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L(+)-Lactic acid production from starch by a novel amylolytic *Lactococcus lactis* subsp. *lactis* B84

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ABSTRACT

A new *Lactococcus lactis* subsp. *lactis* B84, capable of utilizing starch as a sole carbon source and producing L(+)-lactate, was isolated from spontaneously fermented rye sourdough. Aiming at maximum lactic acid productivity, the components of the media and the cultivation conditions were varied. In MRS-starch medium (with absence of yeast and meat extracts), at 33 °C, agitation 200 rpm and pH 6.0 for 6 days complete starch hydrolysis occurred and 5.5 g l^{-1} lactic acid were produced from 18 g l^{-1} starch.

The identification of strain B84 was based on genetic criteria. Amplified ribosomal DNA restriction analysis (ARDRA), PCR with species-specific primers and sequencing of the 16S rDNA proved its species affiliation.

Four genes for enzymes, involved in starch degradation were detected in B84 genome: *amyL*, *amyY*, *glgP* and *apu*, coding cytoplasmic and extracellular α-amylases, glycogen phosphorylase and amylopullulanase, respectively. Reverse transcription PCR experiments showed that both genes, encoding α-amylases (*amyL* and *amyY*) were expressed into mRNAs, whereas *apu* and *glgP* were not. Amylase activity assay was performed at different pH and temperatures. The cell-bond amylase proved to be the key enzyme, involved in the starch hydrolysis with maximum activity at 45 °C and pH 5.4.

1. Introduction

Lactic acid is a valuable product with many practical applications: as a preservative, pH regulator and taste-enhancer in food industry, for implants and suture in the medical practice, as a reagent for polylactic and polyacrylic acids synthesis for biodegradable polymers (Wee et al., 2006). Many efforts have been made to develop biotechnologies for lactate generation from such a cheap, abundant and renewable substrate as the starch. The main problem in starch utilization by LAB, however, is the starch saccharification—a step that could be performed separately prior to fermentation or in parallel with the LA synthesis (Hofvendahl et al., 1999).

One-step process of lactic acid production from starch was achieved by the use of LAB, possessing extracellular amylase activity, such as *Lactobacillus plantarum* A6 (Giraud et al., 1991), *Lactobacillus manihotivorans* (Morlon-Guyot et al., 1998), *Lactobacillus amylophilus* (Vishnu et al., 2002), *Lactobacillus amylovorus* (Zhang and Cheyran, 1991) and *Streptococcus bovis* (Narita et al., 2004). *Lactococcus lactis* is quite desirable for LA industry, because it is homofermentative, secretes only L(+)-lactic acid and is highly productive (Hofvendahl and Hahn-Hägerdal, 1997), but at present its use in starch fermentations is restrained by the absence of amylolytic properties. Most usual overcoming of this problem is the preliminary treatment of the starchy material with α-amylase/amyloglucosidase mixture (Hofvendahl et al., 1999) or hydrolysis by commercial amylolytic enzyme products (Nolasco-Hipolito et al., 2002; Åkerberg et al., 1998). Other approach is co-cultivation of lactococci together with amylolytic strains, such as *Aspergillus awamori* (Roble et al., 2003). In fact, this idea was "borrowed" from the natural microbial communities in the starchy foods, in which the metabolites, produced by the starch-hydrolyzing *Bacillus* spp. may create enough alternative energy sources for LAB (Østergaard et al., 1998). Third solution of the problem is the direct improvement of the *Lactococcus* strain by transformation with vectors, bearing cloned α-amylase gene (Satoh et al., 1997; Okano et al., 2007). Therefore, the isolation of natural lactococci, able to utilize the starch directly for LA production is of special interest.

L. lactis is one of the most used lactic acid bacterium by the dairy industry. It participates in the manufacture of fresh and

Abbreviations: ARDRA, amplified ribosomal DNA restriction analysis; LA, lactic acid; LAB, lactic acid bacteria; RT-PCR, reverse transcription polymerase chain reaction.

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semi-hard cheeses, cream, butter and sour milks, giving the specific taste and texture of the products (Samaržija et al., 2001). Widespread in fermented foods, it could be isolated also from certain sources, containing starch: fermented cereal beverages, such as maize pozol, non-alcoholic drink bushera and sorghum beer (Díaz-Ruiz et al., 2003; Muyanja et al., 2003; Sawadogo-Lingani et al., 2007); sorghum, maize, or wheat sourdough (Hamad et al., 1997; Rocha and Malcata, 1999; Corsetti et al., 2001), cooked fish products and the starchy meal poi, made from the taro plant (Østergaard et al., 1998; Brown and Valiere, 2004). In spite of the starch abundance in these inhabitant niches, very few of the isolated lactococci were found to possess amylolytic properties (Østergaard et al., 1998; Díaz-Ruiz et al., 2003; Muyanja et al., 2003).

On the other hand, the genes encoding several putative amylolytic enzymes (*amyL*, *amyY*, *apu*, *glgP*) were found in the fully sequenced genome of *L. lactis* subsp. *lactis* IL 1403 (Bolotin et al., 2001). Although it has been proven that this strain was unable to produce amylase enzymes (Satoh et al., 1997), the full sequence enables the investigation of the genetic basis of the lactococcal amylolytic activity in general.

In the present study, we report the isolation of a natural strain *L. lactis* subsp. *lactis* B84, which is capable to accomplish full starch saccharification and to produce lactic acid. The purpose of this work is to study the gene expression of the key enzymes, responsible for the starch hydrolysis by *Lactococcus*, using the methods of reverse transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Bacterial strains, media and cultivation conditions

L. lactis subsp. *lactis* B84 was isolated from spontaneously fermenting sourdough, prepared from rye flour (Balvan mill, Bulgaria). It was deposited in the Bulgarian National Collection for Microorganisms and Cell Cultures (http://www.nbimcc.org/en/about.htm), under registration no. 8618. The strain was propagated in MRS medium (Scharlau, Spain) and maintained at 4 °C (subcultured every week), or frozen at −80 °C with 15% (w/w) glycerol added.

Reference strains were supplied from the Belgian Collection of Microorganisms (LMG) and the German Collection of Microorganisms and Cell Cultures (DSMZ): *Enterococcus faecium* LMG 11423, *Enterococcus faecalis* LMG 2937, *Enterococcus durans* LMG 10746, *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893, *Leuconostoc lactis* LMG 8894, *Streptococcus thermophilus* DSM 20617, *L. lactis* subsp. *cremoris* DSM 20069; *L. lactis* subsp. *lactis* DSM 20481. Strain *L. lactis* subsp. *lactis* IL 1403 was provided by T. Sasaki (Meiji Dairies Co., Japan) with the kind permission of A. Chopin (Laboratoire de Génétique Microbienne, INRA, France). The reference strains were cultivated in M17 broth (Merck) or GM17 medium (M17, supplemented with 2% glucose), at optimal temperatures (30 or 37 °C) for 48 h.

2.2. Phenotypic and biochemical characteristics

The strain *L. lactis* subsp. *lactis* B84 was observed for its cell morphology by immersed microscopy and Gram-reaction by the KOH method (Gregersen, 1978). Catalase activity was tested using 20% H_2O_2. Quantitatively L,D-lactic acid isomers were determined using a commercial enzymatic kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.3. Starch fermentation by L. lactis subsp. lactis B84

A number of nutrient media were prepared and the influence of every constituent on the lactic acid production and the cell growth was estimated. Batch cultures were grown in derivatives of Elliker, MRS (Elliker et al., 1956; de Man et al., 1960) and M17 (Oxoid) media with soluble potato starch (Fluka) as a sole carbon source. Three of the fermentation media were found as most useful: M1 had the MRS content, with 20 g l^{-1} starch instead of glucose; M2 medium was M1 without the meat and yeast extracts; M2A contained the components of M2, where the meat peptone was substituted with peptone from casein (Scharlau).

The kinetic experiments without pH control were performed in 500-ml flasks, containing 200 ml medium, inoculated with 2 ml of 24-h precultures, grown in M1 broth, at 33 °C and vigorous agitation (200 rpm), using rotary shaker, New Brunswick Scientific, USA.

Experiments with pH control were carried out in a "New Brunswick Scientific—BIOFLO" fermentor with magnetic stirrer and working volume 450 ml, at 33 °C, stirring at 200 rpm. The pH adjustment was carried out using 5 M NaOH.

Mean values from three independent batches were shown.

2.4. Analytical methods

Cell growth was monitored by a measurement of optical density of the broth (OD_{620}), using a spectrophotometer (UV/vis Spectrophotometer HEλIOS β, UNICOM). Cell dry weight was calculated from the optical density using calibration curve for the strain.

The quantification of glucose and lactic acid concentrations was carried out after filtration of cultures through a membrane filter (0.45 μm pore size, Boeco, Germany) by a high-performance liquid chromatography (HPLC) system equipped with a refractometric detector (Perkin-Elmer chromatograph, series 10). A HPLC column (Aminex HPX-87H, Bio-Rad, Richmond, CA, USA) was used with 5 mM sulfuric acid as the mobile phase at an elution flow rate of 0.6 ml min^{-1}.

The residual starch content was determined by measuring the light absorption of the iodine-starch complex color at wavelength 580 nm (Nakamura, 1981).

2.5. Amylase activity assay

The amylase activity was detected by measurement of the iodine complexing ability of starch according the method of Giraud et al. (1993) at different pH values (4.8–6.4; 0.1 mol l^{-1} citrate-phosphate buffer), and temperatures (33–55 °C). The extracellular and the cell-bond enzymatic activities were determined at the beginning of the stationary phase of the culture, as described by Agati et al. (1998). One enzyme unit was defined as the amount of enzyme hydrolyzing 10 mg starch in 30 min.

2.6. DNA and RNA isolations

Total genomic DNA was isolated from 48-h-old cells of *L. lactis* subsp. *lactis* strains B84 and IL 1403, grown in MRS or M1 media, using GFX Genomic blood DNA purification kit (Amersham Biosciences), following manufacturer's recommendations.

The pure total RNA from the same cultures was obtained from 0.1 g harvested biomass, washed twice in sterile, RNase free bi-distilled water. The microscale procedure of Invisorb®RNA kit I (AJ Roboscreen GmbH) was used. The quantity and purity of the RNA was measured by spectrophotometer at wavelengths of 260 and 280 nm. DNase (Fermentas) treatment was carried out under