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Production and Freeze-Drying of Leben Lactic Starter

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Abstract: The production of two strains of lactic acid bacteria isolated from Tunisian fermented milk (Leben): *Lactococcus lactis* var. *lactis* (SLT6 and SLT10) was investigated in fed-batch process. The final biomass production after 8 h was upper than 10^{10} cells mL⁻¹ for both strains. The strains present an important growth rate (0.95 ± 0.03 h⁻¹) and short generation time. The conversion yield ($Y_{x/s}$) is 0.12 and 0.14 g g⁻¹ for SLT6 and SLT10, respectively. The survival after freeze-drying is 22 and 37% for SLT6 and SLT10, respectively.

Key words: Production, fed-batch, freeze-drying, *Lactococcus*

INTRODUCTION

Lactic acid bacteria are frequently used to start industrial food fermentation and fermented milks. The function of LAB is: the acidification of the milk by the production of lactic acid, the production of flavor ingredients and the production of exopolysaccharides that contribute to the specific rheology and texture of fermented milks products. Leben is traditional cultured milk widely consumed in North Africa and in Middle Eastern countries. It is produced by spontaneous souring of cow's milk followed by churning to separate Leben from butter (Ben Kerroum *et al.*, 2002). The microflora of traditional Tunisian Leben is composed essentially of mesophilic Lactic Acid Bacteria (LAB) represented by *Lactococcus lactis* as a major group (Ben Amor *et al.*, 1998; Jraidi and Guizani, 1998).

A starter culture can be defined as a microbial preparation of large numbers of cells of at least one microorganism to be added to a raw material (Leroy and de Vuyst, 2001). The strain should be genetically stable; it should have good growth properties, to maintain its high viability at processing, freeze-drying and storage. Therefore, good conservation process is required to have a suitable level of the specific strain. Fed-batch fermentation technology allows the obtaining of high cell densities through the continuous supply of fresh medium (Callewaert and de Vuyst, 2000). It requires feeding equipment in addition to the equipment required for batch fermentation and therefore leads to higher fixed costs. However, the process can provide improved productivity as a whole because of the enhanced yield and reduced fermentation time. The main advantage of fed-batch fermentation compared with batch one is the control of carbon source supply and therefore the decrease of stress caused by high concentration of carbon source (Nancib *et al.*, 2006).

The freeze-drying process in the presence of additives was chosen since earlier studies have shown that effective stabilization of enzymes and proteins occurs during treatment. It is suitable for

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the long-term preservation of many types of culture and is particularly suitable for the preservation of vegetative grown cells. Bacteria, while frequently stored by lyophilization, may be susceptible to damage by random mutation (Dietz, 1981).

A recent trend exists in the isolation of wild-type strains from traditional products to be used as starter cultures in food fermentations. In this study fed-batch culture of 2 strains isolated from traditional fermented milk were investigated to determine their kinetical growth parameters and to explore their resistance to the freeze-drying treatment. The earlier research in this area was reported by Hamdi *et al.* (2000) how study the scale up in production of the strain SLT6 and Achour *et al.* (2001) how show the importance of cryoprotectors during the storage of this strain at different temperature.

MATERIALS AND METHODS

Strain and Pre-Culture Conditions

The wild strains (SLT6 and SLT10) used in this study were previously isolated from artisanal Leben (Ben Amor *et al.*, 1998). Strains were grown on MRS medium (Merck) and purified on the same medium. Both cultures were characterized by Gram staining, cell morphology and catalase reaction. Fermentation of carbohydrates was determined using API CHL50 strips according to the manufacturer's instructions (BioMérieux, France). Chromosomal DNA was extracted from both strains. PCR amplification using the 16S rRNA gene was carried out on a Perkin-Elmer DNA thermal cycler 2400. Primers for the PCR were W001: 5'AGAGTTTGATCMTGGCTC3' and W002: 5'GNTACCTTGTTACGACTT3'. Primers used for sequencing 16S rRNA gene were W007: 5'CTCGTTGCGGGACTTAAC3' and W012: 5'TACGCATTTACCKCTACA3'. All primers were obtained from Invitrogen (Invitrogen Life Technologies, California, USA). PCR products were purified before sequencing using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequencing was done by ABI PRISM 310 Genetic sequencer (PE Applied Biosystems). Strains were identified as: *Lactococcus lactis* subsp. *lactis* by comparing sequences in Ribosomal Database Project (<http://rdp.cme.msu.edu/>).

The M17-agar was prepared for the growth, viable count (cells mL⁻¹) and the maintenance of *Lactococcus* strains. A colony from M17-agar was inoculated into a pre-culture medium in 250 mL Erlen Meyer flask filled to 80 % by volume with M17 broth, where lactose has been substituted with 20 g L⁻¹ glucose. This pre-culture (P1) was incubated at 30°C for 16 h. The pre-culture P1 from the exponential growth phase was inoculated into 1 L Erlen Meyer flask filled to 80% with M17-glucose (pre-culture P2).

Fermentation Operations

Fed-batch culture was performed in 20 L stirred fermentor (Biolafite-France, total volume 20 L, working volume 16 L) at 30°C. Two glucose solutions of 20 g L⁻¹ were sterilized. One of the solutions was added to the reactor after sterilization and the second solution was added at fed-batch.

The pre-culture P2 in the exponential phase prepared in 1 L Erlen Meyer flask was added into the culture medium. The temperature of the fermentor was maintained at 30°C and the pH was controlled at 7±0.2 by the addition of 3 N NaOH.

Down-Stream Treatment and Freeze-Drying Process

At the end of fermentation, cells were harvested using a continuous centrifugation process (Sorvall RC 3B, speed 5000 rpm), washed with 0.9% NaCl aqueous solution and recentrifuged. The samples were supplemented with CaCO₃, peptone and saccharose at concentration of 5% of dry mater and malaxed (Fig. 1).

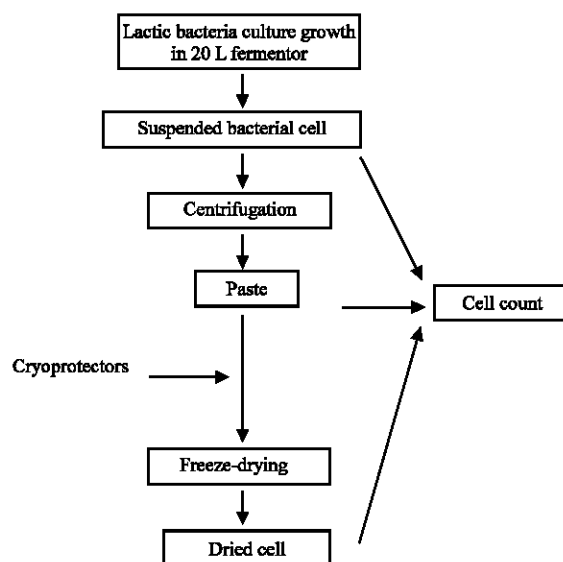


Fig. 1: Experimental approach of lactic bacteria production and freeze-drying

The freeze-dryer used (Louv, model Epsilon 225Dgefriertrocknungsanlagen GmbH, Germany) comprise a rectangular three-shelf chamber with an internal condenser. During freezing, the sample temperature was measured using a thermocouple which is placed inside the sample at each chamber. The drying operation lasted approximately 17-18 h including two main periods; freezing for 6-7 h and sublimation for 11-12 h. During freezing, the sample temperature decreased to a value of -40°C and then progressively increased back to 19-23°C, which correspond to the end of the freeze-drying operation. The vacuum pressure was maintained at 0.6 mbar. The dried samples were stored in metallo-plastic bags maintained at a partial vacuum. Prior to freezing, the samples were stored in a refrigerator.

Analytical Methods

The fermentation broth was centrifuged at 5000 rpm for 10 min. The supernatant was used for glucose analysis and as a Blanc medium for OD₆₀₀ measurement. OD was measured spectrophotometrically at 600 nm to estimate the cell concentration. Viable cells counting method was used to determine the number of cell in the sample capable of forming colonies on M17 or MRS agar. 0.1 mL of diluted sample was spread over the agar surface. The plate was then incubated until the colonies appear. The number of colonies was counted and expressed in cells mL⁻¹.

Glucose concentration was determined enzymatically with a glucose diagnostic kit (Glucose Enzymatique PAP 500, Biomerieux, France).

The freeze-dried samples were rehydrated in 0.9% NaCl aqueous solution and sequential dilutions were prepared. The resulting colonies from were counted. The survival rate after freeze-drying was calculated using the following equation:

$$\text{Survival rate (\%)} = (N'/N) \times 100$$

where, N is the cells number g⁻¹ of dry matter before freeze-drying and N' is the cells number g⁻¹ of dry matter after freeze-drying.

RESULTS AND DISCUSSION

Growth and Kinetics in 20 L Fermentor

The growth of the two strains over time in fed-batch mode is shown in Fig. 2. The study was performed to estimate maximal biomass production and growth parameters of LAB at pH 6.8 and temperature of 30 °C on M17 medium. The agitation was fixed at 100 rpm because it was observed that agitation affected growth and viable cell after freeze-drying for the strains SLT10 and SLT6, especially when the agitation was higher than 150 rpm in 20 L fermentor (Hamdi *et al.*, 2000).

Fermentation lasted 8 h for the lactic strains distributed like follows: 4 h before fed-batch, 4 h after fed-batch. Fed-batch regime was started as soon as glucose was exhausted. The total concentration of glucose used for each fermentation is 40 g L⁻¹, added in two steps, 20 g L⁻¹ in the beginning of fermentation and 20 g L⁻¹ at the time of the fed-batch.

In fed-batch culture, the lactic acid bacteria growth is coupled to the glucose consumption. Both bacterial strains are homofermentative; sugar is only for growth and lactate production. No difference in the growth curve was observed for both strains. It began with a short lag phase followed by an exponential growth phase, then a stationary phase. The fed-batch with glucose resulted in maintaining LAB growth and in increasing in the final cells mL⁻¹. The biomass increases 50 times (from 2.54×10⁸ to 1.3×10¹⁰ cells mL⁻¹) for the strain SLT6 during the first period (before Fed-Batch) and increases twice after Fed-Batch. The kinetic growth before fed-batch operation was higher than after fed-batch.

The same strain SLT6 produced in 20 L fermentor on fed-batch, when yeast extract is replaced by 20 g L⁻¹ of corn steep liquor reached a biomass of 9.4×10⁹ cells mL⁻¹ (Hamdi *et al.*, 2000). Yeast extract appeared to be the most convenient on growth and lactic acid production by LAB (Amrane and

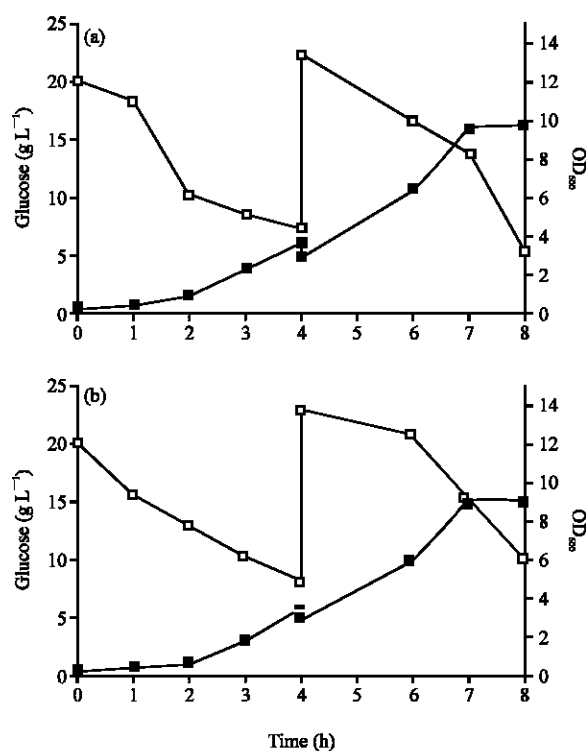


Fig. 2: Time course of OD₆₀₀ (□) and glucose concentration (■) during the fed-batch culture of SLT6 (a) and SLT10 (b) at controlled pH

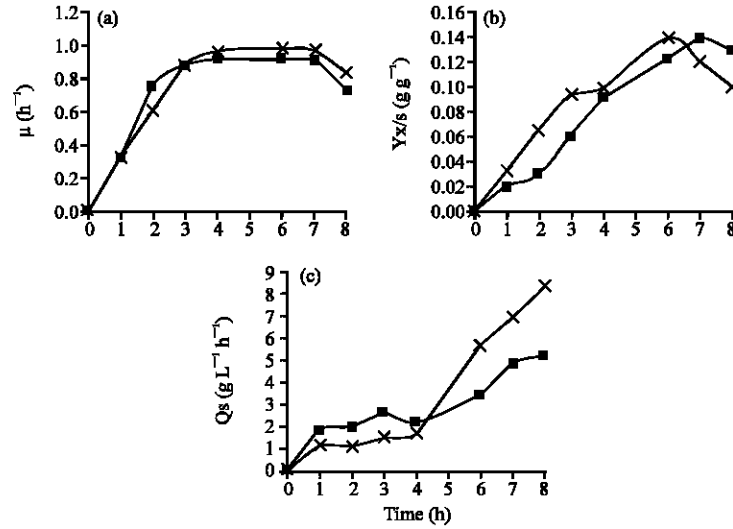


Fig. 3: Time course of (a) specific growth rate μ (h^{-1}), (b) glucose on biomass yield $Y_{x/s}$ (g g^{-1}) and (c) substrate consumption rate Q_s ($\text{g L}^{-1} \text{h}^{-1}$) in 20 L fermentor fed-batch cultures of SLT6 (♦) and SLT10 (■) at controlled pH

Table 1: Growth parameters of SLT6 and SLT10 produced in 20 L fed-batch fermentor

Strain	Fermentation time (h)	OD ₆₀₀ max	Cells mL^{-1} end of fermentation	μ_{max} (h^{-1})	Tg (h)	$Y_{x/s}$ (g g^{-1})
SLT6	8	9.66	2.27×10^{11}	0.98	0.68	0.12
SLT10	8	9.00	2.00×10^{11}	0.92	0.7	0.14

Tg: Generation time

Prigent, 1998), is essential for a good fermentation performance. It is assimilated as the nitrogen source and contains vitamins and cofactors for growth (Aeschlimann and Von Stocar, 1990).

Fed-batch reduces fermentation time, 8 h against 10 h for the same strains in batch culture (data not shown). The fermentation ceases when the accumulation of lactic acid inhibits the fermentation speed and the degree of glucose utilization and when the nitrogen source was exhausted (reduction of the concentration of yeast extract and peptones) (Bibal, 1989).

The specific growth rate (μ), the yield coefficient of biomass on glucose ($Y_{x/s}$) and the substrate consumption rate (Q_s) were calculated and plotted as a function of time (Fig. 3). The specific growth rate (μ) reached the maximum after 3 h of fermentation and then slowly level off to a constant value. The maximum growth rate (μ_{max}) is 0.98 and 0.92 h^{-1} for SLT6 and SLT10, respectively (Table 1).

$Y_{x/s}$ is first stationary then increases with the exponential phase. The maximum is reached when the specific growth rate is maximal. The maximal values is between 0.12 and 0.14 g g^{-1} , some studies showed that on medium enriched in source of nitrogen as the M17 the glucose contributes to the production of the biomass that the production of the lactate, whereas in nitrogen source deficiency the metabolism is directed rather toward the production of the lactate (Baâti, 2000). $Y_{x/s}$ remains constant at the end of culture, although there was not growth in number but rather growth in size that is due to the accumulation of the reserves.

The concentration of the glucose passed at the end of 4 h from 20 to 5.3 g L^{-1} , one addition of substrate is done to continue fermentation. The total glucose consumed is 34.8 and 30 g L^{-1} for SLT6 and SLT10, respectively (Fig. 2). The glucose consumption rate (Q_s) is first weak then increases. After Fed-batch and seen the availability of substrate and the important cell number, Q_s increases

Table 2: Survival rate (%) of SLT6 and SLT10 after freeze-drying operation

Strain	Cells mL ⁻¹ end of fermentation	Cells mL ⁻¹ paste	CDM (%) paste	Cells g ⁻¹ (dried cell)	CDM (%) (dried cell)	Survival rate (%)
SLT6	2.27×10 ¹¹	0.58×10 ¹²	13.6	1.50×10 ¹²	90.7	22
SLT10	2.00×10 ¹¹	0.52×10 ¹²	13.4	1.17×10 ¹²	95.0	37

CDM: Cell dry mater

again. Indeed, both growth and cell densities can be utilized to accelerate the speed of lactic acid production and glucose consumption. Decreased glucose consumption at the end of the fermentation could be due to a deficiency of vitamins, peptides or salts.

Freeze-Drying

After fed-batch operation, culture medium was centrifuged, the paste was malaxed with lyoprotectants (sucrose, peptone and CaCO₃ at reason of 5% of dry matter) than freeze-dried for 18 h. Cell count was performed in paste and lyophilizes to estimate the survival after freeze-drying as shown in Table 2. The lyoprotectants addition is necessary to ameliorate bacterial survival during freeze-drying and stability of the starter (Font de Valdez *et al.*, 1985). However, the mechanisms responsible for these effects on protein stability are rarely understood (D'Andréa *et al.*, 1996). Storage of *Lactococcus lactis* subsp. *lactis* by lyophilization allowed the survival of a sufficiently large numbers of cells and it's better than the immersion in mineral oil that proved to be ineffective (Stoianova and Arkadeva, 2000). Many researchers cited that *Lactococcus* resists to the freeze-drying better than *Lactobacilli* (Tsvetkov and Shishkova, 1982; Bozoglu *et al.*, 1987).

We note that there is no correlation between the final fermentation biomass and the survival after freeze-drying. The growth conditions influence the resistance to the freeze-drying. The survival of LAB increases when cells were cultivated in their optimal growth temperature. The pH regulation between 5.5 and 6.5 increase 5 to 10 times their survival (Desmazeaud, 1992).

The biomass at the end of fermentation and after freeze-drying affects also the stability during storage. Achour *et al.* (2001) show that the stability of the strain SLT6 is found to be greater than SLT10 by applying the accelerated shelf life testing method.

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