioreactor. Since pH was not possible to control in the bioreactor, the pH of the feed was increased to 7.4 to prevent cessation of bacterial growth due to pH drop during lactic acid formation.

Dilution rate was calculated as:

$$D = \frac{F}{V_m} \quad [1/h] \quad (3)$$

where D is dilution rate, F is medium feeding rate and V_m is the working volume of the bioreactor. The maximum specific growth rate in a wash-out experiment was calculated as:

$$\mu_{max} = \frac{\ln(X) - \ln(X_0)}{(t - t_0)} + D \quad [1/h] \quad (4)$$

where μ_{max} is the maximum specific growth rate, X and X_0 are final and initial concentrations of biomass in the bioreactor, t_0 and t are the times at the beginning and end of wash-out, respectively and D is the dilution rate employed in the wash-out experiment.

Analytical methods

Biomass Concentration

Biomass concentration was determined gravimetrically or by measuring the absorbance (at 600 nm), see also Gallazzi et al. [19].

HPLC Determination of Substrates and Products

Lactic acid and glucose concentrations were determined on an Agilent 1200 Series HPLC equipped with a refractometric detector. An IEX H⁺ polymer column (Watrex 250x8 mm) was used for separation. The conditions for HPLC-analysis were as follows: mobile phase: 5 mM H₂SO₄, flow rate: 0.5 mL/min, column temperature: 80 °C, see also Lipovsky et al. [18].

Analysis of Corn Cob Particles Using Scanning Electron Microscopy

Particles withdrawn from different parts of the packed-bed bioreactor were observed using a scanning electron microscope (Hitachi S-4700, Japan). Prior to microscopy, the particles were mounted using double sided sticky carbon tape, dried in a thermostat at 50 °C, sputtered with a thin coat of platinum under vacuum and images were recorded at different magnifications, for details, see Gallazzi et al. [19].

Kinetic parameters

Product yield was calculated according to the equation:

$$Y_{P/S} = \frac{P_f \cdot V_f - P_0 \cdot V_0}{S_0 \cdot V_0 - S_f \cdot V_f} \cdot 100\% \quad [\%] \quad (5)$$

where P_0 and P_f are the initial and final concentrations of lactic acid, respectively, S_0 and S_f are the initial and final concentrations of glucose, respectively and V_0 and V_f are volumes of medium at the beginning and at the end of the cultivation, respectively.

Glucose specific uptake rate was calculated as:

$$\bar{r}_X = \frac{(S_i \cdot X_i) - (S_{i+1} \cdot X_{i+1})}{(t_{i+1} - t_i)} \quad [\text{g/g/h}] \quad (6)$$

where S_i and S_{i+1} are glucose concentrations in samples taken at times t_i and t_{i+1} , respectively and X_i and X_{i+1} are concentrations of dried biomass in the bioreactor measured at times t_i and time t_{i+1} , respectively. Productivity was calculated according to the equation:

$$\bar{p} = \frac{(P_{i+1} \cdot V_{i+1}) - (P_i \cdot V_i)}{(t_{i+1} - t_i)} \cdot \frac{1}{\bar{V}} \quad [\text{g/L/h}] \quad (7)$$

where P_i and P_{i+1} are lactic acid concentrations at times t_i and t_{i+1} , respectively, V_i and V_{i+1} are medium volumes in the bioreactor at times t_i and t_{i+1} , respectively and $\bar{V}$ is the average medium volume over the sampling interval.

Specific growth rate was calculated as:

$$\mu = \frac{\ln X - \ln X_0}{(t - t_0)} \quad [1/\text{h}] \quad (8)$$

where X_0 and X are biomass concentrations in samples taken at times t_0 and t , respectively.

RESULTS

Batch, fed-batch (with three different feeding strategies) and continuous processes using submerged cultures of *L. casei* CCDM 198 or the same strain immobilised on a natural carrier (small particles of corn stover) in a continuously run packed bed bioreactor were performed to compare values of the important parameters achieved in each system.

Batch, fed-batch and continuous systems under model cultivation conditions

Data achieved in batch cultivations in a bioreactor with initial glucose concentrations of 20-70 g/L were used to identify production parameters and to select a suitable initial glucose concentration for fed-batch experiments. As seen from Table 1, the yield, productivity and specific growth rate did not differ significantly for initial glucose concentrations of 40, 50 and 60 g/L, while higher values of specific uptake rates were obtained at lower initial glucose concentrations (20 and 40 g/L). A decrease in specific glucose uptake rate at higher initial glucose concentrations was caused by the longer lag-phase necessary for culture adaptation. Initial glucose concentrations ranging from 40-60 g/L were therefore chosen for fed-batch experiments.

Table 1. Production of L-lactic acid in batch cultivation with different initial concentrations of glucose

Glucose (g/L)	Lactic acid (g/L)	$Y_{P/S}$ (%)	p (g/L/h)	r_x (g/g/h)	μ (1/h)
20.0 $\pm$ 0.30	19.50 $\pm$ 0.10	97.50 $\pm$ 0.10	2.43 $\pm$ 0.01	2.00 $\pm$ 0.01	0.10 $\pm$ 0.01
40.0 $\pm$ 0.50	37.30 $\pm$ 0.10	97.20 $\pm$ 0.20	3.92 $\pm$ 0.02	2.00 $\pm$ 0.03	0.20 $\pm$ 0.01
50.0 $\pm$ 0.40	48.20 $\pm$ 0.10	96.40 $\pm$ 0.30	4.01 $\pm$ 0.02	1.70 $\pm$ 0.02	0.21 $\pm$ 0.01
60.0 $\pm$ 0.90	58.30 $\pm$ 0.10	97.30 $\pm$ 0.20	4.02 $\pm$ 0.02	1.38 $\pm$ 0.03	0.21 $\pm$ 0.02
70.0 $\pm$ 0.90	63.30 $\pm$ 0.10	90.50 $\pm$ 0.10	3.62 $\pm$ 0.01	1.22 $\pm$ 0.02	0.19 $\pm$ 0.02

$Y_{P/S}$ – product (L-lactic acid) yield, p – productivity of L-lactic acid formation, r_x – specific glucose uptake rate; μ – specific growth rate, the calculation equations are given in Material and Methods.

In fed-batch systems, various feeding regimes were tested; repeating pulses, linear or constant feeding of concentrated glucose solutions, beginning at the end of the batch, as signalled on-line by a cessation of neutralizing agent feed. In these experiments, MRS medium was used with 20% NaOH solution for maintaining a constant pH. The total amount of glucose fed into the bioreactors was consumed in all cases and a high yield of lactic acid per consumed glucose (more than 98% w/w) was always achieved. As seen in Table 2, the highest concentration of lactic acid (116.5 g/L) was achieved if pulse feeding was employed while the highest productivity (4.01 g/L/h) was achieved under constant feeding conditions. On the other hand, with linear feeding, moderate results were achieved, most probably because of an underestimation of substrate requirements in the beginning, and an overestimation of them at the end of the feed.

Table 2. Fed-batch production of L-lactic acid using different feeding regimes

Feeding regime	Lactic acid (g/L)	$Y_{P/S}$ (%)	p (g/L/h)	r_x (g/g/h)
Pulse	116.50 $\pm$ 0.50	98.40 $\pm$ 0.10	1.62 $\pm$ 0.13	0.30 $\pm$ 0.05
Constant	91.40 $\pm$ 0.30	98.40 $\pm$ 0.20	4.01 $\pm$ 0.02	1.10 $\pm$ 0.02
Linear	81.70 $\pm$ 0.10	98.50 $\pm$ 0.40	3.50 $\pm$ 1.08	0.86 $\pm$ 0.07

$Y_{P/S}$ – product (L-lactic acid) yield, p – productivity of L-lactic acid formation, r_x – specific glucose uptake rate; the calculation equations are given in Material and Methods.

The production of L-lactic acid was tested using a continuous culture. Chemostat culture with free cells was initially carried out, where the feed rate of MRS medium (20 g/L of glucose) was calculated to achieve dilution rates from 0.05 1/h to 0.24 1/h (maximum specific growth rate calculated from batch data). Data characterising each steady state (Table 3) were measured after 3 volumes of bioreactor were exchanged. As shown in Table 3, by increasing the dilution rate from 0.05 1/h to 0.13 1/h, the steady state concentration of L-lactic acid, as well as the yield and productivity, increased. In both cases, the culture was limited by glucose supply, whose residual concentration was zero. When the dilution rate reached 0.24 $\pm$ 0.01 1/h, non-consumed glucose accumulated in the bioreactor and productivity increased but at the expense of yield (Table 3). Data collected from the wash-out experiment were used to calculate the maximum specific growth rate of this strain (0.235 1/h) as well as the maximum yield of L-lactic acid from glucose (1.04 $\pm$ 0.02 g/g). As seen from this data, the dilution rate of 0.24 1/h was at the boundary of the maximum specific growth rate for this strain, so unconsumed glucose accumulated and the cells were partly washed out from the bioreactor because specific growth rate and dilution rate could not be equilibrated.

Table 3. Production of L-lactic acid in continuous culture at different dilution rates using a submerged chemostat culture and cells immobilised in a packed-bed bioreactor

Submerged culture				Immobilised culture			
Dilution rate (1/h)	0.05 ± 0.01	0.13 ± 0.02	0.24 ± 0.01	Dilution rate (1/h)	0.05 ± 0.01	0.13 ± 0.01	0.25 ± 0.01
Residual glucose (g/L)	0	0	1.37 ± 0.52	Residual glucose (g/L)	0	0	2.51 ± 0.49
Lactic acid (g/L)	14.31 ± 0.09	17.73 ± 0.04	14.19 ± 0.40	Lactic acid (g/L)	20.09 ± 0.09	20.54 ± 0.48	17.18 ± 0.25
$Y_{P/S}$ (%)	91.00 ± 0.07	100.00 ± 0.04	87.00 ± 0.02	$Y_{P/S}$ (%)	101.00 ± 0.12	100.00 ± 0.02	99.00 ± 0.02
P (g/L/h)	0.73 ± 0.05	2.04 ± 0.06	3.61 ± 0.08	P (g/L/h)	1.00 ± 0.16	2.67 ± 0.06	4.30 ± 0.06

$Y_{P/S}$ – product (L-lactic acid) yield, p - productivity of L-lactic acid formation, the calculation equations are given in Materials and Methods.

The strain was then immobilized in a packed bed bioreactor (previously described in [18]) and continuous fermentation was carried out using the same dilution rate as in the chemostat. Since it was not possible to control the pH in this system (it was only measured in the outlet stream), the pH of the medium (the same composition as previously used) was increased to 7.4 to prevent acidification in the upper section of the bioreactor. Values presented in Table 3 were calculated from data obtained from sampling in the outlet of the bioreactor after steady states were achieved (at least 3 cultivation volumes of bioreactor were exchanged). The packed bed system probably yielded an increased level of cells in the bioreactor and thus increased the productivity and the concentration of L-lactic acid compared to the submerged system. The highest concentration of lactic acid (20.54 ± 0.1 g/L) was obtained for a dilution rate of 0.13 1/h. Glucose was fully consumed up to this dilution rate, but not for the highest dilution rate employed (0.25 1/h).

In this case, the residual glucose concentration (2.5 g/L) was determined in the outlet broth accompanied by a drop in the concentration of L-lactic acid produced (17.2 g/L). In this system, the feed flowed through the vertically set up column, so the pH as well as the availability of nutrients was not homogenous along the length of the bioreactor. While at the bottom of the bioreactor, the pH of the feed was 7.4, the pH measured in the outlet stream flowing from the bioreactor was always about 4.0. As seen from the micrographs of cells from different parts of the system (Fig. 1), the density of cells on the corn cob particles in the bottom section of the bioreactor was higher than corn cob particles placed in medium or upper sections (cf. Fig. 1B with Fig. 1C and Fig. 1D).

Figure 1. Micrographs of non-inhabited particles of corn stover (A) and corn stover particles with surface-immobilized cells withdrawn from different sections of a packed – bed bioreactor: bottom (B), middle (C), upper (D).

Production of lactic acid in fed batch using pulse feeding and chicken feather hydrolysate as both a nitrogen source and a neutralizing agent

Based on the comparison of production characteristics described above and using the maximum achieved L-lactic acid concentration as the decisive criterion, the fed-batch using pulse feeding was tested for the production of L-lactic acid using a cheaper cultivation medium in which feather hydrolysate was used as the only complex nitrogen source. The possibility of exchanging all complex nitrogen sources for *L. casei* CCDM 198 in MRS medium with feather hydrolysate has already been demonstrated in flasks [16] and the composition of the feather hydrolysate was described in a previous study [17]. A comparison of L-lactic acid production using standard MRS medium and 20% NaOH as a neutralizing agent with the use of a chicken feather hydrolysate as an alternate complex nitrogen source and neutralizing agent is shown in Fig. 2. At the end of the batch phase of a fed-batch culture (i.e. the start of the feed), L-lactic acid concentrations achieved were 24 and 37 g/L for chicken feather medium and standard MRS, respectively. The maximum concentrations of L-lactic acid after fed-batch cultivations were 116.5 and 56.5 g/L, respectively (Fig. 2) while productivities were 1.6 g/L/h and 0.8 g/L/h and yields were 98 and 85%, respectively. Growth curves in both cultivations correlated with the production of lactic acid, and biomass concentrations of 5.0 g/L and 2.3 g/L were achieved using MRS and chicken feather hydrolysate medium, respectively.

Figure 2. Comparison of pulse fed batch cultivations using standard MRS and feather hydrolysate medium.

Cultivation conditions: 37 °C; stirring rate 300 rpm; constant pH 6.5; neutralizing agent – 20% NaOH or chicken feather hydrolysate for standard MRS and feather hydrolysate media, respectively. Composition of MRS medium for batch phase of the cultivation (in 1 L): 20 g glucose, 10 g/L peptone, 10 g beef extract, 5 g yeast extract, 1 mL Tween 80, 2 g dipotassium hydrogen phosphate, 5 g sodium acetate, 2 g ammonium citrate, 0.2 g magnesium sulphate heptahydrate, 0.2 g manganese sulphate monohydrate. Composition of feather hydrolysate medium (in 1 L): 20 g glucose, 450 mL chicken feather hydrolysate, 1 mL Tween 80, 2 g dipotassium hydrogen phosphate, 5 g sodium acetate, 2 g ammonium citrate, 0.2 g magnesium sulphate heptahydrate, 0.2 g manganese sulphate monohydrate. The arrow shows the start of feeding i.e. end of the batch phase.

DISCUSSION

The highest concentration of L-lactic acid (116.5 g/L) was achieved in a fed-batch system with pulse feeding, while comparably high productivities (about 4.01 g/L/h) were achieved in batch, fed-batch and continuous systems (cf. Table 1, 2 and 3). Although it is generally believed that the highest productivity can be achieved in continuous systems [7,16,17], this was not the case here. In continuous cultivation of *L. casei* using free cells without cell recycle, the maximum productivity derived from the maximum specific growth rate was about 0.24 1/h under the cultivation conditions described. Maximum specific growth rate and maximum productivity of L-lactic acid were the highest for *L. casei* species [5,18,19]. Continuous cultivation with both free and immobilized cells had one significant bottleneck - a low final lactic acid concentration, which would cause problems for subsequent isolation and purification procedures. If the pH in continuous cultivation with immobilized cells could be regulated, the final L-lactic acid concentration, together with other parameters, might be improved. Even though *L. casei* is a species of homofermentative lactic acid bacteria known for its high acid tolerance, and which may survive at pH 4.0 [24], it is probable that both lactic acid productivity and specific growth rate were limited at pH 4.0. Buyukkileci and Harsa [25] demonstrated that *L. casei* production and growth characteristics remained unchanged over pH intervals of 6.5-5.5.

A yield of lactic acid close to the theoretical maximum (100 %) was achieved in almost all cases. It is necessary to consider that MRS cultivation medium contains complex nitrogen sources (peptone, yeast

extract and beef extract) at an overall concentration of 25 g/L, which may be partly utilized as additional carbon sources. Lactic acid as a primary metabolite of homofermentative lactic acid bacteria is associated with growth and energy generation in the form of ATP. Nevertheless, in the study of Hujanen et al. [26] it was observed that in resting cells of *L. casei*, lactic acid can also be formed directly from glucose without other components of the cultivation medium. However, this was probably not the case in cultivations with free cells but might occur in the bioreactor with immobilized cells.

Although the beginning of the pulse fed batch cultivation with chicken feather medium was promising (see Fig. 2 and production curve until 24 h), the final L-lactic acid concentration achieved was about half of that achieved using standard MRS medium. There are two possible explanations for this result. Firstly, use of chicken feather hydrolysate for lactic acid neutralization might cause an accumulation of keratin degradation products such as soluble sulphides [22,23] formed during alkaline hydrolysis, and these might inhibit growth of the bacteria. Secondly, chicken feather hydrolysate may not function in the cultivation medium as a full replacement for all complex nitrogen sources i.e. yeast extract, beef extract and peptone, and this may limit growth and L-lactic acid production through lack of an unknown factor (e.g. a vitamin, amino acid etc.). It is also important to consider that the feather hydrolysate culture medium contained 12.5 % less nitrogen compared to MRS medium (the calculations were performed using data provided by the suppliers of the complex nitrogen sources for MRS medium, and results obtained by us previously [17]).

Until now, replacement of complex nitrogen sources in cultivation medium for *L. casei* using components such as barley sprouts [13], yellow fin tuna head hydrolysate [29] or liquid potato waste [30] have yielded variable results. Replacement of yeast extract may also not be possible [31]. Nevertheless, developing a cheap culture medium for L-lactic acid biosynthesis is of crucial importance for polylactic acid polymer production and chicken feather hydrolysate may function at least as a partial replacement for expensive nitrogen sources, especially after further process optimization.

CONCLUSION

Productivity, yield and final L-lactic acid concentration, representing the main parameters for designing an optimized fermentation process, were compared in batch, fed-batch and continuous cultivation using a model cultivation medium and *Lactobacillus casei* CCDM 198. Surprisingly, almost identical productivities (about 4.0 g/L/h) were found under all conditions tested, contradicting the dogma that a continuous process results in the highest productivity. In addition, the yield of L-lactic acid approached 100% in most cases. As L-lactic acid is a typical example of a primary metabolite, the formation of which is tightly bound with growth, high values of productivity and yield both confirm that the strain chosen for testing was appropriate, was really homofermentative for growth on glucose, and the cultivations were carried out close to the maximum specific growth rate (0.24 1/h). However, in the case of continuous fermentation with surface-immobilized cells on corn stover pieces, the process was carried out under non-ideal conditions due to a pH drop to 4.0 in the upper part of the bioreactor, caused by lack of pH regulation in this type of vessel.

The highest lactic acid concentration (116.5 g/L) was obtained in pulse feeding fed-batch cultivation mode and, considering the importance of final product concentration for isolation and purification, this process arrangement was selected for a cultivation test in which chicken feather hydrolysate was used as an alternative to expensive complex nitrogen sources. Although productivity and yield dropped to half of the values achieved using a model cultivation medium, the use of chicken feather hydrolysate in a more optimized system has potential in this fermentation process.

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