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Original Contribution

OPTIMIZATION OF GROWTH MEDIUM FOR PRODUCTION OF BACTERIOCINS PRODUCED BY *LACTOBACILLUS PLANTARUM* JW3BZ AND JW6BZ, AND *LACTOBACILLUS FERMENTUM* JW11BZ AND JW15BZ ISOLATED FROM BOZA

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SUMMARY

Lactobacillus plantarum JW3BZ and JW6BZ produced bacteriocins bacJW3BZ and bacJW6BZ at 1.80 AU/10^4CFU and 2.69 AU/10^4CFU in MRS broth. *Lactobacillus fermentum* JW11BZ and JW15BZ produced bacJW11BZ and JW15BZ at 2.33 AU/10^4CFU and 3.74 AU/10^4CFU in MRS broth. Growth of *L. plantarum* JW3BZ and JW6BZ, and *L. fermentum* JW11BZ in the presence of tryptone yielded the similar levels of bacteriocin production as reported after 20 h of growth in MRS broth. Higher bacteriocin production was recorded for *L. plantarum* JW6BZ in the presence of tryptone and meat extract. Growth of strain JW3BZ in the presence of 20 g/l maltose and 20 g/l glucose yielded bacteriocin levels of 1.54 AU/10^4CFU and 1.80 AU/10^4CFU, respectively. The highest activity was recorded when strain JW6BZ was grown in the presence of 20.0 g/l mannose or 20.0 g/l glucose. K_2HPO_4 (5.0 g/l) led to doubling of bacteriocin JW3BZ activity. Presence of 10.0 and 20.0 g/l K_2HPO_4 doubled the activity of bacteriocins JW11BZ and JW15BZ. Concentrations of 2.0 to 20.0 g/l KH_2PO_4 led to increased bacteriocin JW3BZ activity. Inclusion of glycerol or exclusion of magnesium and manganese sulphates had a negative effect on bacteriocin production.

Key words: Bacteriocins; *Lactobacillus plantarum*; *Lactobacillus fermentum*; 'boza'.

INTRODUCTION

'Boza', a traditional beverage of the Balkan region, is produced from cereals fermented with lactic acid bacteria of the genera Lactobacillus, *Lactococcus, Pediococcus* and *Leuconostoc*, and yeast [1-5]. Various lactic acid bacteria were isolated from 'boza', including *Lactobacillus acidophilus*, *Lactobacillus coprophilus*, *Lactobacillus fermentum*, *Lactobacillus paracasei, Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus rhamnosus*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides, Leuconostoc raffinolactis* and *Pediococcus pentasaceus* [2, 3, 6, 7]. Bacteriocin-producing strains of *L. paracasei, L. pentosus, L. plantarum, L. rhamnosus*, *L. lactis* subsp. *lactis, Leuconostoc mesenteroides* subsp. *dextranicum* and *P. pentasaceus* have been isolated from the product [6, 7]. As many as 33 strains of lactic acid bacteria with antimicrobial activity against Gram-positive and Gram-negative bacteria have been described [3]. Yeasts thus far isolated from 'boza' have been classified as *Saccharomyces cerevisiae*, *Candida glabrata*, *Geotrichum candidum, Geotrichum penicillatum* [2], *Candida diversa, Candida inconspicua, Candida pararugosa, Issatchenkia orientalis, Pichia fermentans, Pichia guillliermondii, Pichia norvegensis, Rhodotorula mucilaginosa* and *Torulaspora delbrueckii* [5].

Little research has been done on the optimisation of growth medium for the production of bacteriocins. Papers have been published on the optimisation of plantaricin 423 [8], plantaricin ST31 [9], bacteriocin ST194BZ [10], nisin [11, 12], pediocin AcH [13], lactococcin produced by *Lactococcus lactis* 140 NWC [14], and mesenterocin [15]. So far, no study on optimisation of the bacteriocins produced by strains of *Lactobacillus fermentum* has been done. The

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conclusion from these studies is to the effect that incubation temperature and medium pH play an important role in bacteriocin production, with optimal production often recorded under less favourable growth conditions [6, 11, 16-20].

The effect of pH and nutrients, separate and in combination, on production of bacteriocins bacJW3BZ, bacJW6BZ, bacJW11BZ and bacJW15BZ was studied to determine which conditions were required for optimal bacteriocin production and thus extend shelf life of the product. Expression of bacteriocin production as a function of bacterial growth is a valuable point in correct comparison of the bacteriocin production accepting bacterial cell as a "cell factory".

2. MATERIALS AND METHODS

2.1. Strains and bacteriocin assays

Lactobacillus plantarum JW3BZ and JW6BZ, and *Lactobacillus fermentum* JW11BZ and JW15BZ have been described in a previous study [21]. Bacteriocin production was determined by using the agar-spot test and the well diffusion methods [22]. Shortly, sensitive strain was imbedded into soft agar media (containing 1% agar). After solidification of the media 10 µl cell free supernatant containing targeted bacteriocin was spotted on the surface for the agar-spot method or 50 µl cell free supernatant was loaded into previously made holes into agar for the well diffusion method. Growth inhibition from lactic acid was prevented by adjusting all samples to pH 6.0 with sterile 1 M NaOH before testing for activity. Bacteriocin production was expressed as arbitrary units (AU) per ml, calculated as follows: a^b x 100, where "a" represents the dilution factor and "b" the last dilution that produces an inhibition zone of at least 2 mm in diameter. Activity was expressed per ml by multiplication with 100 [23]. *Lactobacillus sakei* DSM 20017 was used as indicator (sensitive test) strain. Viable cell number was determined by plating onto MRS (Biolab) and bacteriocin production expressed as $AU/10^4CFU$ based on ratio between bacteriocin activity (AU/ml) and viable cell number (CFU/ml).

2.2. Bacteriocin production in different growth media and at different initial growth pH

Eighteen-hour-old cultures of strains JW3BZ, JW6BZ, JW11BZ and JW15BZ were inoculated (1%, v/v) into MRS broth (Biolab), BHI broth (Biolab), M17 broth (Merck, Darmstadt, Germany), soy milk (20.0 and 100.0 g/l), skim milk (20.0 and 100.0 g/l) and molasses (20.0 and 100.0 g/l). Incubation was at 30°C for 24 h without agitation. Samples were taken at 1 h intervals and examined for bacterial growth (as determined by changes in optical density measured at 600 nm), changes in pH, and bacteriocin production (AU/ml). The agar-spot test method was used as described before.

The effect of initial medium pH (4.0, 4.5, 5.0, 5.5, 6.0 and 6.5) on bacteriocin production was determined according to the method described by Todorov and Dicks [24]. Hundred ml MRS broth (Biolab) was corrected to pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 with 1M HCl before autoclaving. Modified MRS was inoculated with 1% overnight cultures of *L. plantarum* JW3BZ, *L. plantarum* JW6BZ, *L. fermentum* JW11BZ or *L. fermentum* JW15BZ, respectively and incubated at 30°C. Changes in bacteriocin production ($AU/10^4CFU$) and pH were determined after 20h, as described before.

2.3. Effect of medium composition on bacteriocin production

The four bacteriocin-producing strains were inoculated into 10 ml MRS broth (Biolab) and incubated at 30°C for 18 h. Cells were harvested (8000 x *g*, 10 min, 4°C) and the pellets re-suspended in separate volumes of 10 ml sterile physiological water. From these suspensions, 4 ml was used to inoculate MRS broth (Biolab) and MRS broth [25] modified as listed on **Tables 1-6**: (a) MRS broth [25], 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 without D-glucose, supplemented with single different carbohydrate at 20.0 g/l: fructose, sucrose, lactose, mannose, maltose and gluconate, respectively; (c) MRS broth with 5.0 g/l to 50.0 g/l glucose as sole carbon source; (d) MRS broth with 2.0 g/l to 20.0 g/l K_2HPO_4, or 2.0 g/l to 20.0 g/l KH_2PO_4, or combination of 2.0 g/l K_2HPO_4 and 2.0 g/l KH_2PO_4; (e) MRS broth supplemented with 1.0 g/l to 20.0 g/l glycerol; (f) MRS broth without $MgSO_4$ or without $MnSO_4$; (g) MRS broth without or

supplemented with 5.0 g/l and 10.0 g/l tri-ammonium citrate; and (h) MRS broth without or supplemented with 0.05 ml/l, 0.10 ml/l, 0.15 ml/l and 0.20 ml/l Tween 80.

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/l (final concentration). All cultures were incubated at 30 °C for 24h. Bacteriocin production was determined, as described before, after 20 h of growth at 30 °C.

In a separate experiment, bacteriocin production was studied in modified MRS broth, composed from a combination of nutrients that yielded the highest bacteriocin production when tested individually. Incubation was at 30 ° C. Changes in optical density and pH were recorded every hour for 24 h. Bacteriocin production was recorded, as described before, at every 3 h intervals.

3. RESULTS AND DISCUSSION

All data represent an average of three repeats. The optical density and pH values recorded in each experiment did not vary by more than 5% and standard deviation values were not presented. Identical levels of bacteriocin production (AU/ml; AU/10^4CFU) were recorded for all three repeats.

Low levels of bacteriocin production (0.24 to 0.49 AU/10^4CFU) were recorded in BHI broth and M17 broth. Growth in soy milk (20.0 g/l and 100.0 g/l), skim milk (20.0 g/l and 100.0 g/l) and molasses (20.0 g/l to 100.0 g/l) yielded bacteriocin levels between 0.13 AU/10^4CFU and 0.55 AU/10^4CFU. Good growth was recorded in 20.0 g/l soy milk and 20.0 g/l or 100.0 g/l molasses, but not in skim milk, suggesting that the strains are not adapted to ferment lactose. Highest bacteriocin production (between 1.80 AU/10^4CFU and 3.74 AU/10^4CFU) (**Table 1**) was recorded in MRS broth (Biolab).

Initial medium pH had a major influence on production of bacJW3BZ, bacJW6BZ and JW15BZ. Optimal levels of bacJW3BZ were produced in MRS broth (Biolab) with initial pH values of 5.0 (2.05 AU/10^4CFU), 5.5 (2.25 AU/10^4CFU), 6.0 (2.02 AU/10^4CFU) and 6.5 (2.11 AU/10^4CFU). Similar results for the bacteriocin production were observed for bacJW6BZ produced in MRS broth (Biolab) with initial pH values of 5.0 (3.02 AU/10^4CFU) and 5.5 (3.04 AU/10^4CFU). Low levels of bacJW3BZ (0.73 AU/10^4CFU) and bacJW6BZ (1.06 AU/10^4CFU) were recorded in the same medium adjusted to pH 4.5. High levels of bacJW11BZ were produced at pH 5.0 (2.86 AU/10^4CFU), 5.5 (3.0 AU/10^4CFU), 6.0 (2.53 AU/10^4CFU), and 6.5 (3.03 AU/10^4CFU). Optimal levels of bacJW15BZ were observed at pH 5.5 (3.9 AU/10^4CFU), 6.0 (4.10 AU/10^4CFU) and 6.5 (4.54 AU/10^4CFU) in comparison to lower levels recorded at pH 4.5 (1.95 AU/10^4CFU) and 5.0 (1.81 AU/10^4CFU). Similar results were reported for bacteriocins ST194BZ [10], plantaricin 149 [26], plantaricin ST31 [9] and plantaricin C-11 [27]. The culture pH at the end of fermentation ranged between 3.9 and 4.3, irrespective of initial medium pH. These results suggest that optimal production of bacJW3BZ, bacJW6BZ, bacJW11BZ and bacJW15BZ in MRS broth (Biolab) occurs during early logarithmic growth at a medium pH higher than 4.5.

Growth in MRS broth with glucose replaced by lactose, yielded low bacteriocin levels (**Table 1**). Similar results were recorded when the four strains were grown in skim milk, confirming that bacteriocin production and growth are not stimulated by lactose. This is not surprising, as 'boza' contains no lactose and the strains would probably not have adapted to ferment the substrate. Low levels of bacJW3BZ and bacJW15BZ were produced in MRS broth supplemented with 20.0 g/l mannose and maltose (**Table 1**), suggesting that strains JW3BZ and bacJW15BZ are less well adapted to ferment starch. Higher levels of bacJW6BZ and bacJW11BZ were recorded in the presence of mannose suggesting that the strains have adapted to produce bacteriocins in an environment rich in starch (**Table 1**).

Variable results were obtained when the four strains were grown in MRS broth supplemented with different concentrations of glucose (**Table 1**). Production of bacJW3BZ was repressed in the presence of 5.0 g/l, 10.0 g/l and 50.0 g/l glucose. Production of bacJW6BZ was repressed by glucose concentrations of 5.0 g/l, 10.0 g/l, 30.0 g/l and 50.0 g/l, whereas production of bacJW15BZ increased in the presence of 30.0 g/l and 50.0 g/l glucose. Production of bacJW11BZ increased from 0.26 AU/10^4CFU when strain JW11BZ was grown in the presence of 10.0 g/l glucose to approximately 2.33 AU/10^4CFU when glucose concentrations were increased to 20.0 g/l, 30.0 g/l and 50.0 g/l, respectively (**Table 1**). Further research is needed to determine whether variations in bacteriocin production are due to feedback regulation by

glucose, or the rate at which the culture pH changes with production of lactic acid. All four bacteriocins were produced at low levels (0.11 AU/10^4CFU to 1.23 AU/10^4CFU) in the presence of 5.0 g/l glucose (**Table 1**). Similar results were reported for bacteriocins ST23LD, ST341LD, ST31 and ST194BZ in the presence of different glucose concentrations [9, 10, 27].

Growth of strain JW3BZ in the presence of yeast extract as sole nitrogen source yielded bacJW3BZ levels of 0.75 AU/10^4CFU (**Table 2**). Replacement of yeast extract with tryptone, meat extract, a combination of tryptone with either meat extract or yeast extract, a combination of meat extract and yeast extract, or a combination of all three nitrogen sources, yielded bacJW3BZ production of 1.55 to 2.10 AU/10^4CFU (**Table 2**). These results show that bacJW3BZ production is stimulated by tryptone or meat extract. Growth of JW6BZ in the presence of tryptone, meat extract, yeast extract, a combination of all three nitrogen sources, or meat extract combined with yeast extract, yielded bacJW6BZ levels of 2.49 to 2.93 AU/10^4CFU (**Table 2**). However, a combination of tryptone with either meat extract or yeast extract, yielded bacJW6BZ levels of 4.61 and 4.88 AU/10^4CFU (**Table 2**), respectively. Increased production of bacJW6BZ in the presence of tryptone with either yeast extract or meat extract, compared to lower levels produced in the presence of all three nutrients combined, suggests that production may be controlled by the level of tryptone. Production of bacJW11BZ remained relatively low (0.84 AU/10^4CFU to 2.61 AU/10^4CFU), irrespective of the nitrogen source (**Table 2**). BacJW15BZ levels of 0.55 AU/10^4CFU was recorded in the presence of yeast extract, 0.31 AU/10^4CFU in the presence of a combination of meat and yeast extract and 0.98 AU/10^4CFU in the presence of meat extract, but increased to 3.74 AU/10^4CFU when tryptone was added (**Table 2**). Compared to bacJW3BZ, bacJW6BZ and bacJW11BZ, production of bacJW15BZ is less stimulated by organic nitrogen. Similar results have been recorded for plantaricin 423, produced by *L. plantarum* 423 isolated from sorghum beer [8]. Higher levels of plantaricin 423 activities were recorded in medium with tryptone compared to medium with meat extract as sole nitrogen source [8]. In another study [24], *L. plantarum* ST341LD produced higher levels of bacteriocin when grown in the presence of tryptone.

Optimal levels of bacJW3BZ were recorded in the presence of 2.0 g/l KH_2PO_4 (3.94 AU/10^4CFU), 5.0 g/l KH_2PO_4 (4.53 AU/10^4CFU), 10.0 g/l KH_2PO_4 (3.85 AU/10^4CFU), 20.0 g/l KH_2PO_4 (4.20 AU/10^4CFU) and 5.0 g/l K_2HPO_4 (3.46 AU/10^4CFU). A combination of KH_2PO_4 and K_2HPO_4 yielded 1.71 AU/10^4CFU. K_2HPO_4 added at 20.0 g/l led to lower (1.32 AU/10^4CFU) production of bacJW3BZ (**Table 3**). In the case of strains JW6BZ and JW15BZ, growth in the presence of 10.0 or 20.0 g/l KH_2PO_4 led to increased bacteriocin production. Production of bacJW15BZ was also stimulated in the presence of 5.0 or 10.0 g/l K_2HPO_4 (**Table 3**). Highest production of bacJW11BZ was obtained in medium supplemented with 10.0 g/l or 20.0 g/l K_2HPO_4 (**Table 3**). Changes in activity observed for bacJW3BZ, bacJW6BZ, bacJW11BZ and bacJW15BZ cannot be ascribed to pH changes, as all media were adjusted to pH 6.5 before inoculation.

Exclusion of magnesium sulphate and manganese sulphate from MRS broth led to a decrease in production of bacJW6BZ and bacJW11BZ, compared to production in unmodified MRS (**Table 4**). No changes in production of bacJW3BZ and bacJW15BZ were recorded in the absence or presence of magnesium sulphate. BacJW3BZ and JW15BZ levels increased in the absence of manganese sulphate. No increase in production of bacJW3BZ, bacJW6BZ and bacJW11BZ was recorded in MRS broth supplemented with 5.0 g/l tri-ammonium citrate (**Table 4**), whereas bacJW15BZ levels increased. Lower bacteriocin levels were recorded when strains JW3BZ, JW6BZ and JW11BZ were grown in MRS supplemented with 10.0 g/l tri-ammonium citrate or MRS without tri-ammonium citrate (**Table 4**). In the case of JW15BZ higher levels of bacJW15BZ were observed in the presence of 10.0 g/l tri-ammonium citrate. A decrease in bacJW15BZ production was recorded in the absence of tri-ammonium citrate. The effect of ammonium and citrate on bacteriocin production is not known.

Activity of bacJW3BZ, bacJW6BZ and bacJW15BZ increased in the presence of Tween 80 (**Table 5**). Similar results were recorded for lactacin B [28], lactocin 705 [29] and plantaricin 423 [8].

***Table 1**. Effect of sugars on the production of bacteriocins* JW3BZ, JW6BZ, *JW11BZ and JW15BZ*

	pH	**AU/ml**	**CFU/ml**	**AU/10^{4}CFU**	**pH**	**AU/ml**	**CFU/ml**	**AU/10^{4}CFU**
	L. plantarum JW3BZ				*L. plantarum JW6BZ*			
MRS, Biolab (unmodified):[a]	3.79	25 600	1.42 x 10^8	1.80	3.74	25 600	9.50 x 10^7	2.69
Carbohydrates (g/l):								
Fructose (20.0)	4.03	3 200	7.71 x 10^7	0.42	4.04	12 800	4.91 x 10^7	2.61
Lactose (20.0)	4.12	3 200	1.20 x 10^8	0.27	4.10	6 400	8.35 x 10^7	0.77
Mannose (20.0)	3.82	12 800	1.48 x 10^8	0.87	3.81	25 600	9.25 x 10^7	2.77
Maltose (20.0)	3.81	25 600	1.66 x 10^8	1.54	3.79	12 800	1.45 x 10^8	0.88
Sucrose (20.0)	3.83	12 800	1.38 x 10^8	0.93	3.81	6 400	1.04 x 10^8	0.62
Gluconate (20.0)	5.03	6 400	3.22 x 10^8	1.99	5.08	6 400	2.30 x 10^7	2.78
Glucose (5.0)	4.15	6 400	5.22 x 10^8	1.23	4.54	400	3.50 x 10^7	0.11
Glucose (10.0)	4.18	12 800	1.02 x 10^8	1.25	4.15	3 200	5.80 x 10^7	0.55
Glucose (20.0)	3.79	25 600	1.42 x 10^8	1.80	3.74	25 600	9.50 x 10^7	2.69
Glucose (30.0)	3.74	25 600	1.43 x 10^8	1.79	3.75	12 800	7.60 x 10^7	1.68
Glucose (50.0)	3.73	12 800	1.26 x 10^8	1.01	3.73	12 800	9.03 x 10^7	1.42
	L. fermentum JW11BZ				*L. fermentum* JW15BZ			
MRS, Biolab (unmodified):[a]	4.11	6 400	2.75 x 10^7	2.33	4.14	12 800	3.42 x 10^7	3.74
Carbohydrates (g/l):								
Fructose (20.0)	4.26	1 600	2.89 x 10^7	0.55	4.29	3 200	4.31 x 10^7	0.74
Lactose (20.0)	6.19	6 400	3.87 x 10^7	1.65	6.17	6 400	6.47 x 10^7	0.99
Mannose (20.0)	5.18	3 200	1.00 x 10^7	3.20	5.19	1 600	1.22 x 10^7	1.31
Maltose (20.0)	4.34	1 600	2.83 x 10^7	0.57	4.26	1 600	3.26 x 10^7	0.49
Sucrose (20.0)	4.29	3 200	3.63 x 10^7	0.88	4.30	3 200	4.91 x 10^7	0.65
Gluconate (20.0)	6.52	800	3.52 x 10^6	2.27	6.55	400	5.31 x 10^6	0.75
Glucose (5.0)	5.02	400	1.53 x 10^7	0.26	5.03	800	2.17 x 10^7	0.37
Glucose (10.0)	4.65	3 200	2.81 x 10^7	1.14	4.64	3 200	3.35 x 10^7	0.96
Glucose (20.0)	4.11	6 400	2.75 x 10^7	2.33	4.14	12 800	3.42 x 10^7	3.74
Glucose (30.0)	4.01	6 400	2.87 x 10^7	2.23	4.05	12 800	2.20 x 10^7	5.82
Glucose (50.0)	3.97	6 400	3.03 x 10^7	2.11	4.00	25 600	3.53 x 10^7	7.52

(Results as publish by Von Mollendorff et al. [21])

Table 2. *Effect of organic nitrogen sources on the production of bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ*

	pH	***AU/ml***	***CFU/ml***	***AU/10^{4}CFU***	***pH***	***AU/ml***	***CFU/ml***	***AU/10^{4}CFU***
	L. plantarum JW3BZ				*L. plantarum JW6BZ*			
Organic nitrogen (g/l):								
Tryptone (20.0)	4.00	25 600	1.22 x 10^8	2.10	3.90	25 600	8.92 x 10^7	2.87
Meat extract (20.0)	3.81	25 600	1.50 x 10^8	1.71	3.79	25 600	8.73 x 10^7	2.93
Yeast extract (20.0)	3.79	12 800	1.70 x 10^8	0.75	3.77	25 600	9.53 x 10^7	2.69
T + M (12.5 + 7.5)	3.75	25 600	1.65 x 10^8	1.55	3.73	51 200	1.11 x 10^8	4.61
T + Y (12.5 + 7.5)	3.77	25 600	1.54 x 10^8	1.66	3.75	51 200	1.05 x 10^8	4.88
M + Y (10.0 + 10.0)	3.80	25 600	1.65 x 10^8	1.55	3.79	25 600	1.03 x 10^8	2.49
T + M + Y (10.0 + 5.0 + 5.0)	3.79	25 600	1.42 x 10^8	1.80	3.77	25 600	9.50 x 10^7	2.69
	L. fermentum JW11BZ				*L. fermentum* JW15BZ			
Organic nitrogen (g/l):								
Tryptone (20.0)	4.24	6 400	3.19 x 10^7	2.01	4.21	6 400	3.47 x 10^7	1.84
Meat extract (20.0)	4.28	3 200	2.82 x 10^7	1.13	4.31	3 200	3.28 x 10^7	0.98
Yeast extract (20.0)	4.23	3 200	3.22 x 10^7	0.99	4.19	1 600	2.91 x 10^7	0.55
T + M (12.5 + 7.5)	4.18	6 400	2.45 x 10^7	2.61	4.18	6 400	4.30 x 10^7	1.49
T + Y (12.5 + 7.5)	4.15	3 200	2.99 x 10^7	1.07	4.16	3 200	4.53 x 10^7	0.71
M + Y (10.0 + 10.0)	4.23	3 200	3.77 x 10^7	0.84	4.24	1 600	5.17 x 10^7	0.31
T + M + Y (10.0 + 5.0 + 5.0)	4.11	6 400	2.75 x 10^7	2.33	4.14	12 800	3.42 x 10^7	3.74

Table 3. *Effect of potassium on the production of bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ*

	pH	***AU/ml***	***CFU/ml***	***AU/10^{4}CFU***	***pH***	***AU/ml***	***CFU/ml***	***AU/10^{4}CFU***
	L. plantarum JW3BZ				*L. plantarum* JW6BZ			
Hydrogen phosphate (g/l):								
KH_2PO_4 (2.0)	3.75	51 200	1.30 x 10^8	3.94	3.73	1 600	7.81 x 10^7	0.21
KH_2PO_4 (5.0)	3.76	51 200	1.13 x 10^8	4.53	3.74	1 600	7.65 x 10^7	0.21
KH_2PO_4 (10.0)	3.78	51 200	1.33 x 10^8	3.85	3.77	51 200	5.97 x 10^7	8.58
KH_2PO_4 (20.0)	3.81	51 200	1.22 x 10^8	4.20	3.78	51 200	7.38 x 10^7	6.94
K_2HPO_4 (2.0)	3.79	25 600	1.42 x 10^8	1.80	3.77	25 600	9.50 x 10^7	2.69
K_2HPO_4 (5.0)	3.80	51 200	1.48 x 10^8	3.46	3.82	25 600	1.03 x 10^8	2.49
K_2HPO_4 (10.0)	4.00	25 600	1.35 x 10^8	1.91	3.98	6 400	9.04 x 10^7	0.71
K_2HPO_4 (20.0)	4.42	12 800	9.70 x 10^7	1.32	4.75	3 200	6.72 x 10^7	0.48
K_2HPO_4 and KH_2PO_4 (2.0 + 2.0)	3.79	25 600	1.50 x 10^8	1.71	3.96	12 800	9.15 x 10^7	1.41

	L. fermentum JW11BZ				*L. fermentum* JW15BZ			
Hydrogen phosphate (g/l):								
KH_2PO_4 (2.0)	4.02	3 200	1.94×10^7	1.65	4.05	12 800	3.70×10^7	3.49
KH_2PO_4 (5.0)	4.02	6 400	2.27×10^7	2.82	4.08	12 800	3.60×10^7	3.56
KH_2PO_4 (10.0)	4.04	6 400	2.87×10^7	2.23	4.09	25 600	4.69×10^7	5.46
KH_2PO_4 (20.0)	4.07	6 400	3.03×10^7	2.11	4.13	25 600	3.28×10^7	7.80
K_2HPO_4 (2.0)	4.11	6 400	2.75×10^7	2.33	4.14	12 800	3.42×10^7	3.74
K_2HPO_4 (5.0)	4.15	6 400	3.08×10^7	2.08	4.20	25 600	3.47×10^7	7.38
K_2HPO_4 (10.0)	5.52	12 800	2.64×10^7	4.85	4.61	25 600	4.04×10^7	6.34
K_2HPO_4 (20.0)	4.54	12 800	3.47×10^7	3.69	5.57	6 400	2.29×10^7	2.78
K_2HPO_4 and KH_2PO_4 (2.0 + 2.0)	4.06	6 400	2.29×10^7	2.79	4.09	12 800	2.80×10^7	4.57

Table 4. *Effect of magnesium sulphate, manganese sulphate and tri-ammonium citrate on the production of bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ*

	pH	***AU/ml***	***CFU/ml***	***AU/10^4CFU***	***pH***	***AU/ml***	***CFU/ml***	***AU/10^4CFU***
	L. plantarum JW3BZ				*L. plantarum* JW6BZ			
Magnesium and ammonium (g/l):								
Magnesium sulphate (0)	3.66	25 600	2.00×10^8	1.28	3.70	12 800	9.81×10^7	1.30
Manganese sulphate (0)	3.96	25 600	6.00×10^7	4.27	3.93	6 400	4.00×10^7	1.60
Tri-ammoium citrate (5.0)	4.01	25 600	1.75×10^8	1.46	4.00	25 600	8.90×10^7	2.88
Tri-ammoium citrate (10.0)	3.80	6 400	1.58×10^8	4.05	3.78	12 800	1.08×10^8	1.19
Tri-ammonium citrate (0)	3.61	12 800	1.66×10^8	0.77	3.56	12 800	1.22×10^8	1.05
	L. fermentum JW11BZ				*L. fermentum* JW15BZ			
Magnesium and ammonium (g/l):								
Magnesium sulphate (0)	4.06	1 600	2.27×10^7	0.71	4.05	12 800	3.58×10^7	3.58
Manganese sulphate (0)	4.50	3 200	1.41×10^7	2.27	4.44	12 800	2.22×10^7	5.77
Tri-ammoium citrate (5.0)	4.54	6 400	2.36×10^7	2.71	4.53	12 800	2.29×10^7	5.59
Tri-ammoium citrate (10.0)	4.26	3 200	2.83×10^7	1.13	4.25	12 800	2.68×10^7	4.78
Tri-ammonium citrate (0)	3.89	3 200	2.71×10^7	1.18	3.92	6 400	3.51×10^7	1.82

Table 5. *Effect of Tween 80 and glycerol on the production of bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ*

	pH	***AU/ml***	***CFU/ml***	***AU/10^{4}CFU***	***pH***	***AU/ml***	***CFU/ml***	***AU/10^{4}CFU***
	L. plantarum JW3BZ				*L. plantarum* JW6BZ			
Tween 80 (ml/l):								
0	3.71	3 200	1.49 x 10^8	0.22	3.69	3 200	9.23 x 10^7	0.35
0.05	3.69	25 600	1.46 x 10^8	1.75	3.68	25 600	8.60 x 10^7	3.11
0.10	3.69	25 600	1.73 x 10^8	1.48	3.69	25 600	9.80 x 10^7	2.61
0.15	3.70	51 200	1.72 x 10^8	3.11	3.69	51 200	1.05 x 10^8	4.89
0.20	3.71	51 200	1.33 x 10^8	3.85	3.69	51 200	1.04 x 10^8	4.92
Glycerol (g/l):								
0	3.79	25 600	1.42 x 10^8	1.80	3.77	25 600	9.50 x 10^7	2.69
1.0	3.74	12 800	1.33 x 10^8	0.96	3.75	25 600	8.21 x 10^7	3.12
2.0	3.73	12 800	1.62 x 10^8	0.79	3.73	25 600	8.89 x 10^7	2.88
5.0	3.74	6 400	1.34 x 10^8	0.48	3.73	12 800	9.44 x 10^7	1.36
10.0	3.74	6 400	1.51 x 10^8	0.42	3.73	3 200	9.36 x 10^7	0.34
20.0	3.75	6 400	1.13 x 10^8	0.57	3.73	1 600	9.34 x 10^7	0.17
	L. fermentum JW11BZ				*L. fermentum* JW15BZ			
Tween 80 (ml/l):								
0	4.15	6 400	2.41 x 10^7	2.33	4.13	3 200	3.05 x 10^7	1.05
0.05	4.10	6 400	3.29 x 10^7	1.95	4.12	12 800	3.12 x 10^7	4.10
0.10	4.10	6 400	3.66 x 10^7	1.75	4.12	12 800	4.64 x 10^7	2.76
0.15	4.11	6 400	3.06 x 10^7	2.09	4.12	25 600	3.21 x 10^7	7.98
0.20	4.11	6 400	3.08 x 10^7	2.08	4.12	25 600	3.86 x 10^7	6.63
Glycerol (g/l):								
0	4.11	6 400	2.75 x 10^7	2.33	4.14	12 800	3.42 x 10^7	3.74
1.0	4.06	1 600	2.73 x 10^7	0.59	4.07	6 400	4.04 x 10^7	1.58
2.0	4.06	1 600	2.64 x 10^7	0.61	4.07	6 400	2.43 x 10^7	2.63
5.0	4.06	1 600	2.68 x 10^7	0.61	4.07	6 400	3.30 x 10^7	1.94
10.0	4.06	1 600	3.41 x 10^7	0.47	4.08	6 400	3.44 x 10^7	1.86
20.0	4.06	800	2.60 x 10^7	0.31	4.09	6 400	3.33 x 10^7	1.92

Table 6. *Effect of vitamins on the production of bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ*

	pH	***AU/ml***	***CFU/ml***	***AU/10^{4}CFU***	***pH***	***AU/ml***	***CFU/ml***	***AU/10^{4}CFU***
	L. plantarum JW3BZ				*L. plantarum* JW6BZ			
Vitamins (1.0 mg/l):								
L-ascorbic acid (Vit. C)	3.75	25 600	1.47×10^8	1.74	3.74	25 600	8.74×10^7	2.93
Thiamine (Vit. B_1)	3.72	25 600	1.27×10^8	2.02	3.71	3 200	1.11×10^8	0.29
Cyanocobalamin (Vit. B_{12})	3.72	51 200	1.59×10^8	3.22	3.71	25 600	8.82×10^7	2.90
DL-6,8-thiotic acid	3.74	25 600	1.63×10^8	1.57	3.73	12 800	9.33×10^7	1.37
	L. fermentum JW11BZ				*L. fermentum* JW15BZ			
Vitamins (1.0 mg/l):								
L-ascorbic acid (Vit. C)	4.02	3 200	2.29×10^7	1.41	4.08	6 400	3.97×10^7	1.61
Thiamine (Vit. B_1)	4.03	1 600	2.51×10^7	0.64	4.06	6 400	4.04×10^7	1.58
Cyanocobalamin (Vit. B_{12})	4.04	3 200	2.04×10^7	1.57	4.07	6 400	2.06×10^7	3.11
DL-6,8-thiotic acid	4.04	3 200	2.81×10^7	1.14	4.07	3 200	3.03×10^7	1.06

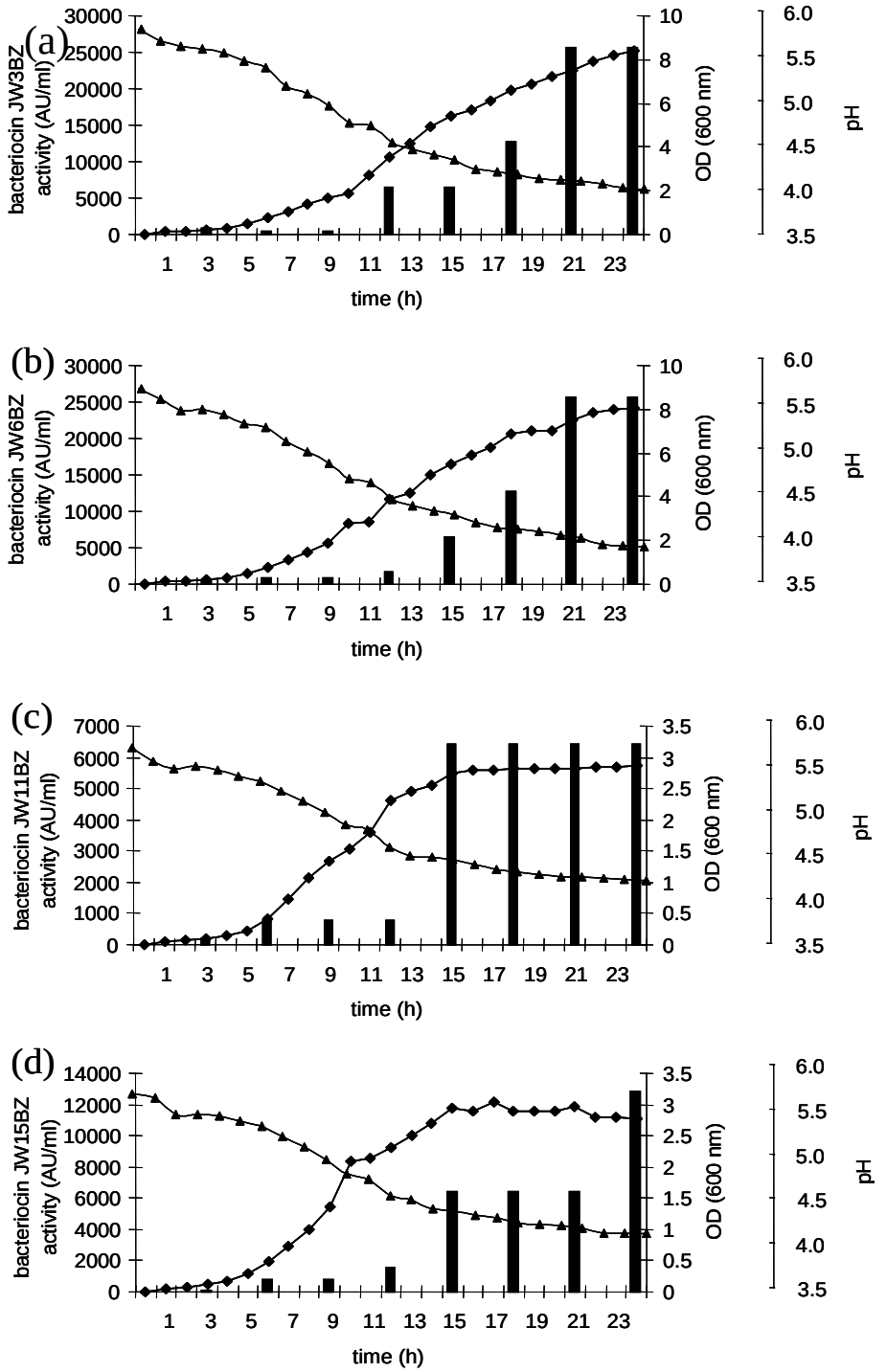

Figure 1. *Growth of strains (a) JW3BZ, (b) JW6BZ, (c) JW11BZ and (d) JW15BZ in modified MRS broth (♦), production of bacteriocins (■) and changes in culture pH (▲)*

Production of bacJW11BZ remained constant (1.75 AU/10^4CFU to 2.33 AU/10^4CFU) in the presence or absence of Tween 80 (**Table 5**). Increased activity may be due to changes in charges on the exterior of the cell wall of producer cells, leading to a discharge of bacteriocins [24].

A reduction in bacteriocin JW3BZ, JW11BZ and JW15BZ activity was recorded when strains were grown in the presence of 1.0 to 20.0 g/l glycerol, whereas a reduction in bacteriocin JW6BZ activity was recorded in medium supplemented with 5.0 to 20.0 g/l glycerol (**Table 5**). In the case of plantaricin ST31 [9], concentrations of 2.0 g/l glycerol and higher led to a decrease in activity, whereas production of bacteriocins ST23LD and ST341LD was repressed in the presence of glycerol [24]. Changes in osmotic stress may be responsible for the decrease in bacteriocin production.

Addition of 1.0 mg/l L-ascorbic acid (Vit. C), 1.0 mg/l thiamine (Vit. B_1), 1.0 mg/l cyanocobalamin (Vit. B_{12}) and 1.0 mg/l DL-

6,8-thioctic acid to MRS broth led to different levels of bacJW3BZ, bacJW6BZ, bacJW11BZ and bacJW15BZ production (**Table 6**). Production of bacJW3BZ increased in the presence of cyanocobalamin and thiamine (**Table 6**). No increase in bacJW6BZ, bacJW11BZ and bacJW15BZ activity was recorded in the presence of 1.0 mg/l cyanocobalamin (Vit. B_{12}), 1.0 mg/l thiamine (Vit. B_1), 1.0 mg/l DL-6,8-thioctic acid and 1.0 mg/l L-ascorbic acid (Vit. C) (**Table 6**). Similar results were reported for bacteriocin ST194BZ, produced by *L. plantarum* ST194BZ [10] and bacteriocins ST23LD and ST341LD produced by *L. plantarum* ST23LD and ST341LD [24].

Optimal production of the four bacteriocins in modified MRS broth composed of 20.0 g/l glucose, 10.0 g/l tryptone, 5.0 g/l meat extract, 5.0 g/l yeast extract, 20.0 g/l KH_2PO_4, 5.0 g/l tri-ammonium citrate, 0.20 g/l Tween 80 and 1.0 mg/l L-ascorbic acid, pH6.5 (**Figure. 1a-d**) was the same as recorded in unmodified MRS broth (**Table 1**). Slight variations were recorded in the rate at which the four bacteriocins were produced in the two growth media. Different results may be obtained in fed-batch fermentations with controlled levels of a specific nutrient. The optimised media was with reduced composition compared with the commercially available MRS broth [25] and this will have an impact on the commercial cost of the production of the studied bacteriocins.

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