Amylolytic *Lactobacillus* Strains from Bulgarian Fermented Beverage Boza

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The lactic acid fermentation is a worldwide method for cereal processing. Great diversity of fermented foods and drinks is produced with the participation of amylolytic lactic acid bacteria (ALAB). In the present study the ALAB content of the Bulgarian cereal beverage "boza" was investigated. Two strains, Bom 816 and N3, were found to possess significant amylolytic activity. The strains' identification was based on genetic criteria, namely amplified ribosomal DNA restriction analysis (ARDRA) and sequencing of the 16S rDNA. The strain Bom 816 belongs to the species *Lactobacillus plantarum* and N3 to *Lactobacillus pentosus*, being the first amylolytic representative of this species.

Optimization of the media composition with starch as a sole carbon source was done. The starch hydrolysis was most efficient in medium containing 4 g/l yeast and 8 g/l meat extracts. Thus, *L. plantarum* Bom 816 consumed 14 g/l starch, while *L. pentosus* N3 consumed 17 g/l. The highest values of lactic acid reached were 9.5 g/l produced by Bom 816 and 5.5 g/l produced by N3. In the presence of yeast extract *L. pentosus* N3 formed 0.8–1 g/l succinic acid.

Both strains produced mainly cell-bound enzymes with amylase activity, at a pH optimum of 5.5, ranging from 3–4 to 21 U/ml for *L. pentosus* N3 and from 0.5 to 11.5 U/ml for *L. plantarum* Bom 816, in dependence of the assay conditions.

Key words: Lactobacillus, Boza, Amylase Activity

Introduction

The term amylolytic lactic acid bacteria (ALAB) unifies the group of lactic acid bacteria (LAB), which are capable to utilize starch as a sole carbon source and could convert it into different products (mainly lactic acid) in a single step of fermentation. Usually ALAB were isolated from fermented foods and beverages, derived from cassava sour starch (Nwankwo *et al*., 1989; Morlon-Guyot *et al*., 1998), fish and rice products (Olympia *et al.,* 1995; Østergaard *et al*., 1998), maize sourdough (Agati *et al*., 1998), or rye sourdough (Petrov *et al*., 2008).

Used for centuries in the natural preservation of food, ALAB played also other roles in food processing: they perform a partial hydrolysis of the starchy material, making it easily digestible (Brown and Valiere, 2004; Nguyen *et al*., 2007), and contribute to the flavour and taste of over 90 different fermented products (Blandino *et al*., 2003).

Boza is a low-pH and low-alcohol cereal-based beverage produced in the Balkan Peninsula. It is a malt drink, made from maize and wheat in Albania, fermented wheat in Turkey, and wheat or millet in Bulgaria and Romania. It has a thick consistency and a low alcohol content (usually around 1%), and has a slightly acidic sweet flavour.

The proved probiotic effects of boza consummation prompted the increasing interest to the microflora, taking part in its fermentation. Boza is a rich source of probiotic LAB that survive conditions simulating the gastrointestinal tract and produce bacteriocins active against a number of pathogens (Todorov *et al*., 2008). The species diversity of the *Lactobacillus* strains isolated from boza include *L. sanfrancisco*, *L. coryniformis*, *L. fermentum*, *L. confusus* (Hancioglu and Karapinar, 1997), *L. plantarum* (Gotcheva *et al*., 2000), *L. paracasei*, *L. pentosus*, *L. brevis*, and *L. rhamnosus* (Botes *et al*., 2007).

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Until now, the attendance of LAB in boza was attributed to the glucose formation, increasing in the first 48 h of beverage fermentation (Gotcheva *et al.*, 2001). The amylolytic properties of LAB isolated from boza have not been investigated and the possibility of direct consumption of the grains' starch by them was not even discussed. The purpose of the present study was to demonstrate the presence of ALAB in boza. Here we describe the starch utilization capacity of two different *Lactobacillus* species, their amylase activity, and final metabolites spectrum.

Materials and Methods

Bacterial strains, media and cultivation conditions

The new amylolytic *Lactobacillus* strains involved in this study were isolated from the Bulgarian traditional cereal drink boza from two distant regions – Sofia and Gotse Delchev town (Bulgaria). The strains were isolated from decimal dilutions of boza probes and plated in MRS-starch medium containing: 10 g/l peptone, 5 g/l yeast extract, 10 g/l meat extract, 2 g/l K_2HPO_4, 1 g/l Tween 80, 5 g/l sodium acetate, 2.0 g/l triammonium citrate, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l $MnSO_4 \cdot H_2O$, and 20 g/l starch instead 20 g/l glucose (pH 6.0–6.5). They formed small, white colonies. Only the pure cultures, derived from one cell and capable to acidify the liquid MRS-starch medium, were further analyzed. They were preserved frozen at –80 °C with 15% (w/w) glycerol added.

L. plantarum Bom 816 and *L. pentosus* N3 are deposited in the Bulgarian National Collection for Microorganisms and Cell Cultures (http://www.nbimcc.org/en/about.htm). The reference strains *Lactobacillus plantarum* ATCC 14917^T and *Lactobacillus pentosus* ATCC 8041^T were supplied by The American Type Culture Collection. They were cultivated in MRS broth (De Man *et al.*, 1960), at 30 °C.

Phenotypic and biochemical characteristics

The new isolated *Lactobacillus* strains were observed for their cell morphology by immersed microscopy and Gram reaction using the KOH method (Gregersen, 1978). The catalase activity was tested using 20% H_2O_2. The stereoisomers of lactic acid (L, D) were determined quantitatively using a commercial enzymatic kit (Roche Diagnostics GmbH, Mannheim, Germany).

Starch fermentation

Four different media based on MRS (De Man *et al.*, 1960) with soluble potato starch (Fluka) as a sole carbon source were used. All of them contained 10 g/l bactopeptone (Difco) and the common salts of MRS, and differed in the amounts of yeast (YE) and meat (ME) extracts: M1 was a medium without YE and ME; M2 contained only 4 g/l YE; M3 contained 2 g/l YE and 4 g/l ME; M4 contained 4 g/l YE and 8 g/l ME.

The kinetic experiments were performed in M1–M4 broths (containing 1% inoculum) at 30–37 °C, vigorous agitation (200 rpm), and under anaerobic conditions (in 250-ml sealed bottles, with 200 ml medium). Mean values from two independent batches were determined.

Analytical methods

The cell growth was monitored by measuring the optical density of the broth at 600 nm (OD_{600}) using a spectrophotometer (UV/VIS Spectrophotometer HEλIOS β, UNICOM).

The quantification of the glucose and lactic acid concentrations was carried out after filtration of the 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.

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

Amylase activity assay

The amylase activity was determined by measuring the iodine complexing ability of starch according to the method of Giraud *et al.* (1993) at different pH values (5–6.5, using K_2HPO_4/NaH_2PO_4 buffer) and temperatures (37–45 °C). The extracellular and the cell-bound 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.

DNA isolation

Total genomic DNA was isolated from 48-h-old cells grown in M4 medium, using GFX genomic blood DNA purification kit (Amersham Biosciences, Buckinghamshire, UK), following the manufacturer's recommendations.

PCR amplification of 16S rRNA genes and ARDRA analysis

All polymerase chain reactions (PCR) presented here were prepared with PuReTaq™ Ready to Go™ PCR beads (Amersham Biosciences Europe GmbH, Freiburg, Germany), according to the manufacturer's instructions, in a total volume of 50 μl and final primer concentrations of 0.5 pmol/μl (all purchased from MWG-Biotech AG, Ebersberg, Germany). The PCR amplifications were done in a Progene termocycler (Techne, Hampshire, UK).

Amplification of the 16S rRNA gene for the ARDRA (amplified ribosomal DNA restriction analysis) and sequence analyses was performed with the universal eubacterial primer pair: forward primer fD1, 5'-AGA GTT TGA TCC TGG CTC AG-3'; reverse primer rD1, 5'-AAG GAG GTG ATC CAG CC-3' (Weisburg *et al.*, 1991). The final concentration of the template DNA was 1–2 ng/μl. The temperature profile was: 95 °C for 5 min, 35 cycles consisting of 94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 2 min, followed by a final elongation at 72 ºC for 5 min. The 1.5-kb PCR products were visualized in 1% agarose gel (Sambrook and Russel, 2001).

The enzymatic digest of the amplified DNA for the ARDRA analysis was done using the endonuclease *Hae*III (New England BioLabs, Ipswich, UK), in a final volume of 20 μl, and incubation for 1 h at 37 ºC.

DNA sequencing and phylogenetic analysis

The 16S rDNA amplification products were purified using GFX PCR DNA and gel band purification kits (Amersham Biosciences). Macrogen Inc. (Korea) determined the nucleotide sequences by an automated sequencer (Applied Biosystems 3700, Applied Biosystems, Inc., Foster City, CA, USA) and the dye-deoxy termination procedure. The primer pair used for sequencing was the described above fD1/rD1. The sequence analysis was performed using Chromas and CAP3 Sequence Assembly Programs (http://genome.cs.mtu.edu/cap). Sequence comparison was done using BLAST (Altschul *et al.*, 1997) and Clustal W programs (Thompson *et al.*, 1994). The phylogenetic tree was constructed with the neighbour-joining method after multiple sequence alignments, using Clustal W and BioEdit programs.

Results

Identification of the new isolated amylolytic strains

Morphological and biochemical features

More than 20 amylolytic LAB strains from four different types of boza were isolated. The majority of them possessed visible, but weak amylolytic activity. Only two strains were able to degrade significant amounts of starch and were subjected to further analyses – Bom 816 and N3. Their cells

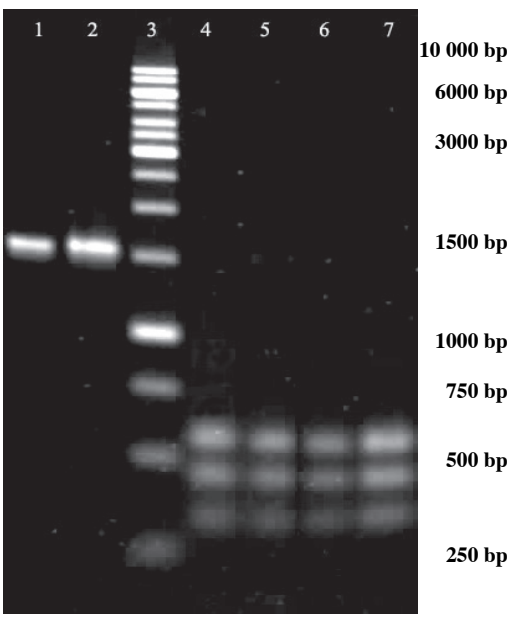

Fig. 1. ARDRA of 16S rDNA of strains *L. plantarum* Bom 816 and *L. pentosus* N3. Lane 1, Bom 816 – 16S rDNA, PCR product; lane 2, N3 – 16S rDNA, PCR product; lane 3, DNA ladder; lane 4, *L. plantarum* ATCC 14917 – 16S rDNA/*Hae*III; lane 5, *L. pentosus* ATCC 8041 – 16S rDNA/*Hae*III; lane 6, Bom 816 – 16S rDNA/*Hae*III; lane 7, N3 – 16S rDNA/*Hae*III.

Table I. Comparison of the amylolytic abilities and acids production of *L. plantarum* Bom 816 and *L. pentosus* N3, grown in media containing 30 g/l starch and different amounts of yeast (YE) and meat (ME) extracts. The maximal values after 96 h of cultivation are presented.

Medium	Starch consumed [g/l]		Lactic acid [g/l]		Succinic acid [g/l]	
	Bom 816	N3	Bom 816	N3	Bom 816	N3
M1	9.5	8.9	8.4	5.5	–	–
M2	11.1	13.1	9.2	4.9	–	0.9
M3	13.3	16.2	9.4	5.5	–	0.8
M4	13.8	16.7	9.5	5.4	–	1.0

M1, medium without YE and ME; M2, 4 g/l YE only; M3, 2 g/l YE and 4 g/l ME; M4, 4 g/l YE and 8 g/l ME.

were rod-shaped (these of N3 were longer and formed short chains), non-motile, Gram-positive, and catalase-negative. Both strains formed a (D/L)-racemic mixture of optical isomers of lactic acid.

In order to identify the new isolated strains, a genomic DNA was used for amplification of the 16S rDNA, and then the product with the approximate size 1.5 kb was digested with the endonuclease *Hae*III. This enzyme recognizes four base pairs (GGCC) and was chosen based on preliminary studies of the reference strains. As it is shown at Fig. 1, the ARDRA profile of the *Lactobacillus plantarum*/*Lactobacillus pentosus* group consisted of three typical bands with size 570, 460 and 360 bp, proving the affiliation of the isolates to one of these species.

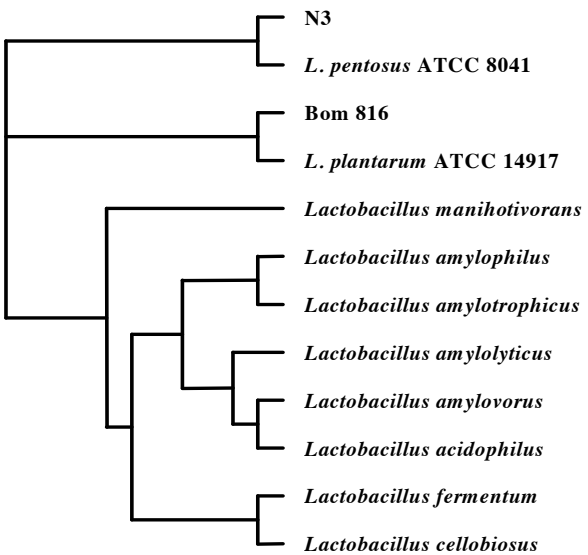

Fig. 2. Phylogenetic tree derived from 16S rDNA sequence analysis of the amylolytic representatives of the genus *Lactobacillus*. The neighbour-joining method applied after multiple sequence alignment was used. The sequences were retrieved by the GenBank database. As a 16S sequence of amylolytic *L. plantarum* was not available, the type strain's sequence was used.

16S sequencing

As the result of ARDRA was not sufficient for identification at the species level, complete 16S rRNA gene sequencing was performed. The complete 16S rDNA sequences, obtained in this study, were deposited in the NCBI GenBank under the accession numbers GU253891 (Bom 816) and GU253892 (N3).

The sequences' comparison showed 100% identity with the type strains 16S rDNA (ATCC 14917^T, ATCC 8041^T), thus proving that the strain Bom 816 belongs to *L. plantarum* subsp. *plantarum* and N3 to *L. pentosus*.

The 16S rDNA sequences comparison of all *Lactobacillus* species, possessing amylolytic activity, allowed the creation of the phylogenetic tree of this peculiar group of bacteria (Fig. 2). The new strains were clustered in the *L. plantarum* group, and were quite phylogenetically distant from the other amylolytic lactobacilli. Importantly, the strain N3 is the first representative of the species *L. pentosus* with amylolytic properties.

Batch cultivation of the new amylolytic strains

Influence of the media content on starch hydrolysis

Aiming at optimization of the growth conditions and quantification of the fermentative products, batch experiments using four different media (M1–M4) with increasing amounts of yeast (YE) and meat (ME) extracts were performed. The maximal values of consumed starch (starting from 30 g/l) and the obtained metabolites are presented in Table I.

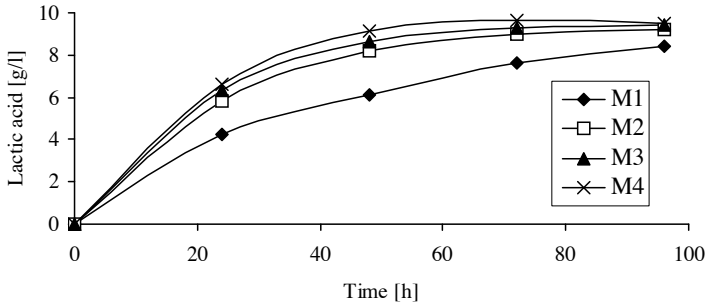

Fig. 3. Time profiles of lactic acid production from 30 g/l starch by *L. plantarum* Bom 816 grown in media with gradual increase of the yeast and meat extracts concentrations (see Materials and Methods).

In the minimal medium M1 (lacking YE and ME) starch hydrolysis occurred partially even after a long period of cultivation (96 h). The results suggested that the YE had slightly higher significance on the process than the ME. In general, the starch hydrolysis by lactobacilli was as more efficient, as richer was the medium. In M4 medium, both strains hydrolyzed high amounts of starch (Bom 816 about 14 g/l, N3 about 17 g/l). Surprisingly, the biomass growth remained almost the same in batches carried out in M1–M4 media (OD_{600} was around 1.7 for Bom 816, and 1.6 for N3 at 24–72 h of cultivation independently of the media content).

Starch fermentation products

The fermentation profiles revealed that the new isolates were homo-fermentative and formed mainly lactic acid as a final product, ranging from 8.4 to 9.5 g/l for Bom 816 and between 4.9 and 5.5 g/l for N3 (Table I).

Comparative kinetics of products formation in the media M1–M4 is shown in Figs. 3 and 4. For Bom 816, the process of lactic acid synthesis was faster, and the maximal values were higher with the increase of YE and ME share in the nutrient medium. In the presence of YE *L. pentosus* N3 produced 0.8–1 g/l succinic acid in addition to lactic acid. This process was possible only when YE was added (media M2–M4); when YE was excluded (M1), the fermentation was turned to lactic acid only. Other fermentative products were not detected.

Amylase activity assay

As the presence of cell-bound and extracellular amylases in LAB species was reported (Agati *et al.*, 1998; Petrov *et al.*, 2008), an amylase assay, able to distinguish the localization of the enzyme activity, was performed. The cultures were grown in M4 medium to the beginning of the stationary phase. The activities of harvested cells (cell-bound amylase) and of the cell-free liquid supernatants (extracellular amylase) were measured in parallel. The assay strongly suggested that the cell-bound enzyme was mainly responsible for starch hydrolysis by both strains, as the amylase activity of the cell-free supernatants was low, ranging between 0.5 and 1.5 U/ml.

The cell-bound amylase activity of the new strains was tested at different pH values and temperatures (Fig. 5). The pH optimum of the

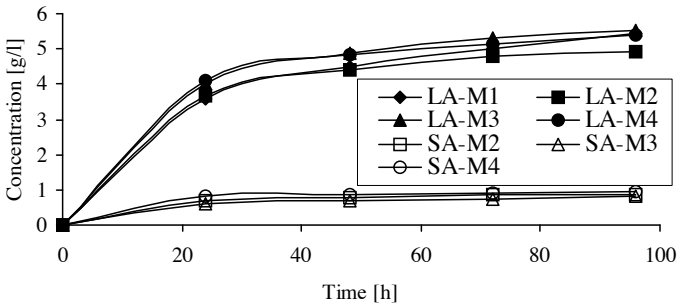

Fig. 4. Time profiles of the lactic (LA) and succinic acid (SA) productions from 30 g/l starch by *L. pentosus* N3 grown in media with gradual increase of the yeast and meat extracts concentrations (see Materials and Methods).

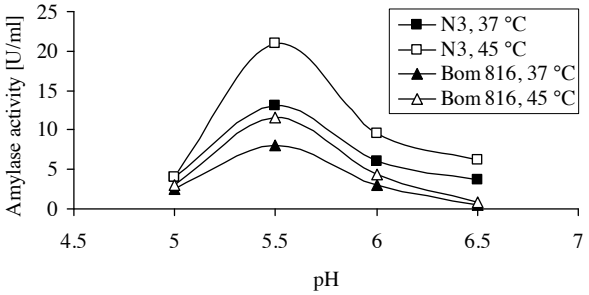

Fig. 5. Amylase activity of cell-bound enzymes of *L. plantarum* Bom 816 and *L. pentosus* N3, assayed at different pH values and temperatures.

cell-bound amylases of both strains Bom 816 and N3 was pH 5.5. At this condition *L. pentosus* N3 showed significant amylase activity, ranging between 13 and 21 U/ml, as the higher activity was obtained at 45 ºC. The activity of *L. plantarum* Bom 816 was 8–11.5 U/ml.

Discussion

Lactic acid fermentation is one of the oldest and most economical methods of producing and preserving food. It contributes towards the safety, nutritional value, shelf life and acceptability of a wide range of cereal-based foods. In spite of the ubiquitous use of LAB in cereals processing the biodiversity of amylolytic lactobacilli is quite limited. Among 120 described *Lactobacillus* species, only nine are able to degrade starch as a sole carbon source (Reddy *et al*., 2008).

Microflora identification of Bulgarian boza showed that it mainly consists of yeasts and LAB, and the LAB are always predominant in the microbial association with an average LAB/yeasts ratio equal to 2.4 (Gotcheva *et al*., 2000). The high LAB content of this cereal-based product inspired the present study, which aimed to answer if the lactobacilli isolated from boza own amylolytic properties or if they use glucose residua, formed by the action of external grain amylases.

From probes of boza taken from distant Bulgarian regions two different amylolytic *Lactobacillus* strains were isolated (Bom 816 and N3), identified as *L. plantarum* and *L. pentosus*. Although *L. plantarum* is a common strain in foods, Bom 816 is just the fourth *L. plantarum* with reported amylolytic activity, together with A6, LMG 18053 and NCIM 2084 (Giraud *et al*., 1991; Krishnan *et al*., 1998). Concerning N3, it is the first amylolytic representative of *L. pentosus*, as this species has not been reported to be able of starch degradation until now (Zanoni *et al.*, 1987; Reddy *et al*., 2008). It can be claimed that the present work expands the family of the ALAB group with a new member.

The culturing of the new isolated strains in media with a gradual increase of yeast and meat extracts revealed that the fastidious lactobacilli need B-complex vitamins and a mixture of amino acids supply for their amylolytic action. Thus they differed from *Lactococcus lactis*, which displayed amylase activity only in minimal medium, without extracts (Petrov *et al.*, 2008).

The succinic acid formation in *L. pentosus* usually occurs in growth conditions with limited glucose content and in the presence of citrate (all media M1–M4 contained 2 g/l triammonium citrate). The succinic acid synthesis resulted in a decrease of lactic acid accumulation, as citrate is metabolized via oxalacetate serving as an H-acceptor in a joint process together with lactic acid (Cselovszky *et al*., 1992). However, the succinic acid synthesis occurred only in the presence of yeast extract. Its exclusion from the nutrient medium allows directing the metabolism of the facultative hetero-fermenter *L. pentosus* towards lactic acid production only.

The comparison of the maximal amylase activities (11.5 and 21 U/ml), obtained by *L. plantarum* Bom 816 and *L. pentosus* N3, showed values in proximity to amylases found in other lactobacilli. They were similar to the cell-bound enzyme, found in *Lactobacillus fermentum* strains, isolated from fermented maize dough (Agati *et al*., 1998), and to the extracellular amylase from *Lactobacillus manihotivorans*, isolated from cassava starch (Guyot *et al*., 2000).

In conclusion, two novel amylolytic *Lactobacillus* strains were isolated from "boza". The estimation of its amylase activity and the quantification of accumulated metabolites contribute to the understanding of the role of ALAB strains for the unique taste and fragrance of the beverage, favorite of people of all ages.

Acknowledgements

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