

## NOTE

# Optimization of Bacteriocin ST311LD Production by *Enterococcus faecium* ST311LD, Isolated from Spoiled Black Olives

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**Bacteriocin ST311LD is approximately 2.3 kDa in size. Low levels of bacteriocin activity were recorded in BHI and M17 broth (800 AU/ml) and in 10% (w/v) soy milk (3,200 AU/ml). No bacteriocin production was recorded in 10% (w/v) molasses, despite good growth. Optimal levels (12,800 AU/ml) were detected in MRS broth which had been supplemented with tryptone (20.0 g/l), saccharose (5.0 or 10.0 g/l) or vitamin C (1 ppm). Increased potassium levels did not result in higher levels of activity, and glycerol (1.0 g/l) inhibited the production of bacteriocin ST311LD.**

**Key words:** *Enterococcus faecium*, bacteriocin ST311LD, black olives

Lactic acid bacteria play important roles in food fermentation, and have also been shown to exert a variety of positive health and nutritional effects (Gilliland, 1990). A host of papers have been published which describe the antagonistic effects of bacteriocins against food spoilage and pathogenic bacteria (Klaenhammer, 1988; Nieto-Lozano *et al.*, 2002; Leroy *et al.*, 2003). As far as we have been able to determine, only a few bacteriocins have been reported to be produced by lactic acid bacteria in olives (Jimenez-Diaz *et al.*, 1993; Ruiz-Barba *et al.*, 1994; Frantz *et al.*, 1996; Lean *et al.*, 1998). Bacteriocins have definitely been detected in conjunction with *Enterococcus faecium*, but these have been isolated from cheese, chicken, nuka (Japanese rice - bran pate), and dry sausages (Audisto *et al.*, 2001; Ennahar *et al.*, 2001; Herranz *et al.*, 2001; Losteinkit *et al.*, 2001; Leroy *et al.*, 2003; Moreno *et al.*, 2003; Saavedra *et al.*, 2003).

Bacteriocin production is frequently regulated by pH and growth temperature, as has been shown in several studies involving the pediocin AcH (Biswas *et al.*, 1991) and pediocin PD-1 (Nel *et al.*, 2001) generated by *Pedococcus* spp., as well as enterocin 1146 (Parente and Ricciardi, 1994), enterocin AS-48 (Abriouel *et al.*, 2001), a bacteriocin produced by *E. faecium* RZS C5 (Leroy *et al.*, 2003) and enterocin P (Herranz *et al.*, 2001), which is generated by *Enterococcus* spp. In specific cases, higher bacteriocin production levels have been recorded at sub-

optimal growth conditions (Parente *et al.*, 1994; Parente and Ricciardi, 1994; Mortvedt-Abildgaard *et al.*, 1995; De Vuyst *et al.*, 1996; Matsusaki *et al.*, 1996; Bogovic-Matijasic and Rogelj, 1998; Aasen *et al.*, 2000; Todorov *et al.*, 2000; Audisto *et al.*, 2001). However, apart from studies involving the effects of nitrogen and carbon sources on the production of enterocin P by *Enterococcus* spp. (Herranz *et al.*, 2001), and the production of a bacteriocin by *E. faecium* CRL 1385 (Audisto *et al.*, 2001), little remains known regarding the growth conditions necessary for optimal bacteriocin production by *E. faecium*.

In this paper, we report on the production of bacteriocin ST311LD by *E. faecium* ST311LD, a strain isolated from spoiled olive brine. We assessed the effects of both nutrients and medium pH values on bacteriocin ST311LD production.

Strain ST311LD was identified as *E. faecium* on the basis of its physiological and biochemical characteristics, API 50 CHL (BioMérieux, France) sugar fermentation reactions, and its DNA banding patterns, which were obtained via PCR with species-specific primers (Todorov and Dicks, 2005). MRS medium (Biolab, South Africa) was employed in all of the experiments in this study, with the exception of the studies focusing on carbon and nitrogen optimization, during which MRS (De Man *et al.*, 1960) was utilized. Incubations were conducted at 30°C.

Bacteriocin bioassays were performed via agar-spot tests and well diffusion methods, as previously described by Ivanova *et al.* (1998). Adjustment of the cell-free supernatant to a pH of 6.0 with 1N NaOH obviated the

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inhibitory effects associated with lactic acid. Antimicrobial activity was expressed in arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition (Ivanova *et al.*, 1998). *Lactobacillus casei* LHS, which originated from our own culture collection, was employed as a sensitive strain, in order to evaluate bacteriocin ST311LD activity. The LHS strain was grown in BHI broth (Biolab, South Africa) at 30°C.

Bacteriocin production levels in different growth media were assessed via the inoculation of an 18 h-old culture of strain ST311LD (2%, v/v) into MRS broth, BHI broth, M17 broth (Merck, Germany), soy milk (10%, w/v, soy flour) and molasses (2 to 10%, w/v, with 2% intervals), respectively. Incubation took place at 30°C and 37°C, respectively, without agitation, for 28 h. Samples were collected every hour and examined for bacterial growth (OD at 600 nm), changes in culture pH, and antimicrobial activity (AU/ml) against *L. casei* LHS. We also conducted agar-spot tests, as was previously described.

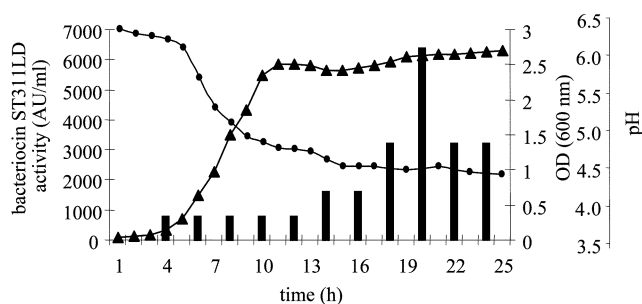
In a separate experiment, we attempted to characterize the effects of initial medium pH values on bacteriocin production. 300 ml volumes of MRS broth were adjusted with 6N HCl or 6N NaOH to pH values of 4.5, 5.0, 5.5, 6.0 and 6.5, and then autoclaved. Each of the flasks was then inoculated with 2% (v/v) of an 18 h-old culture of strain ST311LD, and was incubated at 30°C for 20 h, without agitation. Changes in culture pH and bacteriocin production levels (AU/ml) were assessed every hour, as was previously described.

The effects of different growth components on bacteriocin production levels were determined via the inoculation of 200 ml of the following media with 4 ml of a concentrated ST311LD cell suspension: (a) MRS broth (De Man *et al.*, 1960), 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) or yeast extract (5.0 g/l); (b) MRS broth, i.e. with 20.0 g/l glucose; (c) MRS broth without glucose, supplemented with 20.0 g/l fructose, sucrose, lactose, mannose, or maltose; (d) MRS broth with 0.5 - 40.0 g/l sucrose as a sole carbon source; (e) MRS broth with 2.0 g/l  $K_2HPO_4$  or 2.0 - 50.0 g/l  $KH_2PO_4$ ; and (f) MRS broth supple-

mented with 1.0 - 50.0 g/l glycerol. In a separate experiment, the vitamins cyanocobalamin (Sigma, USA), L-ascorbic acid (BDH Chemicals Ltd, UK), thiamine (Sigma, USA), DL-6,8-thioctic acid (Sigma, USA) and Vitamin K<sub>1</sub> (Fluka Chemie AG, Switzerland) were filter-sterilized and added to the MRS broth to a final concentration of 1 mg/l. Incubations for all tests were conducted for 20 h each, at 30°C. Bacteriocin ST311LD activity levels were determined as was previously described.

The cell-free supernatant of *E. faecium* ST311LD was shown to inhibit the growth of *L. casei* LHS. According to the results of tricine-SDS-PAGE, bacteriocin ST311LD is approximately 2.3 kDa in size (Todorov and Dicks, 2005).

Low levels of bacteriocin activity (800 AU/ml) recorded when the ST311LD strain was grown in BHI and M17 broth, despite the large cell numbers recorded. Relatively good bacteriocin production (3,200 AU/ml) was detected in the presence of 10% (w/v) soy milk. No bacteriocin production was detected in 10% (w/v) molasses, despite good growth. This result suggests that specific nutrients are required for the production of bacteriocin. Furthermore, because higher levels of bacteriocin production were recorded at 30°C (6,400 AU/ml), and relatively lower levels were recorded at 37°C (3,200 AU/ml), growth temperature would appear to also play an important role in this process. Growth temperature and bacteriocin production are frequently correlated, as has been previously observed for lactocin A (Parente *et al.*, 1994), enterocin 1146 (Parente and Ricciardi, 1994), lactocin S (Mortvedt-Abildgaard *et al.*, 1995), amylovorin 1471 (De Vuyst *et al.*, 1996) and nisin Z (Matsusaki *et al.*, 1996).



**Fig. 1.** Growth of *E. faecium* ST311LD in MRS broth under nonregulated pH. Changes in cell numbers (▲), bacteriocin ST311LD (■) and culture pH (●).

**Table 1.** Influence of initial growth pH on the antimicrobial activity of bacteriocin ST311LD

Initial pH	4.5	5.0	5.5	6.0	6.5
Final pH	3.7	3.8	3.8	3.9	3.9
Bacteriocin activity (AU/ml)	800	6,400	6,400	6,400	3,200
Reduction of bacteriocin activity (%)*	87.5	0	0	0	50

\*Compared to the activity in MRS broth (6,400 AU/ml)

The highest levels of bacteriocin activity (6,400 AU/ml) were recorded after 20 h of growth in MRS broth, which then declined to 3,200 AU/ml within the following 2 h (Fig. 1). During the 24 h of growth, the pH of the MRS broth decreased, from a pH of 6.2 to a pH of 4.4. Cell density (OD 600 nm) increased, however, from 0.17 to 2.70 (Fig. 1). Similar results were reported in a study of plantaricin Y (Chin *et al.*, 2001) and the bacteriocins produced by *P. acidilactici* (Nieto-Lozano *et al.*, 2002). Strain ST311LD does not hydrolyze casein on agar plates, thereby indicating that it does not generate extracellular proteases. Bacteriocin ST311LD activity was also not shown to decrease additionally during 48 h of incubation at room temperature (data not shown), suggesting that

specific peptidases are also not generated.

In MRS broth (Biolab, South Africa) adjusted to pH values of 5.0, 5.5, or 6.0, we recorded bacteriocin production levels of 6,400 AU/ml (Table 1). At a pH of 6.5, bacteriocin ST311LD levels were measured at 3,200 AU/ml. However, at a pH of 4.5, we recorded low bacteriocin ST311LD levels (800 AU/ml) (Table 1). Similar results have been reported for other bacteriocins (Daeschel *et al.*, 1990; Jimenez-Diaz *et al.*, 1993; Todorov *et al.*, 2000). The optimal pH for enterocin P production reportedly ranged between pH 5.7 - 6.0. Maximal growth occurred at pH 6.2 - 7.0 (Herranz *et al.*, 2001). Optimal levels of bacteriocin production by *E. faecium* RS C5 were obtained at a pH of 6.5, at temperatures ranging from 20°C to 35°C.

**Table 2.** Influence of organic nitrogen, carbohydrates, potassium and vitamins on bacteriocin ST311LD production

Component	Concentration (g/l)	Activity (AU/ml)	Change in bacteriocin activity (%)*
Tryptone	20.0	12,800	200
Meat extract	20.0	6,400	-
Yeast extract	20.0	3,200	50
Tryptone + meat extract	12.5 + 7.5	6,400	-
Tryptone + yeast extract	12.5 + 7.5	12,800	200
Meat extract + yeast extract	10.0 + 10.0	3,200	50
Tryptone + meat extract + yeast extract	10.0 + 5.0 + 5.0	6,400	-
Glucose	20.0	6,400	-
Fructose	20.0	800	-87.5
Lactose	20.0	3,200	50
Mannose	20.0	1,600	-75
Maltose	20.0	6,400	-
Saccharose	1.0	1,600	-75
"	5.0, 10.0	12,800	200
"	20.0, 30.0	6,400	-
"	40.0	3,200	50
KH <sub>2</sub> PO <sub>4</sub>	2.0	6,400	-
K <sub>2</sub> HPO <sub>4</sub>	2.0	6,400	-
"	5.0, 10.0, 20.0, 50.0	200	50
"	100.0	800	-87.5
Glycerol	0	6,400	-
"	1.0	3,200	50
"	2.0, 5.0, 20.0, 50.0	1,600	-75
Concentration (ppm)			
Cyanocobalamin (Vit. B <sub>12</sub> )	1.0	6,400	-
Thiamine (Vit. B <sub>1</sub> )	1.0	6,400	-
DL-6,8-thioctic acid	1.0	6,400	-
L-ascorbic acid (Vit. C)	1.0	12,800	200
Control	0	6,400	-

\*Compared to the activity in MRS broth (6,400 AU/ml)

At 35°C, enterocin RS C5 activity was detected only between pH 5.5 and 8.0 (Leroy *et al.*, 2003). The end-pH levels of the cultures ranged between 3.7 and 3.9 (Table 1), regardless of the initial growth pH. According to the results obtained in this study, and those reported in the literature (Daeschel *et al.*, 1990; Jimenez-Diaz *et al.*, 1993; Todorov *et al.*, 2000), the optimal production of *E. faecium* bacteriocins occurs during the early logarithmic growth phase, usually at a pH above 4.5.

The addition of Tween 80 to the growth medium increased the production of bacteriocin ST311LD by over 50% (results not shown). This corresponded to the results obtained for plantaricin 423 (Verellen *et al.*, 1998), pediocin AcH (Biswas *et al.*, 1991), and lactocin 705 (Vignolo *et al.*, 1995). No other information is available with regard to the effects of Tween 80 on bacteriocin production by any other *Enterococcus* strains.

The growth of strain ST311LD in the presence of tryptone as a sole nitrogen source resulted in a bacteriocin activity of 12,800 AU/ml, which was double that recorded in the MRS broth (Biolab, South Africa) supplemented with meat extract, yeast extract, or tryptone (Table 2). In the presence of yeast extract or yeast and meat extracts as nitrogen sources, we recorded an activity of 3,200 AU/ml (Table 2). However, in the presence of meat extract (20.0 g/l), or a combination of tryptone and meat extract (1 : 0.6), we recorded production levels of 6,400 AU/ml (Table 2). Growth in MRS broth supplemented with a combination of tryptone and yeast extract (1 : 0.6) resulted in an activity level of 12,800 AU/ml (Table 2). According to these results, the production of bacteriocin ST311LD is stimulated by tryptone, but not by yeast extract or meat extract. Similar results were previously recorded for plantaricin 423 (Verellen *et al.*, 1998).

The growth of strain ST311LD in the presence of 5.0 or 10.0 g/l saccharose resulted in bacteriocin production levels of 12,800 AU/ml (Table 2). In the presence of 20.0 or 30.0 g/l saccharose, these activity levels decreased to 6,400 AU/ml (Table 2). At lower saccharose concentrations (1.0 g/l), bacteriocin levels of 1,600 AU/ml were recorded (Table 2), thereby indicating that saccharose plays an important role in bacteriocin production. A 40.0 g/l saccharose concentration resulted in an activity level of 3,200 AU/ml. As far as we were able to determine, there have been no other results published in other studies regarding saccharose's effects on bacteriocin production by strains of *Enterococcus* spp.

Optimal bacteriocin production levels (6,400 AU/ml) were recorded in the presence of 20.0 g/l glucose and maltose (Table 2). However, in the presence of lactose (20.0 g/l), mannose (20.0 g/l), or fructose (20.0 g/l) as sole carbon sources, much lower levels were recorded: 3,200 AU/ml, 1,600 AU/ml, or 800 AU/ml (Table 2). According to these results, the production of bacteriocin ST311LD is stimulated by glucose, but not in cases in which glucose

is present in a disaccharide form, as in sucrose. The effects of glucose on bacteriocin production have been reported in conjunction with sakacin P (Aasen *et al.*, 2000), enterocin 1146 (Parente *et al.*, 1997), plantaricin UG1 (Enan *et al.*, 1996), and plantaricin ST 31 (Todorov *et al.*, 2000). Maximal bacteriocin activity levels were recorded for *E. faecium* RZS C5 when it was cultured in MRS broth supplemented with lactose (5.0%, w/v), at a pH of 6.5, and incubated at 37°C (Moreno *et al.*, 2003).

Little remains known regarding the influence of potassium ions on the production of bacteriocins. No differences in antibacterial activity were recorded when the ST311LD strain was grown in the presence of 2.0 g/l  $K_2HPO_4$  and 2.0 g/l  $KH_2PO_4$  (Table 2).  $K_2HPO_4$  concentrations between 0.5 g/l and 10.0 g/l resulted in the reduction of bacteriocin ST311LD activity (from 6,400 AU/ml at 0.2 g/l to 3,200 AU/ml at 0.5 g/l to 5.0 g/l and to 800 AU/ml at 10.0 g/l). In the case of plantaricin UG1, a  $K_2HPO_4$  concentration of 7.0 g/l resulted in increased levels of activity (Enan *et al.*, 1996). The optimal  $K_2HPO_4$  level recorded for plantaricin ST31 was between 2.0 and 5.0 g/l (Todorov *et al.*, 2000).

Glycerol concentrations of 1.0 g/l and higher resulted in the inhibition of bacteriocin ST311LD production (Table 2). Similar results were reported with regard to the production of plantaricin ST31, with concentrations in excess of 2.0 g/l resulting in a decrease in activity (Todorov *et al.*, 2000).

Maximal bacteriocin activity (12,800 AU/ml) was detected in MRS broth to which Vitamin C had been added (Table 2). Identical levels of activity (6,400 AU/ml) were recorded when strain ST311LD was grown in MRS broth (Biolab, South Africa) enriched with either 1.0 ppm cyanocobalamin, 1.0 ppm thiamine, or 1.0 ppm DL-6,8-thioctic acid (Table 2).

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