High Cell Density Cultivation of Probiotics and Lactic Acid Production

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Abstract: The commercial interest in functional foods that contain live microorganisms, also named probiotics, is paralleled by the increasing scientific attention to their functionality in the digestive tract. This is especially true of yogurts that contain strains of lactic-acid bacteria of intestinal origin, among these, Lactobacillus delbrueckii ssp. bulgaricus is extensively used in the dairy industry and it has been demonstrated to be a probiotic strain. In this work we describe high cell density cultivations of this microorganism also focusing on the stereospecific production of lactic acid. Key parameters such as medium composition (bactocasitone concentration) and diverse aeration conditions were explored. The results showed that the final concentration of biomass in anaerobic fermentation was lower than the one obtained in microaerophilic conditions, while it gave a very high productivity of lactic acid which was present as a racemic mixture in the permeate. Fermentation experiments carried out with air sparging, even at very low flow-rate, led to the production of the sole L(+)-lactic acid giving sevenfold increase in biomass yield in respect to the batch cultivation. Finally, a mathematical model was developed to describe the microfiltration bioprocess applied in this research considering an inhibition kinetic and elucidating a suitable mathematical description for the decrease of the transmembrane flux. © 2003 Wiley Periodicals, Inc.

Keywords: probiotic; high density cultivation; microfiltration bioreactor; lactic acid

INTRODUCTION

Recently, scientific evidence is accumulating to support the claims of functional foods containing “probiotics,” which can be defined as “living microorganisms, that upon ingestion in certain quantities, exert health benefit beyond inherent basic nutrition” (Fuller, 1989). The commercial value of functional foods has increased with the improved knowledge on the actual beneficial effect on humans such as the decrease on blood pressure and the regulation on cholesterol level (Hamilton-Miller, 2000; Rial, 2000; Tannok, 1997). In addition, it is well established that probiotic-enriched foods and pharmaceutical preparations containing bifidobacteria, can strengthen the organism’s natural defenses against infections in older people thus promoting a better quality of life (Brassart and Schiffirin, 1997; Campieri and Gionchetti, 1999; Marteau, et al., 1990). Within this food cluster, milk-based functional products are particularly interesting both from a scientific and an applicative point of view. Probiotic products usually incorporate intestinal species of Lactobacillus because of their long tradition of safe use in the dairy industry and also because some strains of lactic acid bacteria (LAB) are capable of exerting their beneficial effect by balancing the intestinal flora and eventually competing with pathogens for gut colonization. In particular, Lactobacillus casei and Lactobacillus delbrueckii ssp bulgaricus have been used as starters for the production of drinking yogurt (Vaughan et al., 1999). In this respect, research on LAB has intensified in the last decades exploring their metabolism, eventually applying genetic engineering and flux analysis to improve production of some interesting molecules such as exopolysaccharides, alanine, and flavoring compounds (van Kranenburgh et al., 1999). In addition, a number of research groups have focused their attention on improving LAB biomass production via fermentation by exploiting novel bioreactors that permit the exchange of the medium to prevent LA accumulation and therefore growth inhibition. The most interesting results have been achieved by Kamoshita et al. (1998) that exploited a stirred ceramic membrane reactor to grow Lactococcus lactis yielding 140 g · L⁻¹ of biomass. A bioreactor coupled to a microfiltration module was exploited for the production of an enzyme, superoxide dismutase from Streptococcus lactis giving a 4.3-fold increase in productivity (Taniguchi et al., 1989). In particular, the medium exchange rate (dilution rate) was demonstrated to
Acid has been exploited in leather treatments and dyeing processes; it is frequently applied as acidulant and bacteriostatic agent; it is also used for food preservation (Morris, 1991; Ray and Daeschel, 1992) and as component in pharmaceutical preparations. However, the most interesting recent application of LA is the synthesis of biodegradable polymers for medical use with application ranging from surgery to prosthesis, to tissue engineering (Bostman, 1995; Bucholz, 1994; Eitenmuller et al., 1996), and also as slow release system (Singh et al. 1997). For this last purpose the ratio between L(+) and D(−) lactic acid results very important, because it seems to affect the polymer properties. Also when used in food preparation, lactic acid should be in the L(+) form, as only this isomer can be metabolized by humans. These are the reasons for the increased scientific interest in the stereospecific biotechnological production of this short chain organic acid (Eyal and Lehnhardt, 1998). Currently, 80% of the total world production is due to microbial biotechnology, nevertheless there is scientific and industrial interest for the development of novel bioprocesses which could improve yield and productivity and further simplify the production cycle.

Bearing in mind that LA is a primary metabolite, it is easily recognized that its production is strictly dependent on cell growth and final biomass, therefore in the framework of our research we developed an innovative integrated bioprocess based on a membrane bioreactor and studied the fermentation of Lactobacillus delbrueckii ssp. bulgaricus with the aim of obtaining dense culture of this probiotic strain also improving productivity of stereospecifically pure lactic acid.

**MATERIALS AND METHODS**

**Materials**

Yeast nitrogen base without aminoacids and bacto casitone peptone were purchased from Difco (Becton Dickinson, Le Pont De Claix, France). De Man, Rogosa, and Sharpe Medium (M.R.S.), bacteriological agar and the atmosphere generation system AnaeroGen Compact for solid-state incubation on petri dishes were from Oxoid (Basingstoke, England). All the other chemicals needed to prepare the semi-defined medium and buffers were acquired from Sigma-Aldrich (Milan, Italy). A kit containing acetic acid, lactic acid, citric acid, butyric acid iso-butyric acid, succinic acid, oxalic acid, maleic acid was obtained by Supelco (Milan, Italy) for analytical quantification of organic acids. An enzymatic kit from Sigma-Aldrich (Catalogue #735-10) was also used to determine the L(+-)lactic acid.

**Microfiltration Bioreactor Configuration**

Two polypropylene microfiltration (MF) modules (0.023 m² filtering area), assembled as previously described (Schiraldi et al., 2000), were inserted into specific stainless steel holders which were fixed to two baffles inside the fermentation vessel, and they were connected through a tube to a peristaltic pump (Model 313U; Watson Marlow, England) which supplied the driving force for transmembrane flux. Prior to fermentation, the MF modules were sterilized in situ together with the medium. After cell harvesting and vessel washing, the MF modules were cleaned with de-ionized water at 80°C for 2 h reversing flux direction, the standard restoring procedure was performed successively (Schiraldi et al., 1999). During cultivation backflushings were operated by simply inverting flux for 1–2 min every 30 min during the first 8 h of the microfiltration phase; successively, a solution containing the salts of the medium recipe was pumped reversing the flux to increase the cleaning efficiency. Transmembrane flux was measured on an hourly basis to calculate the influence of cell density and eventually fouling on medium exchange rate.

**Microorganism and Media**

Lactobacillus delbrueckii ssp. bulgaricus (ATCC 11842; DSM, 20081) was obtained by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The lyophilized culture was re-vitalized into the typical Rogosa’s Medium (MRS) (De Man et al., 1960), then petri dishes were inoculated and successively incubated in strictly anaerobic or micro-aerophilic conditions at 37°C. The microorganism was maintained on MRS agar at 4°C and as suspended culture (stabs) at –80°C using glycerol (15% w/v) as cryoprotectant. These stabs were used to inoculate either flasks (1 L) containing 250 mL of medium or pyrex bottles (250 mL) filled up to the top to study cell growth and lactic acid production under different aeration conditions.
conditions over a period of 24–30 h at temperatures ranging from 37 to 40°C, in a rotary shaker (HT Aquatron, Infors, Switzerland) at 160 rpm. The semi-defined medium used for cultivations is reported in Table I (Kimmel and Roberts, 1998).

When fermentor experiments had to be performed the precultures were inoculated with the stabs prepared as previously described and they were grown for 4–5 h at the same temperature of the fermentation experiment, samples were taken every hour.

### Fermentor Experiments

The fermentor used was a Biostat CT, Braun Biotech International (Melsungen, Germany), 2-L working volume, equipped with a DCU and connected to a PC for remote control via MFCS-win software. An INNOVA System for on-line detection of exhaust gas concentration was also used. This system is based on an acoustic detector and can monitor CO2, O2, and volatile organic compounds (VOC) simultaneously giving important information on the metabolism. *Lactobacillus delbrueckii* ssp. *bulgaricus* was grown at T = 37°C, pH = 6.5, the stirring velocity was initially set at 100–200 rpm and the aeration rate at 0.2–0.5 L/min−1 (air) equivalent to 0.1vvm and 0.25 vvm, respectively. Stirring was sometimes increased during experiments to improve the turbulent flow around the membrane tubes, while aeration was maintained at a very slow rate to provide a proper microaerophilic environment. Oxygen partial pressure was kept lower than 4% of saturation—the latter was calibrated with pure oxygen fed at a rate of 1 vvm at 500 rpm.

When anaerobiosis experiments were performed the medium was sparged with nitrogen after sterilization prior to inoculation for at least 30 min.

Experiments in batch mode were carried out using the medium compositions described in Table I, controlling the pH by addition of NH4OH (2.5M) either in microaerophilic or in anaerobic condition by sparging N2 before inoculation and/or during culture.

The fed-batch experiments started in batch mode, using semi-defined or defined medium, then a concentrated nutrient solution containing 400 g · L−1 glucose, 80 g · L−1 bactocasitone and 40 g · L−1 yeast nitrogen base was added in the late exponential phase (6–8 h) following either a step or a linear profile (implemented through the digital control unit, DCU), to increase the rate of glucose addition from 0.5 g L−1 h−1 in the initial phase up to 3.5 g L−1 h−1 when microfiltration started.

The MF experiments started in batch mode, after 8–10 h they switched to fed-batch and approximately 4 h later to MF mode. Each phase-duration was settled upon evaluation of lactate formation, glucose consumption rate, and their influence on specific growth rate. The same volume of fresh medium, as filtrate, was replaced via a pump which was coupled to a level controller to maintain a constant volume in the bioreactor, while microorganisms were held in the vessel and were fed through appropriate profiles (e.g., step or linear, generally ranging from 1–5 g L−1 h−1) to maintain the nutrient concentration as low as possible while keeping an acceptable doubling time (Nordberg Karlsson et al., 1999; Schiraldi et al., 2000). Throughout the MF experiments few samples, opportunely diluted, were plated on MRS agar to prove the cell viability.

### Analytical Methods

Cell growth was followed during experiments by measuring absorbance at 600 nm on a Beckman DU 640 Spectrophotometer (Milan, Italy). Cell counting by direct microscopic analysis was not always reliable because this kind of bacteria happens to agglomerate during growth, due especially to polysaccharides formation—therefore, cell density estimate can be affected by an error. However, throughout the experiment a few samples were extruded through a syringe needle (2–4 times), diluted, and the number of cell per unit volume was counted in a Burker chamber using an optical microscope. In addition, to evaluate cell viability, the corresponding cell suspension was plated at increasing dilution on MRS agar and colony forming units (CFU) were evaluated after 48–72 h incubation at 37°C. Samples collected every hour were spun down in an ALC PK 131R centrifuge at 2000g; wet weight was measured after centrifugation and washing in saline solution (0.9% NaCl w/v). A calibration curve was completed to relate the absorbance value to the cell dry weight. The latter was measured by drying the washed pellet overnight (16–18 h) at 85°C. One gram per litter of dry cell weight corresponded to 1.9 OD600, this correlation was extrapolated on eight different fermentation experiments (15 samples each). The supernatant (1 mL) was ultrafiltered on centrifron tube (10 KDa MW cutoff, Millipore) at 5000g to prepare the samples for analytical quantification.

The concentration of glucose, the main carbon source, was frequently measured through HPAEC-PAD analysis performed with a Dionex chromatographer (model DX 500) using a Carbopac PA100 column coupled with a pulsed amperometric detector (PAD), as previously described (Schiraldi et al., 2000; 2001). A quick off-line determination was obtained by using the Haemo-Glukotest 20-800 strips (Boehringer-Manheim, In vitro diagnosticum).

### Table I. Semidefined medium (SDM) composition.

<table>
<thead>
<tr>
<th>Composition</th>
<th>g · L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>20.0</td>
</tr>
<tr>
<td>Tween 80</td>
<td>1.0</td>
</tr>
<tr>
<td>(NH₄)₂H₂C₂H₂O₇</td>
<td>2.0</td>
</tr>
<tr>
<td>C₆H₄O₂Na</td>
<td>5.0</td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>0.1</td>
</tr>
<tr>
<td>MnSO₄ · 4H₂O</td>
<td>0.05</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.0</td>
</tr>
<tr>
<td>Yeast nitrogen base w/o aminoacids</td>
<td>5.0</td>
</tr>
<tr>
<td>Bactocasitone peptone</td>
<td>10.0</td>
</tr>
</tbody>
</table>
The organic acids from the culture broth and the permeate solutions were analyzed by isocratic elution with phosphoric acid at 0.1% v/v, on a Supelcogel TM C-610 H (30 cm × 7.8 mm i.d.) equipped with a precarrtridge Supelgard C610H (5 cm × 4.6 mm i.d.) at 32°C, using a Dionex-HPLC supplied with a P580 pump and an UV detector 170 S (210 nm). Several analytical-grade organic acids were used as standards to cover all the most common primary metabolites possibly produced during fermentation of LAB. In particular, standards contained citric acid with elution time (ET) 11.7 min, lactic acid (ET 17.7), acetic acid (ET 21 min), isobutyric acid (ET 28.5 min), butyric acid (ET 30.8 min).

In addition, lactate purified from the fermentation broth by precipitation of a calcium salt was resuspended in H3PO4 and analyzed at the polarimeter (Jasco P1010) to evaluate the optical purity.

**Mass Balance Equations**

The glucose concentration in the fermentor and in the permeate solution was measured as described in Materials and Methods. Since it is very difficult to evaluate the concentration of the complex components (e.g., bactocasitone and yeast nitrogen base), and the initial ratio of glucose to these components is 1 to 0.5 and 1 to 0.25, respectively, and it is lowered in the concentrated nutrient solution. All the calculations on substrates consumption were made on the main carbon source (glucose G). The mass balance on glucose is straightforward for batch experiments, while, for the microfiltration experiments, it takes into account the total amount of permeate (P) and the overall feeding (F).

**Batch Experiment**

Initial conditions: \( V = 1.8 \text{ L}, \ c_G = 20-25 \text{ g/L}^{-1} \);
Final conditions: \( V = 1.8 \text{ L}, \ c_G = 0-2 \text{ g/L}^{-1} \);
Overall mass balance: substrate consumption = \( V \cdot (c_G-c_f) \);
Substrate waste = \( V \cdot c_f \).

Obviously, if the batch is repeated \( N \) times to produce a certain amount of biomass the equation for glucose consumption needs to be multiplied by \( N \).

**Yield Coefficients Estimation**

\( Y_{xs} = \) yield coefficient biomass/substrate
\[ Y_{xs} = \frac{\left( \text{OD}_2 - \text{OD}_1 \right)}{(C_{G1} - C_{G2})} \cdot 0.52 \]
\( Y_{ps} = \) yield coefficient product (LA)/substrate (G)
\[ Y_{ps} = \frac{(LA_2 - LA_1)}{(C_{G1} - C_{G2})} \]

Productivity (\( \varphi \)) is evaluated as the biomass or the LA production per unit time (h) per unit culture volume (L).

\[ \varphi_{\text{biomass}} = \frac{(\text{OD}_2 - \text{OD}_1)}{(t_2 - t_1)} \]
\[ \varphi_{\text{lacticacid}} = \frac{(LA_2 - LA_1)}{(t_2 - t_1)} \]

**Microfiltration Experiment**

Initial conditions: \( V = 1.8 \text{ L}, \ c_{G_i} = 20 \text{ g/L}^{-1}, \ P = 0 \text{ L} \);
Feeding solution: \( V_F = 0.6-0.8 \text{ L}, \ c_{F-G} = 400 \text{ g/L}^{-1} \);
Final conditions: \( V = 1.8 \text{ L}, \ c_{F-G} = 1-2 \text{ g/L}^{-1}, \ V_P = 8-10 \text{ L}, \ c_{P-G} = 4-8 \text{ g/L}^{-1} \);
Overall mass balance: substrate consumption = \( V \cdot (c_{G_i} - c_{G_f}) + V_F \cdot c_{F-G} \cdot V_P \cdot c_{P-G} \);
Substrate waste = \( V \cdot c_f \).

Yield coefficients have been evaluated for each fermentation phase thus considering the overall production of LA and the consumption of glucose.

**Mathematical Model**

The mathematical model that describes the MF-fermentation has to take into account a decrease in transmembrane flux, mainly due to the adhesion properties of this microorganism that secretes polysaccharides, the inhibition kinetic for the batch phase and also the variation of the yield coefficients with the different fermentation phases. Mathcad 2000 was used for integrating the following system of first order differential equation:

\[ \frac{dX}{dt} = \mu \cdot X \]
\[ \frac{dP}{dt} = Y_{ps} \cdot \mu \cdot X \]
\[ \frac{dS}{dt} = \frac{1}{Y_{xs}} \cdot \mu \cdot X - \frac{1}{Y_{ps}} \cdot \mu \cdot X \cdot Y_{ps} + \frac{F}{V} \cdot c_p \]
\[ \frac{dG}{dt} = \frac{P}{V} \cdot c_p \]

Also, the equation for noncompetitive inhibition kinetic has to be described in the evaluation of specific growth rate that would be:

\[ \mu = \mu_{\text{max}} \cdot \frac{S}{K_s + S} \cdot \frac{K_p}{K_p + P} \]

The yield coefficients \( Y_{xs}, Y_{ps}, \) and \( Y_{xp}, \) were calculated for the different phases by averaging the data of 10 MF experiments, \( F \) and \( P \) were described in the model as function of time, initially their values were 0.5 \( g \cdot L^{-1} \cdot h^{-1} \) and 0.85 \( g \cdot L^{-1} \), respectively. The Monod constant \( K_s \) and the inhibition constant \( K_p \) were obtained by fitting of the experimental data.
RESULTS

Shake-Flask Experiments

Shake-flask cultivations were performed to evaluate the influence of aeration and bactocasitone concentration on both growth rate and LA production. Figure 1a reports the growth curve for *L. delbrueckii* ssp. *bulgaricus* under anaerobic conditions and it can be seen that the increase in complex nutrients is positively affecting the final yield. Cell density is equal to about 0.9 g dw L⁻¹ in the 10 g · L⁻¹ bactocasitone experiment while it achieved 1.6 g · L⁻¹ when using the richer medium, the corresponding LA production was 3 g · L⁻¹ and 4.5 g · L⁻¹, respectively (Fig. 1b). The microaerophilic experiments (Fig. 2a, 2b) were able to improve biomass yield of 60%, comparing BC10 in microaerophilic and anaerobic conditions, the increase in bactocasitone concentration led to a marked increase in cell density but the correlation we found was not linear. In fact, BC20 resulted in a 50% increase with respect to the final biomass of the corresponding anaerobic experiment while for the highest concentration, the increment was calculated to be approximately of 44%. The LA production was indeed influenced by the aeration condition. In fact, anaerobic metabolism drives the flux prevalently vs. the LA formation in homolactic fermentation, while in microaerophilic conditions the faster increase in cell mass is coupled to CO₂ formation, the carbon flux is deviated towards the common glycolysis pathway therefore yielding a lower amount of acids. As it can be seen from Table II, the specific growth rate in anaerobic conditions is averages 80% of the specific growth rate in the presence of air.

Fermentor Experiments

**Batch Fermentation**

Initially, batch experiments were performed to evaluate the yield coefficients in the two different aeration conditions of interest. When anaerobic fermentations were performed the vessel was sparged with nitrogen before inoculation, the final biomass concentration was about 2–2.3 g cdw L⁻¹ and LA was produced to the extent of 15–18 g · L⁻¹ (Table II) in 22–26 h, while glucose was not completely consumed. En-
zymatic tests were performed to evaluate the composition of the total organic acid produced, the results showed that LA was composed of 60% of L(+) isomer of lactic acid and 40% of the D(−). The corresponding batch experiment in microaerophilic conditions was yielding approximately 2.6–3 g cdw L−1, the final concentration of LA was about 8–10 g/L−1 (Table II), the final cell density was increased by 25–30%; moreover, only L(+)-LA was produced which is very important to simplifying the recovery and purification processes. The yield coefficient $Y_{xs}$ was calculated to be 0.12 in microaerophilic conditions and 0.1 in anaerobic conditions while the $Y_{ps}$ were 0.3 and 0.7, respectively. In both experiments a serious decrease in specific growth rate was observed after 12–14 h of fermentation when the stationary phase was approached, indicating that LA concentration is influencing cell growth rate even at low concentrations.

**MF Experiments**

The MF experiments lasted 50–65 h. They ended either when the specific growth rate was found to decrease down to 10–20% of the maximal one, or if the high density achieved, together with the accumulation of sticky compounds (e.g., exopolysaccharides; Kimmel and Roberts, 1998) on the membrane module, were slowing the medium exchange at 30% of the maximum rate.

After the batch phase, during the anaerobic MF cultivation, the feeding strategy was able to maintain the glucose concentration between 3–5 g/L−1 (Fig. 3); only during the last 5 h was observed an accumulation, probably due to both a lower dilution rate and a higher doubling time. The growth curve and the LA concentration in the fermentor are shown in Figure 3. During this experiment a final cell density of 9.5 g/L−1 was achieved and the final LA concentration was reaching 38 g/L−1. In comparison to batch culture both the products, biomass and LA, resulted in better yield and productivity (Table II).

In general, the MF experiments in microaerophilic conditions further improved the production. In fact, a comparison between the two different environmental conditions clearly shows that the biomass yield was twofold superior when low air sparging was applied to the system (Table II). The growth curves in batch and MF cultivation are drawn in Figure 4, where it can be seen that 24 h in traditional batch cultures led to a cell density corresponding to 2.8 g cdw L−1, while MF cultivation resulted in a biomass concentration higher than 18 g cdw L−1.

This is the first time that high cell density cultivation has been applied to *Lactobacillus delbrueckii ssp. bulgaricus*, however very good results have been obtained in similar reactor configurations on *Lactobacillus casei* and *Lactococcus lactis*. The former yielded up to 40 g/L−1 corresponding to 10^{11} CFU/L−1, and the latter achieved the highest cell mass concentration ever reported with 141 g · L−1 of dry biomass. However, in both cases a huge amount of filtering medium was used, in fact, 214 L for *L. casei* (Hayakawa et.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Highest cell density [g · L−1]</th>
<th>$\varphi_{\text{biomass}}$ [g · L−1 · h−1]</th>
<th>Maximum LA-concentration [g · L−1]</th>
<th>$\varphi_{\text{LA}}$ [g · L−1 · h−1]</th>
<th>L(+) LA [%]</th>
<th>$Y_{xs}$-Averaged [g · g−1]</th>
<th>$Y_{ps}$-Averaged [g · g−1]</th>
<th>Relative yield MF/batch Biomass</th>
<th>Relative yield MF/batch LA</th>
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<tr>
<td>Micro-aerophilic conditions</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Batch</td>
<td>2.8</td>
<td>0.115</td>
<td>9</td>
<td>0.34</td>
<td>100%</td>
<td>0.12</td>
<td>0.3</td>
<td>6.6</td>
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<tr>
<td>MF</td>
<td>18.6</td>
<td>0.310</td>
<td>29</td>
<td>1.86</td>
<td>98%</td>
<td>0.13</td>
<td>0.7</td>
<td>4.1</td>
<td>(10)</td>
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<tr>
<td>Anaerobic conditions</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Batch</td>
<td>2.3</td>
<td>0.09</td>
<td>16</td>
<td>0.56</td>
<td>60%</td>
<td>0.10</td>
<td>0.7</td>
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<tr>
<td>MF</td>
<td>9.5</td>
<td>0.182</td>
<td>38</td>
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<td>54%</td>
<td>0.08</td>
<td>0.9</td>
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</tbody>
</table>

![Figure 3. MF fermentation under anaerobic conditions. Batch phase up to 10 h, fed-batch up to 16 h, MF phase is concluded at 45 h.](image-url)
al., 1990) and 142 L for *L. lactis* (Suzuki, 1996). In our fermentation experiments the permeate volume was ranging from 8 to 10 L. By applying an overall mass balance as described in Material and Methods it can be easily verified that more than 6–7 batches in microaerophilic conditions are necessary to obtain the same amount of biomass, while the MF permitted a higher productivity of LA, in fact, to obtain an analogous production 12 batches would be needed. In both cases, 2–5% of the supplied glucose will be lost but far less bactocasitone and yeast nitrogen base will be needed. In fact, the nutrient split feeding strategy is using the minimum amount of concentrated nutrient solution, which is mainly composed of glucose and the two complex components in ratios of ¼ and ⅛ with respect to its concentration. During the whole experiment the glucose concentration was maintained at a very low level thus resulting in a reduction of medium waste. The LA productivity was monitored and calculated according to the equation described in Materials and Methods on an hourly basis, the corresponding values are shown in Figure 5; the dotted vertical lines represent the transition between batch and MF cultures. During the initial phase, in both conditions the productivity increases together with the increase in cell density. In the MF phases this coupling is not so evident but especially in microaerophilic conditions a constant productivity could be maintained for 30–35 h. The decrease observed in anaerobic conditions most probably can be ascribed to the higher LA concentration in the fermentor (e.g., average of 20 g/L−1 vs. 35 g/L−1). Nevertheless, it can be noticed that the anaerobic conditions led to an average LA productivity which was two- to threefold higher with respect to the microaerophilic conditions.

Furthermore, vitality tests showed that even at high cell densities an active biomass is produced. A comparison between the cell counted at the microscope and the corresponding CFU per unit sample volume is shown in Figure 6a and 6b. At the end of the experiments a vitality of 92% was measured in microaerophilic conditions, while it was 95% in the corresponding samples from the anaerobic experiments.

**Modeling**

For the determination of the kinetic parameters as a function of initial cell concentration and aeration conditions a Lineweaver-Burk form of the Monod equation was used, the saturation constant *K*ₚ was found to be 4.8 g/L−1, which was higher than the ones previously reported (Burgos-Rubio et al., 2000), while *μ*ₚ was 0.7 h⁻¹. LA was previously reported as a noncompetitive inhibitor of microbial growth (Luedeking and Piret, 2000), therefore its concentration did not influencing *K*ₚ because the inhibitor does not affect the binding of the substrate with the enzyme while it affects the growth rate. Despite a number of equations that describe the correlation of the inhibitor level to cell growth in the literature (Bailey and Ollis, 1986; Bugos-Rubio et al., 2000) in our simulation we used the simple Luedeking and Piret equation (2000).

The numerical integration of the mathematical model (see Materials and Methods) was obtained following a subdivision of different phases, batch, fed-batch, and finally microfiltration. In fact, each phase was characterized by yield coefficients specifically calculated from 10 different MF experiments in microaerophilic conditions. As reported in Table II, batch and MF yield coefficients were quite different, for this reason the model varied the parameter values when switching from the first to the second phase. The permeate rate, and therefore the dilution rate (*D* = permeate flowrate/fermentor working volume) was also changed over time, then experimental data were interpolated and the resulting curve expressed *D* (h⁻¹) as a function of time. The results predictive model were plotted in comparison to the
experimental values; this resulted in a good fit of the data points (Fig. 7). The oscillation observed for LA derives from the changing in D (h⁻¹), occurring during the night when no back-flushings could be performed.

DISCUSSION

The development of a suitable medium plays a central role in the attempt at improving biomass and related product yield in a fermentation process. In particular, LAB have limited biosynthetic abilities (Kask et al., 1999), and therefore vitamins and aminoacids often need to be added to the medium. Nevertheless, the possibility of obtaining high biomass and LA yield from a low cost medium is very challenging. In these experiments we used a semidefined medium based on yeast nitrogen base without aminoacids, and bactocasitone peptone at 1.5% concentration, the formulation was developed by Kimmel and Roberts (1998) in view of simplifying the quantification of exopolysaccharides produced by their *L. bulgaricus* RR. However, a basic study using shake flasks to evaluate the influence of a complex key component such as bactocasitone on growth rate, final cell density, and also LA production was not available in literature. For this reason we performed an extensive set of experiments to obtain the fundamental information for subsequent use of the medium in the fermentor cultivations. As shown in Figures 1 and 2, bactocasitone concentration increase led to a superior production of both biomass and LA, nevertheless the impact was not linear with the concentration, bearing these results in mind (also considering the importance of containing medium costs in fermentation processes) we preferred to use the 10 g/L⁻¹ bactocasitone medium in all the successive experiments.

Production of biomasses and commodity chemicals require simple and robust processes able to deliver high product yields at low cost. In general, biotechnological production via fermentation are characterized by low productivities mainly due to the multiple factors that are affecting microbial growth, therefore the design of innovative bioprocesses is necessary for industrial purposes. The rational design of a fermentation process is built on the evaluation of key physiological parameters, such as yield coefficients, glucose uptake rate, specific growth rate, and so on. In the last few years a number of research groups have modeled the growth kinetics of many strains of LAB, however, frequently the behavior even of similar microorganism results are very different.

A number of batch experiments were performed to evaluate the key parameters for designing the MF bioprocess, such as maximal specific growth rate, influence of oxygen on production rate, and LA purity. The most important result was achieved in relation to the LA composition. In fact, for several reasons the selective production of the L(+)-LA would be of key importance, and in our research we highlighted that the aeration conditions play a fundamental role in driving the LA production vs. an optically pure product in

![Figure 6](image-url). Cell counted at the optical microscope and colony forming units at increasing cell densities in MF experiments in microaerophilic (a) and anaerobic (b) conditions.

![Figure 7](image-url). Comparison of the experimental measurements of biomass (cdw g/L) ($R^2 = 0.995$), glucose ($R^2 = 0.970$), and lactic acid ($R^2 = 0.927$) and their expected value for the mathematical model.
the mathematical model developed fit the experimental data viability of 90% was demonstrated (Fig. 6 a, 6b). Finally, preparations. In fact, even at the end of the experiment a cell and vital biomass that can be potentially used in probiotic to prove that the highest cell density achieved in the MF but again it was as high as 10-fold for LA production, even improvement in biomass final concentration was about 5-fold, concerning the anaerobic experiments the im- increase in LA throughput were achieved in microaerophilic fact, a 7-fold increase in biomass yield and a 12-fold in- process. As it can be seen in Figures 3 and 4 and also in Table II, the MF experiments had very effective results. In Table II, the MF experiments had very effective results. In fact, a 7-fold increase in biomass yield and a 12-fold increase in LA throughput were achieved in microaerophilic conditions. Concerning the anaerobic experiments the im- provement in biomass final concentration was about 5-fold, but again it was as high as 10-fold for LA production, even if a racemic mixture was obtained.

Vitality tests were performed throughout the experiment to prove that the highest cell density achieved in the MF fermentation of this microorganism corresponds to an active and vital biomass that can be potentially used in probiotic preparations. In fact, even at the end of the experiment a cell viability of 90% was demonstrated (Fig. 6 a, 6b). Finally, the mathematical model developed fit the experimental data quite well when the parameters of the inhibition kinetic Ks and Kp were 4.8 g/L−1 and 38 g/L−1, respectively. In addition the modeling work was essential to evaluate the dra- matic influence that dilution rate has on LA production, and also the effect of the yield coefficient that was changing with respect to the cultivation phase. Tackling the problem from another point of view the growth-related LA produc- tion was actually occurring only in the exponential phase of batch phase, while in the MF phase it happened that a slower growth rate corresponded to a constant LA produc- tivity (Fig. 5).

This variation had been experienced before in the batch cultivation of Lactobacillus plantarum where the Yxs was decreasing from 0.25 to 0.1 within 15 h, while Yps was also diminishing by approximately 20%. The optimization of lactic acid production has been studied following different approaches—recycling low-cost waste materials (whey), with or without further addition of complex nutrients, exploit- ing stirred tank reactor in a recycle mode with a mem- brane separation step (Kwon et al., 2000; Persson et al., 2000; increase Vickroy, 1985; Viniegra-Gonzales and Gomez, 1984). However, our approach allowed for cell mass production while at the same time obtaining good productivity of stereospecifically pure L(+)-LA by exploit- ing a novel membrane bioreactor which is easily scalable to larger plants. In addition, simple mass balances demon- strated that the MF fermentation was decreasing waste es- pecially of complex components, and would reduce the du- ration of production cycles in comparison to repeated batch experiments.

CONCLUSION

An extensive study was completed on L. delbrueckii ssp. bulgaricus correlating all the growth parameters (biomass and product yield coefficient, oxygen requirement, biomass production) to the fermentation conditions (microaerophilic or anaerobic conditions, initial substrate concentration). The microaerophilic environment was found to improve growth considerably, in particular in the MF experiments, also lead- ing to the production of optically pure L(+)-lactic acid. Seven batch experiments are needed to achieve the average biomass yield of a MF experiment, which in contrast last only 24–30 h longer than traditional batches. This difference is accentuated when considering the LA production.

The experimental work we completed in this project highlighted the capacity of the MF bioprocess to improve biomass yield in probiotic cultivations, in fact, by constantly refreshing the growth medium it was possible to keep the lactic acid concentration lower than the toxic level of 35 g/L−1, thus avoiding inhibition. The mathematical simulation resulted in good agreement with the experimental data; therefore it will be helpful in developing high density for- mulation processes based on the MF techniques for other probiotic strains of interest in the food industry.

References


