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Bacteriocin Production by *Enterococcus faecalis* TS9S17 in MRS Medium with Tuna Condensate as a Nitrogen Source and Its Characteristics

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Abstract

The purpose of this study was to optimize bacteriocin production by *Enterococcus faecalis* TS9S17 isolated from a mangrove forest. *Ent. faecalils* TS9S17 produced 40 AU/ml bacteriocin in MRS medium at an initial pH of 6.5 and at 37 °C for 24 h, which inhibited *Lactobacillus sakei* subsp. *sakei* JCM 1157 and *Listeria monacytogenes*. When 26.5 g/l of tuna condensate was used instead of peptone, beef extract, and yeast extract in the MRS medium, *Ent. faecalils* TS9S17 also produced 40 AU/ml of bacteriocin. The maximum bacteriocin activity, 160 AU/ml, was obtained at pH 6.5 and at 25 °C. Crude bacteriocin displayed the highest activity at pH 6 and remained active over a wide pH range. It was active after heating at 60 °C for 60 min, but showed little activity after heating at 121 °C for 15 min. It could be kept at –20 °C for more than 4 weeks. This study shows that tuna condensate could be used as a low cost nitrogen source for bacteriocin production.

Keywords: Bacteriocin, lactic acid bacteria, tuna condensate, *Enterococcus faecalis*

Introduction

Lactic acid bacteria (LAB) are Gram-positive bacteria and are considered to be GRAS (Generally Recognized as Safe) microorganisms [1]. They can produce many kinds of anti-microbial compounds, such as lactic and acetic acids, hydrogen peroxide, carbon dioxide, and bacteriocin. LAB and their bacteriocins are applied to enhance the safety and extend the shelf life of food products [2]. Food applications of bacteriocins from LAB are considered to be biopreservative compounds, and are used to satisfy increasing consumer demand [3]. Bacteriocins of LAB offer potential biotechnological applications because they are stable at low pH values, easy to produce, free of adverse effects, and sensitive to proteases [4]. Bacteriocins produced by enterococci have become the subject of interest, because bacteriocin producer strains can be isolated from a variety of fermented foods and silage, and many are active towards food borne pathogens. The production of bacteriocins depends largely on the source of nutrients, the pH, and the temperature [5], as has been shown in several studies, including the enterocin AS-48 [3] and enterocin P [6], as well as bacteriocins produced by *Enterococcus faecium* B3L31 [7] and *Ent. faecium* DB1 [8].

Most media used for the production of bacteriocins by LAB have a relatively high cost and are unsuitable for large-scale production. Therefore, some cheap raw materials, such as whey, sugar molasses, and mussel-processing wastes, have been use in culture media for bacteriocin production [4,9]. Mirhosseini and Emtiazi [9] reported that *Ent. faecium* strains produced maximum bacteriocin in cheese whey with supplements of yeast extract 10 g/l. Ananou *et al.* [3] reported that enterocin AS-48 produced by *Ent. faecalis* A-48-32 showed the maximum bacteriocin activity of 360 AU/ml in the medium including 5 % whey-derived substrate (Esprion-300) and 1 % glucose. In addition, the growth of

Lactobacillus casei ssp. *rhamnosus* (SN_{11}) showed the highest bacteriocin production in MRS medium containing sucrose 1 % as a carbon source and diluted tuna condensate 50 % as a nitrogen source [10].

The purpose of this study was to optimize the bacteriocin production by *Ent. faecalis* TS9S17 in the MRS medium using tuna condensate as a nitrogen source. The characterization of the crude bacteriocin produced by this strain was also studied.

Materials and methods

Bacterial strain and growth condition

Ent. faecalis TS9S17 was isolated from soil in the mangrove forest of southern Thailand [11]. This strain was grown at 37 °C in de Man, Rogosa, and Sharpe (MRS) medium (HiMedia Laboratories Pvt Ltd., India) for 18 h, and was used as the starter culture. *Lactobacillus sakei* subsp. *sakei* JCM 1157 was used as an indicator organism to determine bacteriocin activity. It was grown in MRS medium at 37 °C for 18 h. *Listeria monocytogenes, Salmonella* sp., *Staphylococcus aureus*, and *Vibrio parahaemolyticus* obtained from the department stock culture were cultivated in BHI broth (HiMedia Laboratories Pvt Ltd.,) at 37 °C for 18 h.

Confirmation of bacteriocin producing strain

Ent. faecalis TS9S17 was grown in MRS broth at 37 °C for 18 h. The cell free supernatant (CFS) was obtained by centrifugation (8,000×*g*, 15 min, 4 °C). The CFS was adjusted to pH 6.5 with 6 N NaOH and treated with catalase 300 U/ml (Fluka, Buchs, Switzerland), trypsin 1 mg/ml (Sigma, St. Louis, USA), or α-chymotrypsin 1 mg/ml (Sigma, St. Louis, USA) at 37 °C for 3 h. The CFS was heated at 95 °C for 10 min to deactivate the enzyme activity, and the bacteriocin activity was checked by agar well diffusion assay against *Lb. sakei* subsp. *sakei* JCM 1157 [12].

Determination of bacteriocin activity by agar well diffusion assay

Ent. faecalis TS9S17 was grown in MRS broth at 37 °C for 18 h. CFS was obtained by centrifugation (8,000×*g*, 15 min, 4 °C), adjusted to pH 6.5 with 6 N NaOH, and heated at 95 °C for 10 min. Then, 2-fold serial dilution was prepared with sterile distilled water. The MRS soft agar (0.75 % agar) inoculated with *Lb. sakei* subsp. *sakei* JCM 1157 (10^6 CFU/ml), was poured into plates. The wells of the agar plates were cut by a sterile tube. The aliquots of 50 µl from each dilution were placed in wells in plates. Plates were incubated at 37 °C for 24 h and were observed for the inhibition zone. The bacteriocin was defined as an arbitrary unit per milliliter (AU/ml). One AU was defined as the reciprocal of the highest dilution factor that showed inhibition of the indicator strain [13,14].

The antimicrobial activity of the bacteriocin against other pathogenic bacteria was performed by agar well diffusion assay, but the pathogen was used instead of *Lb. sakei* subsp. *sakei* JCM 1157.

Optimization of bacteriocin production

Selection of nitrogen source for bacteriocin production

In this experiment, tuna condensate (Songkla Canning Public Co., Ltd., Songkhla, Thailand) was used as an organic nitrogen source in MRS medium. Tuna condensate was freeze dried and the total nitrogen was analyzed by using CN analyzer (Central Equipment Division, Faculty of Science, Prince of Songkla University). The salt composition was analyzed by AOAC method [15] and the phosphorous, potassium, iron, zinc, calcium and magnesium were analyzed by the Scientific Equipment Center, Prince of Songkla University.

One ml of the starter culture was inoculated into 100 ml of the modified MRS broths pH 6.5 and incubated at 37 °C without agitation for 24 h. The freeze-dried tuna condensate was used instead of peptone, beef extract and/or yeast extract in the MRS medium. The compositions of the MRS, modified MRS 1, 2, 3 media are shown in **Table 1**. Growth was monitored by measuring the optical density (OD) at 650 nm. The bacteriocin activity was measured by agar well diffusion assay. All experiments were done in duplicate.

Table 1 Composition of modified MRS media.

Composition (g/l)	MRS* (control)	Modified MRS 1	Modified MRS 2	Modified MRS 3
Glucose	20	20	20	20
Peptone	10	-	-	-
Beef extract	10	10	-	-
Yeast extract	5	5	5	-
Tuna condensate	-	13.5	26.5	26.5
Polysorbate 80	1	1	1	1
Ammonium citrate	2	2	2	2
Sodium acetate	5	5	5	5
Magnesium sulfate	0.1	0.1	0.1	0.1
Manganese sulfate	0.05	0.05	0.05	0.05
Dipotassium phosphate	2	2	2	2

*Source: [16]

Effect of temperature and initial pH on bacteriocin production

The effect of temperature on growth and bacteriocin production by *Ent. faecalis* TS9S17 was studied in the selected modified MRS medium at pH 6.5 and incubated at 25, 30, and 37 °C, respectively, without agitation. Samples were taken every 12 h and examined for bacterial growth (OD 650 nm), pH, and bacteriocin activity.

The effect of an initial medium pH on growth and bacteriocin production was studied by adjusting the selected modified MRS medium to pH 5, 6, 7, 8, and 9, with 6 N HCl or 6 N NaOH, and then autoclaved before inoculation with 1 % starter culture and incubated at the optimum temperature bacteriocin production. The bacterial growth, pH, and bacteriocin activity were determined.

Partial purification of bacteriocin

For preparation of crude bacteriocin, *Ent. faecalis* TS9S17 was grown in the selected modified MRS medium (1000 ml) for 24 h at 25 °C. The CFS was obtained by centrifugation at 8,000×*g* 4 °C for 20 min and pH was adjusted to 6.5, with 6 N NaOH. Ammonium sulfate added slowly to the supernatant while being stirred to a final saturation of 70 %, and then stirred overnight at 4 °C. The suspension was centrifuged at 8,000×*g* 4 °C 30 min, and the precipitate was resuspended in a minimum amount of sterile distilled water and dialyzed for 24 h at 4 °C, using membrane with a molecular weight cut-off 1 kDa. The dialysate was determined for protein content by the Lowry method [17] and for bacteriocin activity, and then kept at −20 °C [18].

Characterization of bacteriocin

Effect of pH on bacteriocin activity

The effect of pH on bacteriocin activity was determined by adjusting the pH of 1 ml of crude bacteriocin (2,560 AU/ml) to pH 3 - 8 with sterile 3 N HCl or 3 N NaOH. Then, the residual bacteriocin activity was determined.

Thermal stability of bacteriocin

The effect of temperature on bacteriocin activity was examined by incubating 1 ml of crude bacteriocin (2,560 AU/ml) at 37, 60, 80, and 100 °C for 3 h, and 110 °C for 20 min and 121 °C for 15 min. Then, the bacteriocin activity was determined.

Effect of storage temperature on bacteriocin activity

The crude bacteriocin (2,560 AU/ml) was stored at −20, 4, and 37 °C for 5 weeks. The samples were taken every week to determine bacteriocin activity.

Results and discussion

Confirmation of bacteriocin producing strain

Ent. faecalis TS9S17 was isolated from the mangrove forest [11], having activity against *Lb. sakei* subsp. *sakei* JCM 1157. The CFS of *Ent. faecalis* TS9S17 was adjusted to pH 6.5 and the activity decreased, which meant some inhibitory activity was related to the acid in the solution. After treatment with catalase, the activity did not decrease, indicating that the activity was not related to hydrogen peroxide. However, the inhibition zone of CFS was decreased by adjustment to pH 6.5, and had no activity when treated with α-chymotrypsin (**Table 2**).Therefore, the CFS of *Ent. faecalis* TS9S17 contained bacteriocin. Similar results were obtained by Hwanhlem *et al.* [11]. They reported that the antibacterial activities of supernatants of *Lactococcus lactis* subsp. *lactis* KT2W2L, *Ent. faecalils* KT2W2G, *Ent. faecalils* TS9S17, and *Ent. faecalils* TS9S19 were made completely inactive by treatment with α-chymotrypsin.

Antimicrobial activity of bacteriocin

The CFS of *Ent. faecalis* TS9S17 was tested with pathogenic bacteria including *L. monocytogenes, Salmonella* sp, *S. aureus*, and *V. parahaemolyticus.* Only *L. monocytogenes* was inhibited. The inhibition of *Ent. faecalils* TS9S17 was associated with properties of bacteriocin from enterococci, or class II, bacteriocins, that displayed anti-listeria activity [9]. The other studies show that most bacteriocin produced by *Ent. faecalils* had inhibitory activity against Gram positive bacteria [9,11,12,19].

Optimization of bacteriocin production

Selection of carbon source and nitrogen source

Chemical compositions of the freeze-dried tuna condensate are shown in **Table 3**. The powder had pH 7.2, a total nitrogen content of 8.5 % and a total salt content of 11.09 %. Thus, the use of the freeze-dried tuna condensate 13.5 g/l would had the total nitrogen content almost equal to 10 g/l of peptone in MRS medium.

Ent. faecalis TS9S17 was grown in the modified MRS media with an initial pH of 6.5 at 37 °C; growth reached the stationary phase at 12 h (**Figure 1A**). When peptone was replaced with 13.5 g/l of tuna condensate (modified MRS 1), its growth was similar to that of the control (MRS medium). When peptone and beef extract was replaced with 26.5 g/l of tuna condensate in MRS medium, with and without yeast extract (modified MRS 2 and modified MRS 3, respectively), the growth was decreased. However, the bacteriocin production in the modified MRS 3 medium was still at 40 AU/ml at 12 - 48 h, which was better than in the other modified MRS media (**Figure 1B**).

Juntraporn [10] studied the optimization of medium composition for bacteriocin production by *Lb. casei* ssp. *rhamnosus* (SN 11), using tuna condensate instead of yeast extract. The growth and bacteriocin activity were not different with the control (MRS medium), due to the tuna condensate being full of several nitrogen compounds and minerals. On the contrary, Junudom [20] reported that *Lb. casei* ssp. *rhamnosus* (SN 11) grown in the modified MRS medium containing tuna condensate did not produce bacteriocin activity after the stationary phase. The loss of bacteriocin activity was affected by proteolytic degradation [21] and adsorption of bacteriocin to cells [18].

Table 2 Inhibition activity of cell free supernatants from *Enterococcus faecalis* TS9S17 against *Lactobacillus sakei* subsp. *sakei* JCM 1157.

Treatment	Inhibition zone (mm)
CFS	3.45±0.06
CFS + adj. pH 6.5	2.25±0.02
CFS + adj. pH 6.5 + catalase	2.25±0.08
CFS + adj. pH 6.5 + catalase + trypsin	0.60±0.35
CFS + adj. pH 6.5 + catalase + α-chymotrypsin	0.00

CFS = cell free supernatant, adj. = adjusted to

Table 3 Chemical composition of freeze-dried tuna condensate.

Composition	
Total protein (%w/w)	8.50
Salt (%w/w)	11.09
Phosphorus (% w/w)	2.18
Potassium (% w/w)	2.014
Iron (% w/w)	0.0033
Zinc (% w/w)	0.0028
Calcium (% w/w)	0.819
Magnesium (% w/w)	1.945

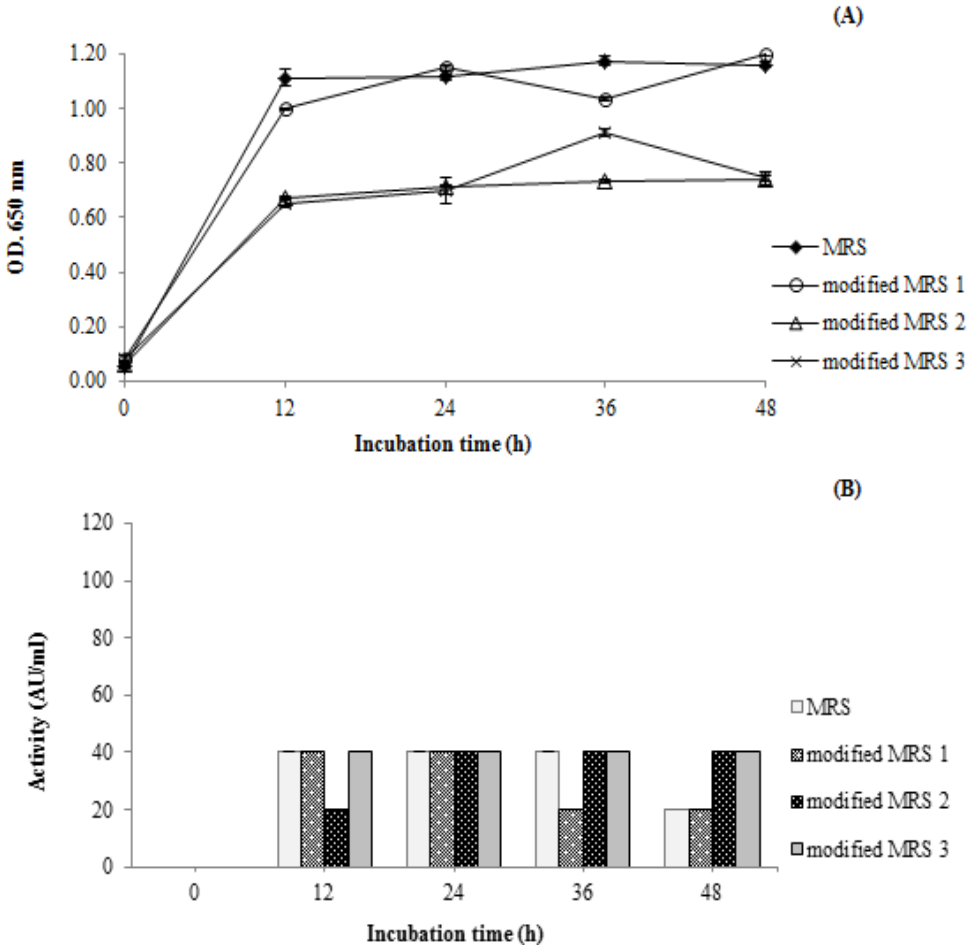

Figure 1 Effect of nitrogen sources on growth (A) and bacteriocin activity (B) of *Enterococcus faecalis* TS9S17 in different media at 37 °C.

Effect of temperature on growth and bacteriocin production

Ent. faecalis TS9S17 was grown in the modified MRS 3 medium with an initial pH of 6.5 at 25, 30, and 37 °C. *Ent. faecalis* TS9S17 entered the stationary phase at 12 h, and growth was highest at 37 °C (**Figure 2A**). However, the highest bacteriocin activity was obtained when *Ent. faecalis* TS9S17 was grown at 25 °C for 24 to 48 h (**Figure 2B**). The optimum temperature for bacteriocin production depended on cellular environment regulation and growth associated processes [22]. Hwanhlem *et al.* [11] reported that *Ent. faecalis* KT2W2G grew well at 37 °C, but gave the highest bacteriocin activity at 25.29 °C. However, the temperature for growth and bacteriocin production of *Ent. faecium* strain B3L3 was optimum at 37 °C [7].

Effect of initial pH on growth and bacteriocin production

Ent. faecalis TS9S17 was grown in the modified MRS 3 medium with different initial pH levels at 25 °C (**Figure 3A**). The growth of *Ent. faecalis* TS9S17 was highest at an initial pH of 9 at 12 h with 0.86 OD_{650}. However, the highest bacteriocin production (160 AU/ml) was in the medium with an initial pH of 7.0 at 24 - 48 h (**Figure 3B**). The optimum pH for growth of *Ent. faecalis* TS9S17 was at an alkali pH, but the optimum pH for bacteriocin production occurred at a neutral pH (pH 7). This result is similar to

other studies for bacteriocin production by *Ent. faecalis* A-48-32 [3], *Ent. faecalis* P13 [6], and *Ent. mundtii* QU 2 [23], where the optimal pH for bacteriocin production was lower that the optimal pH for the growth of these bacteria.

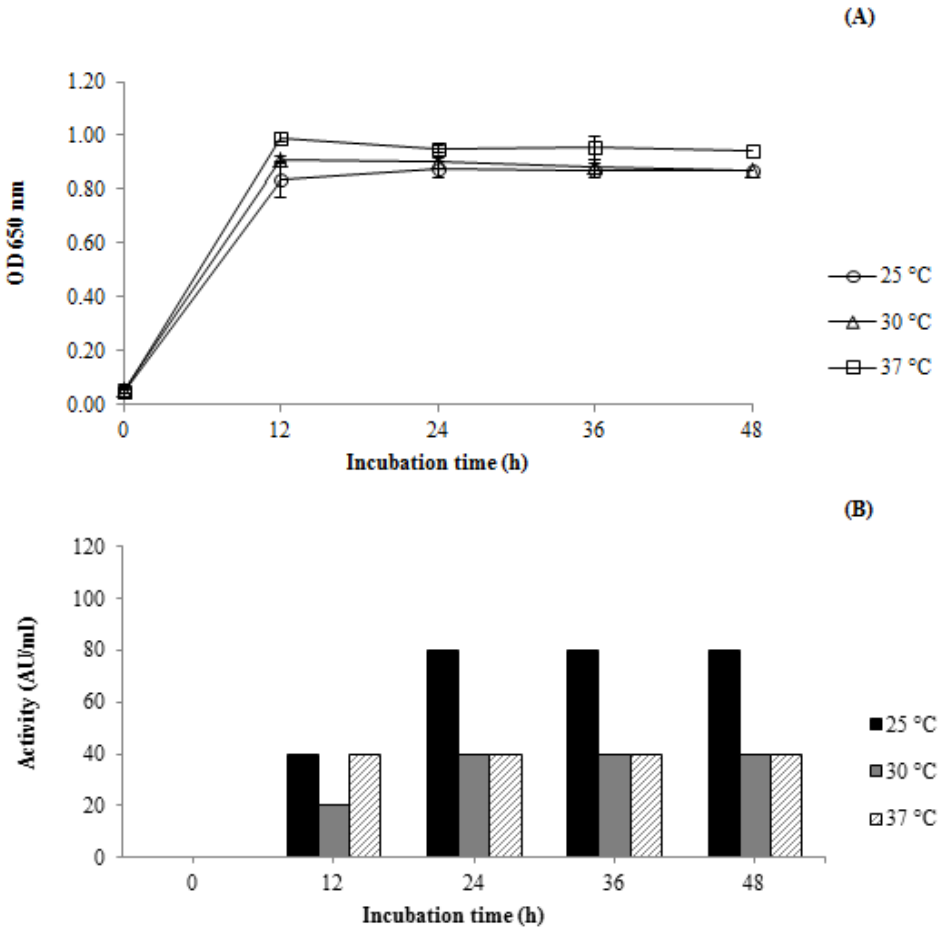

Figure 2 Effect of temperature on growth (A) and bacteriocin activity (B) of *Enterococcus faecalis* TS9S17 in modified MRS 3.

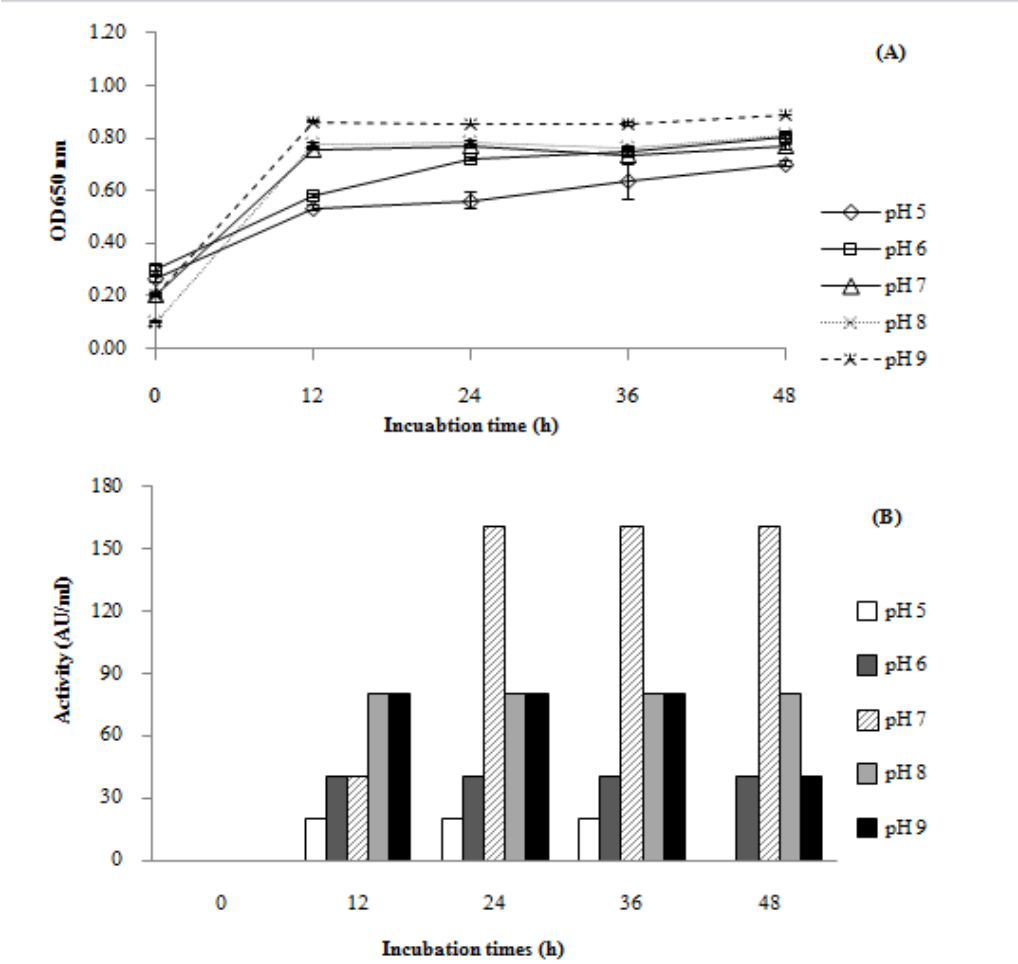

Figure 3 Effect of initial pH on growth (A) and bacteriocin activity (B) of *Enterococcus faecalis* TS9S17 in modified MRS 3 at 25 °C.

Characterization of the bacteriocin

Effect of pH on bacteriocin activity

Crude bacteriocin was obtained by precipitation with ammonium sulfate at 70 %. The activity of the crude bacteriocin was increased to 2,560 AU/ml (**Table 4**). The effect of pH on the bacteriocin activity was studied by adjusting the pH of the crude bacteriocin to pH 3 - 8 and determining the activity. The optimum pH for bacteriocin activity was at pH 6, while the activity decreased at more acidic or alkaline pH levels (**Table 5**). This loss of activity might be due to protein aggregation [21]. Other studies showed different effects of pH on bacteriocin activity. The bacteriocin produced by *Ent. faecium* MMRA remained active over a wide pH range, from 2 - 12 at 4 °C [24]. From the bacteriocins produced by *Ent. faecium* BFE 1072, BFE 1170, and BFE 1228, *Ent. faecalis* 1229 and 1263 showed the highest activity at pH 6 [18]. Moreover, *Lc. lactis* subsp. *lactis* KT2W2L was active over a wide range of pH, from 2 - 10, but no activity was found at pH 12 [25].

Table 4 Partial purification steps of bacteriocin produced by *Enterococcus faecalis* TS9S17.

Steps of purification	Total volume (ml)	Activity (AU/ml)	Protein (mg/ml)	Total activity (AU)	Total protein (mg)	Specific activity (AU/ml)	Yield (%)	Purification (fold)
Cell-free supernatant	1,000	160	2.24	160,000	2,240	71.42	100	1
$(NH_4)_2SO_4$ precipitate	70	2,560	1.78	179,200	125	1,438.20	112	20.13

Table 5 Effect of pH on activity of crude bacteriocin produced by *Enterococcus faecalis* TS9S7 against *Lactobacillus sakei* subsp. *sakei* JCM 1157.

pH	Bacteriocin activity (AU/ml)
Control (pH 6.5)	2,560
3	320
4	320
5	320
6	2560
7	1280
8	640

Thermal stability of bacteriocin activity

Bacteriocin activity was not different to the control after incubation at 37 °C for 3 h. At 60 °C, the bacteriocin activity was stable in the first hour, but decreased by half at 2 h. Most activity was lost at higher temperatures. The bacteriocin activity was retained at 80 AU/ml after heating at 110 °C for 20 min and 121 °C for 15 min. The results are shown in **Figure 4**.

The bacteriocin produced by *Ent. faecium* ST5Ha was stable at 25 - 100 °C for 2 h and decreased by half at 121 °C for 20 min [4]. From the bacteriocin produced by *Ent. faecium* BFE 1072, BFE 1170, and BFE 1228, *Ent. faecalis* 1229 and 1263 [18] and *Ent. faecium* MMRA [25] were stable at 121 °C for 15 min. However, the bacteriocin produced by *Lc. lactis* subsp. *lactis* KT2W2L was lost at 100 °C for 30 min and 121 °C for 15 min [25].

Effect of storage temperature on bacteriocin activity

Bacteriocin activity was stable at –20 °C for 4 weeks of storage, but at 4 °C, the activity was slightly lost in the first week, and remained stable to 3 weeks. In the case of storage at 37 °C, the bacteriocin activity was lost in the first week (**Table 6**). The results demonstrated that this bacteriocin could be better kept at low temperatures than at high temperatures for long storage. Most bacteriocins were still active after storage at low temperatures. The bacteriocin produced by *Ent. faecium* MMRA remained stable during storage at –20 °C for at least 12 months [25]. Bacteriocin produced by *Lb. fermentum* retained its activity for 3 to 5 months at 0 - 4 °C and 1 year at –20 °C [26]. Bacteriocin produced by *Lc. lactis* subsp. *lactis* KT2W2L was fully active after 8 weeks of storage at –20 °C [25].

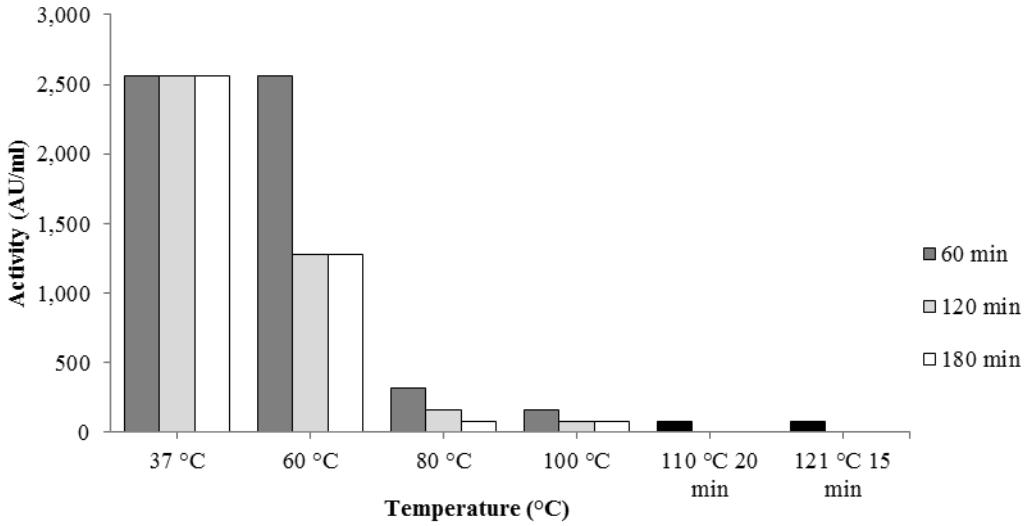

Figure 4 Effect of temperature on activity of crude bacteriocin produced by *Entrococcus faecalis* TS9S17 against *Lactobacillus sakei* subsp. *sakei* JCM 1157.

Table 6 Effect of storage temperature on activity of crude bacteriocin produced by *Enterococcus faecalis* TS9S17 against *Lactobacillus sakei* subsp. *sakei* JCM 1157.

Temperature (°C)	Bacteriocin activity (AU/ml) at different time (weeks)					
	0	**1**	**2**	**3**	**4**	**5**
–20	2,560	2,560	2,560	2,560	2,560	1,280
4	2,560	1,280	1,280	1,280	640	640
37	2,560	0	0	0	0	0

Conclusions

Tuna condensate is a cheap nitrogen source. It could be used instead of peptone, beef extract, and yeast extract in MRS broth for bacteriocin production by *Ent. faecalis* TS9S17. The crude bacteriocin of *Ent. faecalis* TS9S17 showed high activity at pH 6, and was stable at 60 °C for 1 h. It could be used as a biopreservative strain to increase the safety and to extend the shelf-life of food products.

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