BACTERIOCIN PRODUCTION BY LACTOBACILLUS PENTOSUS ST712BZ ISOLATED FROM BOZA

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ABSTRACT
Bacteriocin ST712BZ (14.0kDa in size) inhibits the growth of Lactobacillus casei, Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumoniae and Lactobacillus curvatus. Growth of strain ST712BZ in BHI, M17, soy milk and molasses was similar to growth in MRS, with optimal bacteriocin production (12800AU/mL) recorded in MRS after 24h. The same level of bacteriocin production (12800AU/mL) was recorded in MRS broth with an initial pH of 6.5, 6.0 and 5.5. However, MRS broth (pH 6.5) supplemented with 1mM EDTA, yielded only 6400AU/mL. Low levels of bacteriocin activity were recorded in MRS broth with an initial pH of 5.0 and 4.5. Of all media compositions tested, MRS supplemented with tryptone (20.0g/L), glucose (20.0 to 40.0g/L), mannose (20.0g/L), vitamin B12, or vitamin C yielded 12800AU/mL. Glycerol concentrations of 1.0g/L and higher repressed bacteriocin production. Maximal bacteriocin activity (25600AU/mL) was recorded in MRS supplemented with Vit. B1 or DL-6,8-thioctic acid.

Key words: Bacteriocin ST712BZ; Lactobacillus pentosus; boza

INTRODUCTION
Cereals are fermented in most regions of the world and a variety of raw materials and fermentation conditions are used, mostly with lactic acid bacteria and yeast as starter cultures. Lactic acid bacteria play an important role in the preservation, microbiological stability and production of aroma compounds in these products (27).

A few papers have been published on the microbial composition of boza, a beverage traditionally produced in Bulgaria and prepared from a combination of different cereals (14,12,3,35). Most of the lactic acid bacteria that have been isolated from boza belong to the genera Lactobacillus spp., Lactococcus spp. and Leuconostoc spp. and as many as 33 strains revealed antibacterial activity against a number of Gram-positive bacteria, including Listeria innocua, and a few Gram-negative bacteria, including Escherichia coli (14). In most of these studies, the antimicrobial properties of the strains have been ascribed to the production of bacteriocins (27,33), defined as small proteins or peptides with bactericidal or bacteriostatic activity against genetically closely related species (17).

Few bacteriocin-producing strains of Lactobacillus pentosus have been reported, namely bacteriocins produced by strains 191 and 204, isolated from sucuk (8); bacteriocins ST151BR and ST112BR produced by strains ST151BR and ST112BR, respectively (29,30); a bacteriocin produced by a commercial starter culture (25); and pentocin TV35b, produced by strain TV35b isolated from vaginal secretions (26).

In recent papers (20,23), specific environmental conditions, including those found in food, have been studied to determine their effect on the production of bacteriocins. Bacteriocin production changes dramatically upon altering of environmental conditions and optimum production may require a specific combination of environmental parameters (19). Little is known about the interactions these factors have on the production of a bacteriocin, especially in a complex food environment.

Two papers have reported on the effect of nitrogen and carbon in the medium on the production of bacteriocins ST151BR and ST112BR produced by L. pentosus (29,30).
Studies on bacteriocins from other lactic acid bacteria, e.g. pediocin PD-1 (24), enterocin AS-48 (2), enterocin P (13), sakacinP (1), bacteriocins produced by L. mesenteroides L124 (21), plantaricin ST31 (31) and plantaricin UG1 (11) have suggested that production is often regulated by growth pH and temperature. In some cases, higher bacteriocin activity has been recorded at sub-optimal growth conditions (22,5,18,1,31).

The aim of this study was to determine the conditions needed for optimal production of bacteriocin ST712BZ produced by L. pentosus ST712BZ isolated from boza.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

Strain ST712BZ, isolated from boza, was classified as *L. pentosus* based on phenotypic and genotypic characteristics (33). The strain was cultured in MRS medium (Biolab, Biolab Diagnostics, Midrand, SA) at 30°C and stored at -80°C in spent MRS broth, supplemented with 15% (v/v) glycerol. MRS broth (Biolab) was used in all experiments, except growth optimization, in which case MRS broth (10) was modified as indicated.

**Bacteriocin bioassay**

Bacteriocin screening was performed by using the agar-spot-test method (32). Correction of the cell-free supernatant to pH 6.0 with 1M NaOH prevented the inhibitory effect of lactic acid. Antimicrobial activity was expressed as arbitrary units (AU/mL), calculated as a x 100, where “a” represents the dilution factor and “b” the last dilution that produces an inhibition zone of at least 2mm in diameter. Activity is expressed per mL by multiplication with 100. One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (32). *Lactobacillus casei* LHS was used as indicator strain.

**Molecular size of the bacteriocins**

*L. pentosus* ST712BZ was cultured in MRS broth (Biolab) for 20h at 30°C. The cells were harvested (8000xg, 10 min, 4°C) and the bacteriocins precipitated from the cell-free supernatants with 60% saturated ammonium sulphate. The precipitate was re-suspended in one tenth volume 25mM ammonium acetate (pH 6.5), desalted against distilled water by using a 1000Da cut-off dialysis membrane (Spectrum Inc., CA, USA) and then separated by tricine-SDS-PAGE, as described by Schägger and Von Jagow (28). A low molecular-weight marker with sizes ranging from 2.5 to 45.0kDa (Amersham International) was used. The gels were fixed and one half overlaid with *L. casei* LHS (10°CFU/mL), embedded in Brain Heart Infusion (BHI) agar (Biolab) to determine the position of the active bacteriocin, as described by Todorov and Dicks (29).

**Bacteriocin production in different growth media and at different initial growth pH**

An 18h-old culture of strain ST712BZ was inoculated (2%, v/v) into MRS broth (Biolab), BHI broth, M17 broth (Merck), soy milk (10%, v/w, soy flour) and molasses (10%, w/v), respectively. Incubation was at 30°C and 37°C, respectively, without agitation, for 25h. Samples were taken every hour and examined for bacterial growth (OD at 600 nm), changes in culture pH, and production of bacteriocins (AU/mL). The agar-spot-test method was used, with *L. casei* LHS as target organism.

In a separate experiment, the effect of initial medium pH on the production of bacteriocin ST712BZ was determined. Volumes of 300 mL MRS broth were adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.5, respectively, with 6M HCl or 6M NaOH and then autoclaved. Each flask was inoculated with 2% (v/v) of an 18h-old culture of *L. pentosus* ST712BZ and incubated at 30°C for 24h, without agitation. Changes in culture pH and production of bacteriocin ST712BZ, expressed as AU/mL, were determined every hour as described elsewhere. All experiments were done in triplicate.

**Effect of medium composition on bacteriocin production**

*L. pentosus* ST712BZ was grown in 10 mL MRS broth (Biolab) for 18h at 30°C, the cells harvested by centrifugation (8000xg, 10 min, 4°C), and the pellet re-suspended in 10 mL sterile peptone water. Four mL of the cell suspension was used to inoculate 200 mL of the following media: (a) MRS broth (10), without organic nutrients, supplemented with tryptone (20.0 g/L), meat extract (20.0 g/L), yeast extract (20.0 g/L), tryptone (12.5 g/L) plus meat extract (7.5 g/L), tryptone (12.5 g/L) plus yeast extract (7.5 g/L), meat extract (10.0 g/L) plus yeast extract (10.0 g/L), or a combination of tryptone (10.0 g/L), meat extract (5.0 g/L) and yeast extract (5.0 g/L), respectively; (b) MRS broth, i.e. with 20.0 g/L D-glucose; (c) MRS broth without D-glucose, supplemented with 20.0 g/L fructose, sucrose, lactose, mannose, and maltose, respectively; (d) MRS broth with 1.0 to 40.0 g/L glucose as sole carbon source; (e) MRS broth with 2.0 to 100.0 g/L K2HPO4; and (f) MRS broth supplemented with 1.0 to 50.0 g/L glycerol.

In a separate experiment, the vitamins cyanocobalamin (Sigma, St. Louis, Mo.), L-ascorbic acid (BDH Chemicals Ltd), thiamine (Sigma) and DL-6,8-thioctic acid (Sigma) were filter-sterilised and added to MRS broth at 1.0 mg/mL (final concentration). All cultures were incubated at 30°C for 24h. Activity levels of bacteriocin ST712BZ were determined as described elsewhere. All experiments were done in triplicate.

**Plasmid isolation**

Plasmid DNA was isolated according to the method described by Burger and Dicks (6), followed by CsCl density gradient centrifugation (4). The DNA was separated on an agarose gel, according to Ausubel et al. (4).
RESULTS AND DISCUSSION

All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5% and single data points are presented in the figures without standard deviation bars.

The cell-free supernatant of *L. pentosus* ST712BZ inhibited the growth of *L. casei, E. coli, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumoniae* and *Lactobacillus curvatus* (33). According to tricine-SDS-PAGE, bacteriocin ST712BZ is approximately 14.0kDa (Fig. 1). No plasmid DNA was isolated.

Growth of *L. pentosus* ST712BZ in BHI, M17, soy milk and molasses was very similar to growth in MRS (Fig. 2). The cell density of both strains increased from OD$_{600nm}$ 0.02 to approximately 9.0 (dilution factor taken into calculation) during 25h (Fig. 2). Low levels of bacteriocin ST712BZ activity (less than 200AU/mL) were detected after 6h of growth in MRS broth (Fig. 2). Optimal production of bacteriocin ST712BZ (12800AU/mL) was recorded after 24h (Fig. 2), and only when incubated at 30°C. Only 6400AU/mL for ST712BZ was recorded when the cells were incubated at 37°C (not shown). Bacteriocin ST712BZ is a primary metabolite. Similar results have been reported for bacteriocin ST151BR (29), plantaricin Y (7) and bacteriocins produced by *P. acidilactici* (25).

The activity of bacteriocin ST712BZ did not decrease during 72h of incubation at 25°C, suggesting that extracellular proteases have not been produced. Optimal bacteriocin production (12800AU/mL) was recorded in MRS broth with an initial pH of 6.5, 6.0 and 5.5 (Table 1). In MRS broth (pH 6.5) supplemented with 1 mM EDTA, bacteriocin ST712BZ production was 6400AU/mL. Low levels of bacteriocin activity were recorded when the strains were cultured in MRS broth with an initial pH of 5.0 and 4.5 (Table 1). The culture pH after 24h growth was between 3.52 and 3.75. Similar results have been reported for other bacteriocins produced by *L. pentosus* (29,30) and *L. plantarum* (9,15,31).

**Table 1.** Influence of initial medium pH on production of bacteriocin ST712BZ in MRS broth (Biolab).

| Initial pH | 4.5 | 5.0 | 5.5 | 6.0 | 6.5 |
|------------|-----|-----|-----|-----|-----|
| Final pH   | 3.5 | 3.6 | 3.7 | 3.7 | 3.8 |
| Δ pH       | 1.0 | 1.4 | 1.9 | 2.3 | 2.8 |
| Bacteriocin activity (AU/mL) | 3200 | 6400 | 12800 | 12800 | 12800 |
| OD$_{600nm}$ | 8.3 | 8.8 | 9.2 | 9.1 | 9.4 |
| Specific bacteriocin activity (AU/mL/OD$_{600nm}$) | 386 | 727 | 1391 | 1407 | 1362 |
| Changes in bacteriocin activity (%)* | 25 | 50 | 100 | 100 | 100 |

*Compared to 12 800AU/mL recorded in MRS broth (Biolab).
Growth of strain ST712BZ in BHI broth or M17 broth adjusted to pH 6.5 yielded only 400 AU/mL of bacteriocin ST712BZ (Table 2). No bacteriocin production was recorded in 10% (w/v) soy milk (Table 2). Low levels of bacteriocin ST712BZ (200 AU/mL) were recorded when the strains were grown in 10% (w/v) molasses (Table 2). Specific nutrients are required for the production of the bacteriocin ST712BZ. This phenomenon has been observed for other bacteriocins, e.g. bacteriocins ST151BR and ST112BR (29,30).

Tryptone (20 g/L), or a combination of tryptone and yeast extract (1:0.6), added to basal MRS medium yielded a bacteriocin level of 12 800 AU/mL (Table 2). Growth in the presence of a combination of tryptone and meat extract (1:0.6) reduced bacteriocin production with 50%. Growth in the presence of

### Table 2. Influence of organic nitrogen, carbohydrates, growth medium and potassium on the production of bacteriocin ST712BZ

| Component                  | Concentration (g/L) | pH  | Activity (AU/mL) | Changes in bacteriocin activity (%)* |
|----------------------------|---------------------|-----|------------------|-------------------------------------|
| Tryptone                   | 20.0                | 3.8 | 1280             | 100                                 |
| Meat extract               | 20.0                | 3.7 | 1600             | 13                                  |
| Yeast extract              | 20.0                | 3.7 | 3200             | 25                                  |
| Tryptone + meat extract    | 12.5 + 7.5          | 3.7 | 6400             | 50                                  |
| Tryptone + yeast extract   | 12.5 + 7.5          | 3.7 | 12800            | 100                                 |
| Meat extract + yeast extract| 10.0 + 10.0        | 3.8 | 3200             | 25                                  |
| Tryptone + meat extract + yeast extract | 10.0 + 5.0 + 5.0 | 3.8 | 12800            | 100                                 |
| Glucose                    | 20.0                | 3.7 | 12800            | 100                                 |
| Fructose                   | 20.0                | 4.0 | 1600             | 13                                  |
| Sucrose                    | 20.0                | 3.8 | 6400             | 50                                  |
| Maltose                    | 20.0                | 3.8 | 3200             | 25                                  |
| Mannose                    | 20.0                | 3.7 | 12800            | 100                                 |
| Lactose                    | 20.0                | 4.0 | 3200             | 25                                  |
| Gluconate                  | 20.0                | 5.2 | 3200             | 25                                  |
| Glycerol                   | 0                   | 3.8 | 12800            | 100                                 |
|                            | 1.0                 | 3.8 | 6400             | 50                                  |
|                            | 5.0                 | 3.8 | 6400             | 50                                  |
|                            | 10.0                | 3.8 | 6400             | 50                                  |
|                            | 20.0                | 3.8 | 3200             | 25                                  |
|                            | 50.0                | 3.8 | 3200             | 25                                  |
| MRS                        | 50.0                | 3.8 | 12800            | 100                                 |
| BHI                        | 37.0                | 6.1 | 400              | 3                                   |
| M17                        | 42.5                | 6.0 | 400              | 3                                   |
| Soy flour                  | 100.0               | 4.3 | 0                | 0                                   |
| Molasses                   | 100.0               | 4.1 | 200              | 2                                   |
| K₂HPO₄                     | 2.0                 | 3.8 | 12800            | 100                                 |
| KH₂PO₄                     | 2.0                 | 3.8 | 12800            | 100                                 |
| (mg/mL)                    | Cyanocobalamin (Vit. B₁₂) | 1.0 | 4.0             | 12800                               | 100 |
|                            | Thiamine (Vit. B₁)  | 1.0 | 4.0             | 25600                               | 200 |
|                            | DL-6,8-thioctic acid| 1.0 | 4.0             | 25600                               | 200 |
|                            | L-ascorbic acid (Vit. C) | 1.0 | 4.0             | 12800                               | 100 |
|                            | Control             | 0   | 3.9             | 12800                               | 100 |

*Compared to 12 800 AU/mL recorded in MRS broth (Biolab).
either yeast extract, or a combination of meat extract and yeast extract (1:1), resulted in bacteriocin production of 3200AU/mL (Table 2). Growth in the presence of only meat extract yielded only 1600AU/mL.

Tryptone is the key nitrogen source needed for optimal production of bacteriocin ST712BZ. Similar results have been reported for the production of plantaricin 423 (34) and for bacteriocins ST151BR and ST112BR (29,30). In the case of plantaricin 423, optimal bacteriocin production was recorded in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract. Stimulation of bacteriocin production by yeast extract and meat extract has been reported for helveticin J (16). As far as we could determine, this is the first indication that tryptone is the key nitrogen source needed in the production of L. pentosus bacteriocins.

Growth of strain ST712BZ in the presence of glucose (20.0, 30.0 and 40.0 g/L) yielded 12800AU/mL of bacteriocin ST712BZ (Fig. 3A). Lower concentrations of glucose (1.0 g/L, 5.0 g/L and 10.0 g/L) yielded 800AU/mL, 800AU/mL and 6400AU/mL, respectively (Fig. 3A). Growth in presence of mannose (20 g/L) yielded the same activity as 20 g/L glucose (Table 2). Growth in the presence of sucrose (20.0 g/L) reduced bacteriocin production by 50%, i.e to 6400AU/mL. Gluconate, maltose and lactose (20.0 g/L), on the other hand, yielded 3200AU/mL (Table 2). Low activity (1600AU/mL) was recorded in the presence of 20.0 g/L fructose. Based on these results, the production of bacteriocin ST712BZ is stimulated when cells are grown in medium supplemented with 20.0 to 40.0 g/L glucose.

Bacteriocin ST712BZ production remained at 12800AU/mL when strain ST712BZ was grown in the presence of K$_2$HPO$_4$ or KH$_2$PO$_4$ (Fig. 3B). Growth in the presence of 10.0 g/L or 20.0 g/L yielded 6400AU/mL bacteriocin ST712BZ (Fig. 3B). In the case of plantaricin UG1, 7.0 g/L K$_2$HPO$_4$ resulted in increased activity (11). Concentrations of 10.0 and 20.0 g/L K$_2$HPO$_4$ lowered bacteriocin ST151BR activity by 50% (29). In the case of bacteriocin ST112BR, higher levels of activity were recorded when the medium contained 5.0 g/L, 10.0 g/L and 20.0 g/L KH$_2$PO$_4$ (30). The optimal concentration of K$_2$HPO$_4$ required for plantaricin ST31 production was between 2.0 g/L and 5.0 g/L (31).

Production of bacteriocin ST712BZ was the highest (128000AU/mL) in the absence of glycerol (Table 2). Glycerol concentrations of 1.0 g/L and higher (up to 50.0 g/L) led to decreased levels of bacteriocin ST712BZ production (Table 2). Similar results were reported for the production of bacteriocins ST151BR, ST112BR and plantaricin ST31 (29,30,31). An increase in glycerol leads to a lowering in water activity. The production of bacteriocin ST712BZ may be influenced by osmotic stress.

Bacteriocin ST712BZ production of 12800AU/mL was recorded in the presence or absence of Vit. B$_12$ or Vit. C (Table 2). A increase in production levels was recorded in MRS supplemented with Vit. B$_1$ or DL-6,8-thioctic acid up to 25600AU/mL (Table 2). In the case of bacteriocin ST151BR, produced by L. pentosus ST151BR, the latter vitamins had no effect on production levels (29). However, these vitamins led to a 50% decrease in the production of bacteriocin ST112BR, produced by L. pentosus ST112BR (30).

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RESUMO

Bacteriocina produzida por Lactobacillus pentosus ST712BZ isolado de boza

A bacteriocina ST712BZ produzida par Lactobacillus pentosus (peso molecular de 14,0kDa) inibe o crescimento de Lactobacillus casei, Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumoniae e Lactobacillus curvatus. O crescimento de L. pentosus ST712BZ
Bacteriocin production by L. pentosus

Palavras-chave: Bacteriocina ST712BZ, Lactobacillus pentosus, boza

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