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Partial characterisation of two bacteriocins produced by *Lactobacillus paracasei* subsp. *paracasei* ST242BZ and ST284BZ and the effect of medium components on their production

Svetoslav Dimitrov TODOROV*, Leon Milner Theodore DICKS

Department of Microbiology, University of Stellenbosch, 7600 Stellenbosch, South Africa

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Abstract - The influence of medium components on production of bacteriocins ST242BZ (10.0 kDa) and ST284BZ (3.5 kDa) by *Lactobacillus paracasei* subsp. *paracasei* ST242BZ and ST284BZ have been studied. Growth in MRS broth (pH of 6.5) yielded bacteriocin levels of 12800 AU/ml. Modified MRS with tryptone as the only nitrogen source, MRS supplemented with KH_2PO_4 (10-100 g/l), or MRS supplemented with thiamine increased bacteriocin ST242BZ production to 25600 AU/ml. Tryptone, combinations of tryptone, meat extract and yeast extract, or thiamine did not increase bacteriocin ST284BZ production. However, MRS supplemented with K_2HPO_4 (50-100 g/l) increased bacteriocin ST284BZ production up to 25600 AU/ml. Our results suggest that production of bacteriocins ST242BZ and ST284BZ are stimulated by potassium ions.

Key words: bacteriocins ST242BZ and ST284BZ, *Lactobacillus paracasei* subsp. *paracasei,* boza.

INTRODUCTION

Countries of the Balkan region in Europe are famous for the production of food and beverages fermented with lactic acid bacteria. Boza is produced from the fermentation of different cereals by yeast and lactic acid bacteria. The microbial composition of boza consists mainly of *Lactobacillus*, *Lactococcus* and *Leuconostoc* spp. (Gotcheva *et al.*, 2000; Arici and Daglioglu, 2002; Zorba *et al.*, 2003). A bacteriocin produced by one of these strains, *Lactococcus lactis* subsp. *lactis* 14, was partially characterised (Ivanova *et al.,* 2000). A few subsequent papers reported on boza strains with activity against Gram-positive bacteria, including *Listeria innocua*, and Gram-negative bacteria, including *Escherichia coli* (Ivanova *et al.*, 2000; Kabadjova *et al*., 2000). Todorov and Dicks (2004) described mesentericin ST99, produced by *Leuconostoc mesenteroides* subsp. *dextranicum* ST99. The latter bacteriocin, and pediocin ST18 produced by *Pediococcus pentosaceus* ST18, revealed good anti-*Listeria* activity (Todorov and Dicks, 2005a). Bacteriocin ST194BZ, produced by *Lactobacillus plantarum* ST194BZ, is active against *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Todorov and Dicks, 2005b).

Lactic acid bacteria are fastidious and require complex growth media for optimal bacteriocin production (De Vuyst *et al.*, 1996; Todorov *et al.*, 2000). Growth conditions have been optimised for enterocin AS-48, produced by *Enterococcus faecalis* (Abriouel *et al.*, 2001); enterocins 1146 and P, produced by *Enterococcus faecium* (Parente and Ricciardi, 1994; Herranz *et al.*, 2001); plantaricins ST31, bacST8KF and plantaricin 423 (Verellen *et al.*, 1998; Todorov *et al.*, 2000; Powell et *al*., 2007); unclassified bacteriocins, produced by *Pediococcus acidilactici* (Calderon-Santoyo *et al.*, 2001); bacST712BZ, produced by *Lactobacillus pentosus* ST712BZ (Todorov and Dicks, 2007); and pediocin PD-1, produced by *Pediococcus damnosus* (Nel *et al.*, 2001).

Bacteriocin production is strongly dependent on pH, nutrient sources and incubation temperature. Activity levels do not always correlate with cell mass or growth rate of the producer strain (Bogovic-Matijasic and Rogelj, 1998). Higher bacteriocin levels are often obtained at temperatures, nutrient sources and pH values lower than required for optimal growth (De Vuyst *et al.*, 1996; Krier *et al.*, 1998; Todorov *et al.*, 2000).

Several bacteriocin-producing strains of *Lactobacillus paracasei* subsp. *paracasei* have been isolated from various habitats, *viz*. raw milk (Rodriguez *et al.*, 2000), cheese (Gardiner *et al.*, 1998; Atanassova *et al.*, 2001; Caridi, 2002), barley beer (Todorov *et al.*, 2005) and healthy oral cavities (Sookkhe *et al.*, 2001). Little is known about the conditions required for production of bacteriocins by *Lactobacillus paracasei*.

In this paper we report on bacteriocins ST242BZ and ST284BZ, produced by *L. paracasei* subsp. *paracasei* ST242BZ and ST284BZ, respectively, isolated from boza. The effect of medium components on the production of bacteriocins ST242BZ and ST284BZ have been studied.

* Corresponding author. Phone: +27-21-808 5850; Fax: +27-21-808 5846; E-mail: todorov@sun.ac.za

MATERIALS AND METHODS

Bacterial strains and growth media. Strains ST242BZ and ST284BZ were isolated from boza and were classified as *Lactobacillus paracasei* subsp. *paracasei*, based on phenotypic and genotypic characteristics (Todorov and Dicks, 2005c). The strains were cultured in MRS medium (Biolab, Biolab Diagnostics, Midrand, South Africa) at 30 °C and stored at –80 °C in spent MRS broth in the presence of 15% glycerol. MRS broth (Biolab) was used for all experiments, except in growth optimisation, in which case MRS broth (De Man *et al.*, 1960) was modified.

Bacteriocin assay. Bacteriocin screening was performed by using the agar-spot test method (Shillinger and Lücke, 1989). Adjustment of the cell-free supernatant to pH 6.0 with 1.0 M NaOH prevented the inhibitory effect of lactic acid. Antimicrobial activity was expressed as arbitrary units (AU)/ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (Shillinger and Lücke, 1989; Todorov and Dicks, 2005a). *Lactobacillus casei* LHS, isolated from wine (culture collection of the Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa) was used as indicator strain.

Molecular size of the bacteriocins ST242BZ and ST284BZ. Strains ST242BZ and ST284BZ were grown in MRS broth at 30 °C for 20 h. The cells were harvested by centrifugation (8000 *x g*, 10 min, 4 ºC) and the bacteriocin precipitated from the cell-free supernatant with 70% saturated ammonium sulphate. The precipitate was resuspended in one tenth volume 25 mmol/l ammonium acetate (pH 6.5), desalted by using a 1000 Da cut-off dialysis membrane (Spectrum Inc., CA, USA) and separated by tricine-SDS-PAGE, as described by Schägger and Von Jagow (1987). A low molecular weight marker with sizes ranging from 2.35 to 46.0 kDa (Amersham International, UK) was used. The gels were fixed and one half stained with Coomassie Blue R250 (Saarchem, Krugersdorp, South Africa). The position of the active peptide band in the gel was determined by overlaying an unstained gel with cells of *L. casei* LHS (10^6 CFU/ml) embedded in BHI agar.

Plasmid isolation. Plasmid DNA was isolated according to the method described by Burger and Dicks (1994), followed by CsCl density gradient centrifugation (Ausubel *et al.*, 1994). The DNA was separated on an agarose gel, according to Ausubel *et al.* (1994).

Production of bacteriocins ST242BZ and ST284BZ in different growth media and at different initial growth pH. An 18-h-old culture of strains ST242BZ and ST284BZ was inoculated (2%, v/v) into MRS broth, BHI broth, M17 broth (Merck, Darmstadt, Germany), soy milk (100 g soy flour/l water), and molasses (sugar cane syrup) of 20-100 g/l (20 g/l intervals), respectively. Incubation was at 30 °C and 37 °C, respectively, without agitation for 28 h. Samples were taken every hour and examined for bacterial growth (Optical density at 600 nm, OD_{600}), changes in culture pH, and antimicrobial activity (AU/ml) against *L. casei* LHS. The agar-spot test method was used as described before.
In a separate experiment, the effect of initial medium pH on the production of bacteriocins ST242BZ and ST284BZ 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 6 M HCl or 6 M NaOH and then autoclaved. Each flask was inoculated with 2% (v/v) of an 18-h-old culture of strains ST242BZ and ST284BZ, respectively, and incubated at 30 °C for 20 h without agitation. Changes in culture pH and production of bacteriocins ST242BZ and ST284BZ, expressed as AU/ml, were determined every hour as described elsewhere. All experiments were done in triplicate.

Effect of medium composition on the production of bacteriocins ST242BZ and ST284BZ. Strains ST242BZ and ST284BZ were grown in 10 ml MRS broth at 30 °C for 18 h, the cells harvested by centrifugation (8000 *x g*, 10 min, 4 °C), and the pellet resuspended in 10 ml sterile peptone water. Four ml of this cell suspension was used to inoculate 200 ml of the following media: (a) MRS broth, without organic nutrients, supplemented with tryptone (20 g/l), meat extract (20 g/l) and yeast extract (20 g/l), respectively, and a combination of 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 g/l) plus yeast extract (10 g/l), and tryptone (10 g/l) plus meat extract (5 g/l) and yeast extract (5 g/l), respectively; (b) MRS broth with 20 g/l glucose; (c) MRS broth without glucose, supplemented with either fructose, sucrose, lactose, mannose or maltose (20 g/l, respectively); (d) MRS broth modified to contain glucose levels of 1, 5, 10, 20, 30 and 40 g/l, respectively, and sucrose as sole carbon source at levels of 1, 5, 10, 20, 30 and 40 g/l, respectively; (e) MRS broth modified to contain 2, 5, 10, 20, 50 and 100 g/l KH_2PO_4 and 2, 5, 10, 20, 50 and 100 g/l K_2HPO_4, respectively; (f) MRS broth supplemented with 5-50 g/l glycerol (5 g/l intervals). In a separate experiment, the vitamins cyanocobalamin (Sigma, St. Louis, Mo.), L-ascorbic acid (BDH Chemicals Ltd, Poole, UK), thiamine (Sigma) and DL-6,8-thioctic acid (Sigma) were filter-sterilised and added to MRS broth at 1.0 mg/ml (final concentration). Incubation for all tests was at 30 °C for 20 h. Activity levels of bacteriocins ST242BZ and ST284BZ were determined as described before. All experiments were done in triplicate.

RESULTS AND DISCUSSION

All data represent an average of three repeats. The pH values recorded in each experiment did not differ by more than 5% variation and standard deviation values were not presented. Identical levels of bacteriocin production (AU/ml) were recorded for all three repeats.

The cell-free supernatant of strains ST242BZ and ST284BZ inhibited the growth of *L. casei*, *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Lactobacillus delbruekii* subsp. *bulgaricus, Lactobacillus sakei, Pseudomonas aeruginosa* and *Enterococcus faecalis* (Todorov and Dicks, 2005c). Bacteriocin ST242BZ inhibited the growth of *Acinetobacter baumanii* and *Staphylococcus aureus*, whereas bacteriocin ST284BZ inhibited *Streptococcus pneumoniae* (Todorov and Dicks, 2005c). Activity against Gram-negative bacteria is an unusual phenomenon and has so far only been reported for thermophylin 81, produced by *Streptococcus thermophilus*, bacteriocins produced by *L. paracasei* subsp. *paracasei* L126 and

L134, a bacteriocin produced by *L. lactis* KCA2386, and plantaricin 35d produced by *L. plantarum* (Ivanova *et al*., 1998; Ko and Ahn, 2000; Messi *et al*., 2001; Caridi, 2002). According to tricine-SDS-PAGE, *L. paracasei* subsp*. paracasei* ST242BZ produces a bacteriocin with a molecular mass of approximately 10.0 kDa and *L. paracasei* subsp*. paracasei* ST284BZ a bacteriocin of approximately 3.5 kDa (Fig. 1). For more accurate size determination, mass spectrometry on pure bacteriocins will have to be performed.

Strains ST242BZ and ST284BZ do not contain plasmids, suggesting that the genes encoding bacteriocin production are located on the chromosome. Similar results were reported for plantaricin ST31 produced by *L. plantarum* ST31 (Todorov *et al.*, 1999). However, in case of plantaricin 423, the genes encoding bacteriocin production are plasmid bound (Van Reenen *et al.*, 1998).

Similar growth was recorded for strains ST242BZ and ST284BZ in MRS broth adjusted to pH 4.5 and 6.5, respectively. Optical density (OD_{600}) readings ranged from 7.5 to 8.5 after 20 h of growth. Bacteriocin ST242BZ was produced at 12800 AU/ml in MRS broth at an initial pH of 6.5 (Table 1). A 50% reduction in bacteriocin ST242BZ activity (6400 AU/ml) was recorded in the same medium adjusted to an initial pH of 5.0 (Table 1). The end pH of both fermentations was 3.6 (Table 1). Production of bacteriocin ST242BZ was stimulated in medium with an initial pH between 5.5 and 6.5 compared to production at lower pH. At an initial medium pH of 4.5, bacteriocin ST242BZ was produced at 3200 AU/ml (Table 1). Slightly different results were recorded for bacteriocin ST284BZ. Optimal bacteriocin ST284BZ production (12800 AU/ml) was recorded in MRS broth with an initial pH of 6.5 (Table 1). Medium with an initial pH of 5.0-6.0 yielded bacteriocin ST284BZ levels of 6400 AU/ml, whereas an initial pH of 4.5 yielded 3200 AU/ml (Table 1). The end pH of all cultures ranged between 3.6 and 3.9 (Table 1), suggesting that bacteriocin ST284BZ is not affected by low pH values. Bacteriocin ST284BZ production is stimulated in growth medium with an initial pH of 6.5, but inhibited at levels between pH 4.5 and 6.0. Similar results were reported for other bacteriocins produced by *L. plantarum* (Daeschel *et al.*, 1990; Todorov *et al.*, 2000).

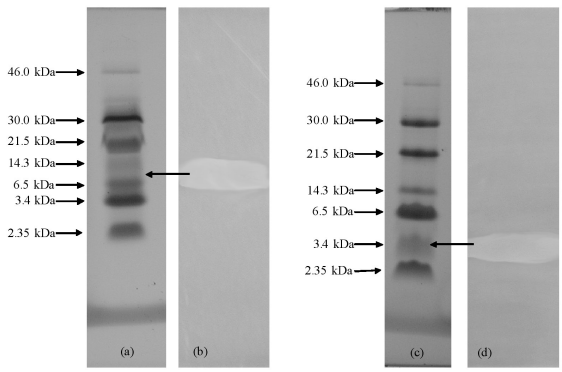

FIG. 1 - Separation of bacteriocins ST242BZ and ST284BZ by tricine-SDS PAGE. Lanes (a) and (c): molecular weight marker. Lanes (b) and (d): zone of growth inhibition, corresponding to the position of the peptide band of bacteriocins ST242BZ and ST284BZ, respectively. The gels were overlaid with BHI agar, seeded with *Lactobacillus casei* LHS (1 x 10^6 CFU/ml), and incubated at 30 °C for 24 h.

Low bacteriocin activity (less than 400 AU/ml) was recorded for strain ST242BZ grown in BHI or M17 broth adjusted to pH 6.5 (Fig. 2a), despite good growth. Bacteriocin ST284BZ production in BHI and M17 broth was 3200 AU/ml and 6400 AU/ml, respectively. Growth of the two strains in 100 g/l soy milk or 100 g/l molasses yielded bacteriocin levels less than 1600 AU/ml (Fg. 2a). The low activity levels recorded in M17 broth, BHI broth, soymilk and molasses, despite relatively good growth, suggests that specific nutrients are required for the production of bacteriocins ST242BZ and ST284BZ.

Production of bacteriocins ST242BZ and ST284BZ was higher at 30 °C (12800 AU/ml) than at 37 °C (6400 AU/ml). Growth temperature and bacteriocin production are often correlated, as observed for lactococcin A (Parente *et al.*, 1994), enterocin 1146 (Parente and Ricciardi, 1994) and amylovorin 1471 (De Vuyst *et al.*, 1996).

In the presence of tryptone as the only nitrogen source, bacteriocin ST242BZ was produced at 25600 AU/ml, whereas combinations of tryptone and meat extract, or tryptone and yeast extract yielded 12800 AU/ml (Fig. 2b). Growth in the presence of meat extract or yeast extract as the only nitrogen source yielded 6400 AU/ml and 3200 AU/ml, respectively (Fig. 2b). Strain ST284BZ grown in the presence of tryptone, or combinations of tryptone, meat extract and yeast extract, yielded bacteriocin levels of 12800 AU/ml (Fig. 2b). Meat extract of yeast extract as sole nitrogen source yielded only 6400 AU/ml and 1600 AU/ml, respectively (Fig. 2b). Tryptone is the key nitrogen source for optimal production of bacteriocins ST242BZ and ST284BZ. Similar results have been reported for the production of plantaricin 423, in which case optimal production was recorded in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract (Verellen *et al.*, 1998). Stimulation of bacteriocin production by yeast extract and meat extract has been reported for pediocin AcH (Bhunia *et al.*, 1988). As far as we could determine, this is the first report of tryptone being the choice of nitrogen source in the production of *L. paracasei* bacteriocins.

TABLE 1 – Influence of initial pH medium (MRS broth, Biolab) on the production of bacteriocins ST242BZ and ST284BZ

	Bacteriocin ST242BZ					Bacteriocin ST284BZ				
Initial pH	4.5	5.0	5.5	6.0	6.5	4.5	5.0	5.5	6.0	6.5
Final pH	3.5	3.6	3.7	3.7	3.8	3.6	3.7	3.8	3.8	3.9
Bacteriocin activity (AU/ml)	3200	6400	12800	12800	12800	3200	6400	6400	6400	12800
Reduction of bacteriocin activity (%)*	75	50	0	0	0	75	50	50	50	0

* Based on the highest activity, recorded as 12800 AU/ml.

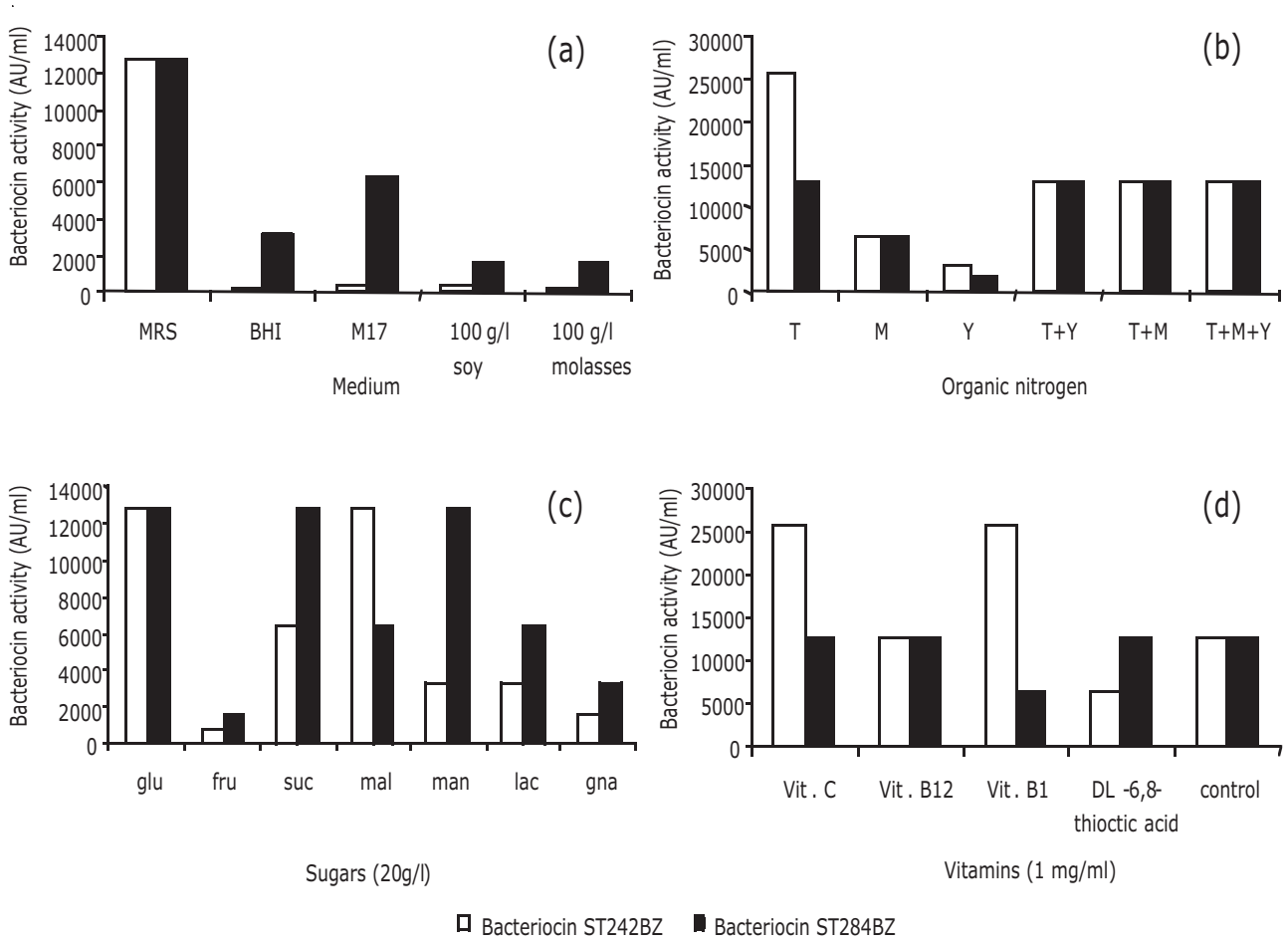

FIG. 2 - Effect of different medium components (a: medium, b: organic nitrogen, c: sugars, d: vitamins) on the activity of bacteriocins ST242BZ and ST284BZ. T = tryptone, M = meat extract, Y = yeast extract, glu = glucose, fru = fructose, suc = sucrose, mal = maltose, man = mannose, lac = lactose, gna = gluconate, control = MRS (Biolab) without the addition of vitamins.

Growth of strain ST242BZ in the presence of glucose (20-40 g/l) yielded 12800 AU/ml (Fig. 2c, Table 2). Maltose (20 g/l) yielded the same bacteriocin level (Fig. 2c). Lower concentrations of glucose (10 g/l) yielded 6400 AU/ml, with even lower activity recorded in the presence of 1 g/l and 5 g/l glucose (Table 2). Growth in the presence of sucrose (20 g/l) yielded 6400 AU/ml, whereas mannose and lactose (20 g/l) yielded only 3200 AU/ml (Fig. 2c). Based on these results, bacteriocin ST242BZ production is stimulated by glucose, but only at concentrations of 20 g/l and higher. Growth in the presence of gluconate yielded the lowest bacteriocin activity (Fig. 2c).

Strain ST284BZ grown in the presence of mannose (20 g/l), sucrose (20 and 30 g/l) or glucose (20 g/l) produced bacteriocin levels of 12800 AU/ml (Fig. 2c, Table 2). Sucrose at 40 g/l yielded 6400 AU/ml, whereas sucrose at 5 g/l yielded less than 3200 AU/ml (Table 2). Bacteriocin ST284BZ may be regulated by levels of sucrose in the medium, with concentrations below 10 g/l and above 30 g/l leading to repression. These findings differ from those reported for plantaricin UG1 (Enan *et al.*, 1996) and plantaricin ST31 (Todorov *et al.*, 2000), in which cases higher glucose levels stimulated bacteriocin production. Glucose levels of 20 g/l and higher, on the other hand, reduced sakacin P (Aasen *et al.*, 2000) and enterocin 1146 production (Parente *et al.*, 1997). Lactose and maltose (20 g/l) yielded bacteriocin ST284BZ levels of 6400 AU/ml. Fructose at these levels yielded 1600 AU/ml, whereas the same concentration of gluconate yielded 3200 AU/ml (Fig. 2c).

Little is known about the influence of K_2HPO_4 or KH_2PO_4 on the production of bacteriocins. In the case of strain ST242BZ, growth in the presence of 2 g/l K_2HPO_4 yielded 12800 AU/ml, and 10, 20, 50 and 100 g/l of KH_2PO_4 yielded 25600 AU/ml (Table 2). Higher potassium concentrations led to increased production of bacteriocin ST242BZ. In the case of strain ST284BZ, K_2HPO_4 concentrations of 2 g/l yielded 12800 AU/ml and KH_2PO_4 of 2 g/l yielded 6400 AU/ml (Table 2). Growth in the presence of 50 or 100 g/l K_2HPO_4 increased bacteriocin ST284BZ production by 100% to 25600 AU/ml (Table 2). In the case of plantaricin UG1, 7 g/l K_2HPO_4 resulted in increased activity (Enan *et al.*, 1996). The optimal level of K_2HPO_4 recorded for plantaricin ST31 was between 2 g/l and 5 g/l (Todorov *et al.*, 2000).

Production of bacteriocin ST242BZ was not affected by the presence of glycerol, whereas bacteriocin ST284BZ production was the highest (12800 AU/ml) in the absence of glycerol. Glycerol concentrations of 1 g/l and higher (up

TABLE 2 - Influence of potassium and carbohydrates on bacteriocins ST242BZ and ST284BZ production

Component	Concentration g/l	Activity (AU/ml)	Change in bacteriocin activity (%)*
		Bacteriocin ST242BZ	
K_2HPO_4	2	12800	0
KH_2PO_4	2	12800	0
"	5	12800	0
"	10	25600	+100
"	20	25600	+100
"	50	25600	+100
"	100	25600	+100
Glucose	1	800	-93.75
"	5	800	-93.75
"	10	6400	-50.00
"	20	12800	0
"	30	12800	0
"	40	12800	0
		Bacteriocin ST284BZ	
KH_2PO_4	2	6400	-50.00
K_2HPO_4	2	12800	0
"	5	12800	0
"	10	12800	0
"	20	12800	0
"	50	25600	+100
"	100	25600	+100
Sucrose	1	800	-93.75
"	5	3200	-75.00
"	10	12800	0
"	20	12800	0
"	30	12800	0
"	40	6400	-50.00

* Based on the highest activity, recorded as 12800 AU/ml

to 50 g/l) led to a decrease in bacteriocins ST242BZ and ST284BZ production (data not shown). Similar results were reported for the production of plantaricin ST31, in which case concentrations higher than 2 g/l led to a decrease in activity (Todorov *et al.*, 2000).

The addition of cyanocobalamin (Vit. B_{12}) in MRS broth (1.0 mg/l) yielded 12800 AU/ml for both bacteriocins. In the presence of thiamine, the activity of bacteriocin ST242BZ increased to 25600 AU/ml, whereas that of bacteriocin ST284BZ decreased to 6400 AU/ml. MRS broth (Biolab) supplemented with L-ascorbic acid (Vit. C) yielded 25600 AU/ml and 12800 AU/ml of bacteriocins ST242BZ and ST284BZ, respectively. Addition of DL-6,8-thioctic acid yielded bacteriocin ST242BZ activity of 6400 AU/ml and bacteriocin ST284BZ activity of 12800 AU/ml (Fig. 2d).

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