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Detection, purification and efficacy of warnerin produced by *Staphylococcus warneri*

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Summary

Three strains of *Staphylococcus warneri* (FM10, FM20 and FM30) isolated from meat samples were investigated for their ability to synthesize bacteriocin. All the tested strains produced warnerin, a new peptide bacteriocin; which inhibits the growth of a large number of Gram-positive and Gram-negative bacteria. The inhibitory effect of warnerin produced by the FM20 isolate was high when compared to the other isolates. The results on the effect of carbon sources, nitrogen sources, pH, temperature, incubation time and surfactant (tween 80) inferred that the bacteriocin production was high in medium supplemented with 1% glucose (12,800 AU/ml), 1% urea (6800 AU/ml), and 0.5% Tween 80 (25,600 AU/ml). The higher productivity of bacteriocin was registered during 12 h of incubation in the medium pH 6.5 at 37 °C temperature. Among the various indicator strains tested, *Staphylococcus aureus* was more sensitive to the bacteriocin activity. Partially purified warnerin exhibited a single band on SDS-PAGE with an apparent molecular weight of 2500 Da. Warnerin, the antibacterial compound was determined as a proteinaceous substance, since it lost its activity when pepsin was added.

Introduction

Gradual increase in the antibiotic resistance among the bactericidal pathogens and intricacy in controlling them is of major concern (Ray 1992). Bacteriocins produced by some bacteria have the ability to inhibit the growth of Gram-positive and Gram-negative bacteria. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a number of different bacteria (Tagg *et al.* 1976), whose activity is generally directed towards species that are closely related or share the same ecological niche (Klaenhammer 1993). Bacteriocins produced by Gram-positive bacteria have attracted much attention because of their potential use as food preservatives. Because of their low antimicrobial activity in foods, applications of bacteriocins as food preservatives have been so far limited. Thus the isolation and screening of new bacteriocins with antimicrobial potency in foods is anticipated. Warnerin, a bacteriocin produced by the specific bacterial strains *Staphylococcus warneri* FM10, FM20 and FM30 were found beneficial for the treatment of typical infections caused by *Staphylococcus aureus* and *Propionibacterium acne* (Kurniasih & Ray 2000). The present study was undertaken to isolate warnerin-producing organisms, and to characterize the inhibitory action of warnerin against various Gram-positive and -negative organisms as well as to determine the molecular mass of the partially purified warnerin. Attempts were also made to find out the optimal cultural conditions for greater bacteriocin yield.

Materials and methods

Isolation and identification of bacteriocin-producing organism, Staphylococcus warneri

In the present study, three strains of *S. warneri* such as FM10, FM20, and FM30 were isolated from meat samples as described by Vanderzant & Splittstoesser (1992). The meat sample was homogenized, diluted and plated on to selective medium, Baird Parker agar (Himedia, India) and incubated at 37 °C for 24 h. After incubation, individual colonies were replicated in duplicate plates, incubated and after appearance of colonies one set of plates was overlaid with 5 ml of 50 °C hot soft agar suspension containing 100 μl of an exponentially growing bacterial culture (10^6 cells per ml) of an indicator strain. The plates were incubated at 37 °C for 24 h and inhibition zones were measured. Corresponding producer strains were isolated from the replica plates. The isolated organisms were characterized by using API STAPH-IDENT kit (Biomerieux, France), the biochemical reaction profiles were compared to determine

the genus and species of each isolate from the information supplied by commercial suppliers. Bacterial strains used as indicator organisms are listed in Table 1. All strains with the addition of *S. warneri* FM10, FM20 and FM30 were cultured aerobically in MRS medium (Himedia, India). The composition of MRS medium was glucose (20 g), peptone (10 g), beef extract (8 g), sodium acetate (5 g), yeast extract (4 g), dipotassium hydrogen phosphate (2 g), triammonium citrate (2 g), $MgSO_4$, $MnSO_4$, sorbitolmanooleate (1.0 ml), and agar (20 g) in 1000 ml distilled water (pH 6.5 ± 0.2). Soft and solid agar media were prepared by adding 1 and 2% agar to the broth respectively.

Bacteriocin detection and activity assay

S. warneri was cultured aerobically in MRS broth having pH 6.5 ± 0.2 for 24 h at 37 °C. The culture supernatant was then purified to eliminate the other antimicrobial compounds such as lactic acid, hydrogen peroxide, diacetyl ions etc. and assayed according to the method of Jimenez-Diaz *et al.* (1993). The antagonistic activity was detected by agar well diffusion assay as described by Tagg & McGiven (1971). Briefly, the stationary phase culture was centrifuged at $2500 \times g$ at 4 °C for 10 min. Cell-free supernatant was serially diluted up to 1:200. Then 50 μl of a two-fold diluted sample was placed on the surface of the MRS agar plate and incubated at 37 °C for 24 h after pouring 5 ml of soft MRS agar containing 8.3×10^9 c.f.u./ml of the indicator strain (*Micrococcus luteus*, MTCC 1541). After incubation the inhibition zone was observed and the antibacterial activity (Arbitrary Unit; AU/ml) was calculated. One arbitrary unit was (AU) defined as reciprocal of the highest dilution showing an inhibition zone.

Effect of medium components and growth conditions on bacteriocin production

The effect of medium ingredients on production of bacteriocin was appraised using complex media. The MRS medium was supplemented with various sugars (1%) such as fructose, mannitol, xylose, raffinose, lactose, maltose and glucose and various nitrogen sources (1%) such as urea, ammonium sulphate and sodium nitrate and with 0.1–0.5% of surfactant (Tween 80). Samples were collected at regular intervals to measure the cell growth of the strain (A_{600}), pH change and bacteriocin activity (AU/ml). The influence of growth conditions such as incubation time, medium pH and incubation temperature on the production of bacteriocin was also carried out by the method described by Balasubramanyam & Varadaraj (1998) and Yildirim & Johnson (1998).

Table 1. Indicator organisms and their origin.

Indicator strains	Strain No	Strain origin
Staphylococcus aureus	MTCC 902	Reference strain
Micrococcus luteus	MTCC 1541	Reference strain
Listeria monocytogenes	MTCC 1143	Reference strain
Klebsiella pneumoniae	MTCC 2405	Reference strain
Salmonella typhi	MTCC 734	Reference strain
Shigella flexneri	MTCC 1457	Reference strain
Vibrio cholerae	MTCC 1738	Reference strain
Pseudomonas aeruginosa	MTCC 2905	Reference strain
Staphylococcus warneri	FM10	Meat
Staphylococcus warneri	FM20	Meat
Staphylococcus warneri	FM30	Meat

Characterization of bacteriocin

The bacteriocin was treated with various enzymes such as pepsin, α-chymotrypsin and lysozyme (Sigma Chemical Co.). Enzymes were filter-sterilized and added to partially purified bacteriocin preparations at final concentration of 1 mg/ml. Following incubation at 37 °C for 12 h, enzyme action was arrested and the bacteriocin activity (AU/ml) was determined as described above.

Growth Inhibitory action of bacteriocin

The bactericidal action of warnerin was performed by the method of Kurniasih & Ray (2000). Exponentially grown cells of *Micrococcus luteus* (MTCC 1541), *Listeria monocytogenes* (MTCC 1143), *Salmonella typhi*, (MTCC 734), *Klebsiella pneumoniae* (MTCC 2405), *Vibrio cholerae* (MTCC 1738), *Staphylococcus aureus* (MTCC 902), *Shigella flexneri* (MTCC 1457), *Bacillus subtilis* (MTCC 1789), *Pseudomonas aeruginosa*, (MTCC 2905) in MRS broth were plated on MRS agar individually. After inoculation, partially purified warnerin was placed over the soft agar lawns of bacteria used to test sensitivity. The plates were incubated at 37 °C overnight and the zone of growth inhibition against each test strain was assessed.

Partial purification of bacteriocin

Bacteriocin produced by *S. warneri* was purified by the scheme of Crupper *et al.* (1997). The isolated culture was inoculated into the MRS media prepared under optimum pH, temperature and incubation period. After incubation, the culture was centrifuged at $10{,}000 \times g$ for 20 min at 4 °C. The cell free culture filtrate was precipitated using 80% ammonium sulphate and pelleted down by centrifuge. The pellet containing protein was resuspended in 10 ml of 10 mM sodium citrate buffer (pH 7.0) and dialyzed against the same buffer for 24 h at 4 °C using a dialysis membrane. The retentate was collected in a sterile container and stored at −20 °C for further studies.

SDS-PAGE electrophoresis

For the molecular weight determination of the synthesized bacteriocin, 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Daba *et al.* (1991).

Results and discussion

Isolation and identification of bacteriocin-producing organism

The initial screening indicated that three strains of *S. warneri*, designated as FM10, FM20, and FM30 produced bacteriocin (warnerin), which was found to be effective against a wide range of indicator strains (Table 1). Microscopic and biochemical examinations revealed that the cells were Gram-positive, spherical, non motile, mostly present in clusters and all were catalase-positive and coagulase-negative (Table 2). Earlier reports by Tagg *et al*. (1976); Sanni *et al*. (1999) and Kurniasih & Ray (2000) have shown that some bacteriocins produced by Gram-positive bacteria have a broad spectrum of antibacterial activity.

Effect of medium components and culture conditions on bacteriocin production

The effect of culture medium components on the production of warnerin by *S. warneri* isolates was performed using *Micrococcus luteus* (MTCC 1541) as an indicator organism. Among the three tested isolates, FM20 showed better activity. Higher amount of warnerin was synthesized when the medium supplemented with 1% glucose, 1% urea, and with 0.5% Tween 80 (Tables 3, 4 and Figures 1–3). On the other hand, the supplementation with other sugar and nitrogen sources had less effect on bacteriocin production. These results inferred that the supplementation of culture media and the concentration of constituents had altered the bacteriocin production. Ogunbanwo *et al*. (2003) obtained similar results in the production of bacteriocin from *Lactobacillus brevis* OG1. They obtained optimum production of bacteriocin when the MRS medium was supplemented with 1% glucose, 0.5% Tween 80, 2–3% yeast extract and 1–2% NaCl. Sanni *et al*. (1999) reported that highest bacteriocin activity in the constituted MRS broth supplemented with 0.25% glucose and 0.5% peptone despite nil bacteriocin activity at 2% glucose and peptone level.

The effect of growth conditions such as incubation time, initial pH and medium temperature on the production of warnerin was also investigated (Figures 4–12). Bacteriocin activity was detected in the growth medium after 8 h of incubation at 37 °C. Maximum bacteriocin activity was recorded after 12 h of incubation with the attainment of maximum cell population and thereafter the activity declined dramatically. Piard

Table 2. Biochemical reaction profile of *Staphylococcus warneri* isolates.

Biochemical tests	*Staphylococcus warneri* isolates		
	FM10	FM20	FM30
Mannose[a]	–	–	–
Mannitol[a]	–	–	–
Trehalose[a]	+	+	+
Salicin[a]	–	–	–
β-Glucoronidase[a]	+	+	+
β-Glucosidase[a]	+	+	+
Phosphatase[a]	+	+	–
Urea	+	+	+
Arginine[a]	+	+	+
β-Galactosidase[a]	–	–	–
Lactose[a]	–	+	–
Nitrate reduction	–	–	–
Catalase	+	+	+
Coagulase	–	–	–

[a]Commercial API STAPH-IDENT Kit.

Table 3. Effect of carbon sources on the production of warnerin by *S. warneri* isolates.

Microbial strains	Carbon sources (1%)	Growth (600 nm)	Final pH	Activity of warnerin (AU/ml)
S. warneri FM10	Fructose	1.5	4.25	6400
	Mannitol	0.8	5.4	1200
	Xylose	0.65	6.2	–
	Raffinose	0.75	6.48	800
	Lactose	0.84	5.26	1800
	Maltose	1.6	4.15	3200
	Glucose	1.8	4.10	6800
S. warneri FM20	Fructose	1.6	4.7	6800
	Mannitol	0.7	6.2	1600
	Xylose	0.56	6.8	–
	Raffinose	0.72	5.8	1200
	Lactose	0.86	5.2	1800
	Maltose	1.8	4.25	10,000
	Glucose	1.9	3.95	12,800
S. warneri FM30	Fructose	1.3	4.5	6400
	Mannitol	0.75	5.8	800
	Xylose	0.62	6.5	–
	Raffinose	0.7	6.2	1400
	Lactose	0.82	5.4	1800
	Maltose	1.5	4.2	6800
	Glucose	1.7	4.0	10,000

Warnerin (bacteriocin) activity after 12 h incubation at 37 °C in the MRS was determined by well diffusion assay.

Table 4. Effect of nitrogen sources on the production of warnerin by *S. warneri* isolates.

Microbial strains	Nitrogen sources (1%)	Growth (600 nm)	Final pH	Activity of warnerin (AU/ml)
S.warneri FM10	Urea	1.4	5.25	1800
	Yeast extract	1.0	6.2	800
	Ammonium sulphate	1.2	5.7	1400
	Sodium nitrate	0.5	6.5	400
S. warneri FM20	Urea	1.7	4.25	6800
	Yeast extract	1.0	6.0	800
	Ammonium sulphate	1.4	5.46	1800
	Sodium nitrate	0.7	6.4	600
S. warneri FM30	Urea	1.2	5.85	1800
	Yeast extract	0.7	6.25	1200
	Ammonium sulphate	1.0	6.0	1600
	Sodium nitrate	0.4	6.55	600

Warnerin (bacteriocin) activity after 12 h incubation at 37 °C in the MRS was determined by well diffusion assay.

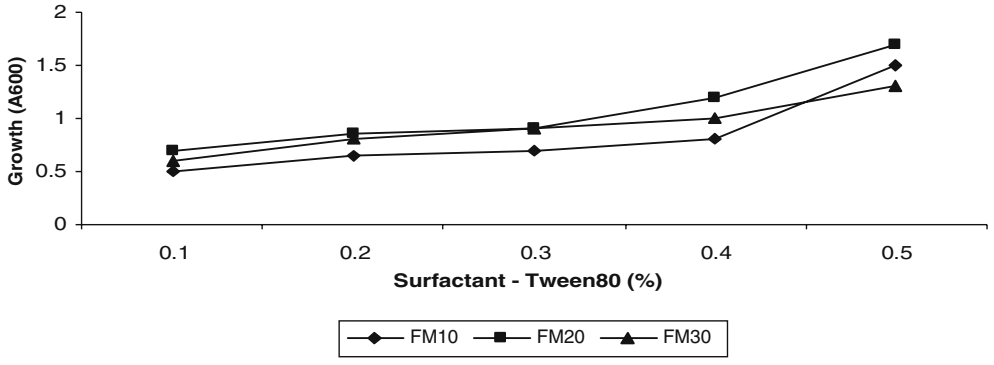

Figure 1. Growth (A_{600}) of *S. warneri* isolates (FM10, FM20 and FM30) during 12 h of incubation at 37 °C as a function of concentration of surfactant.

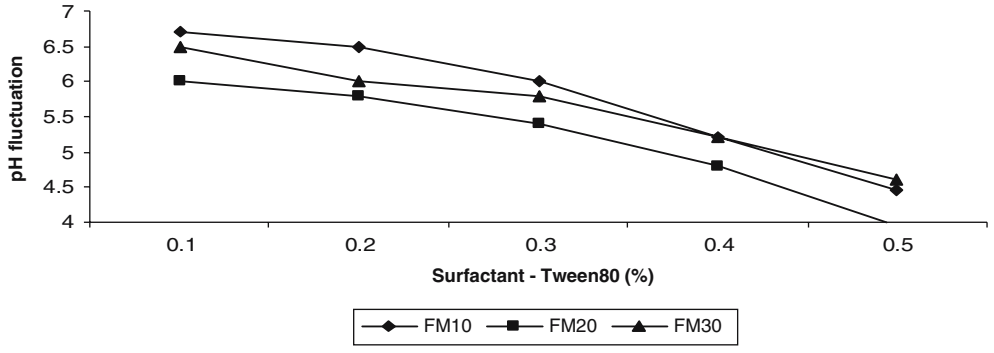

Figure 2. Fluctuation in medium pH of *S. warneri* isolates (FM10, FM20 and FM30) during 12 h of incubation at 37 °C as a function of concentration of surfactant.

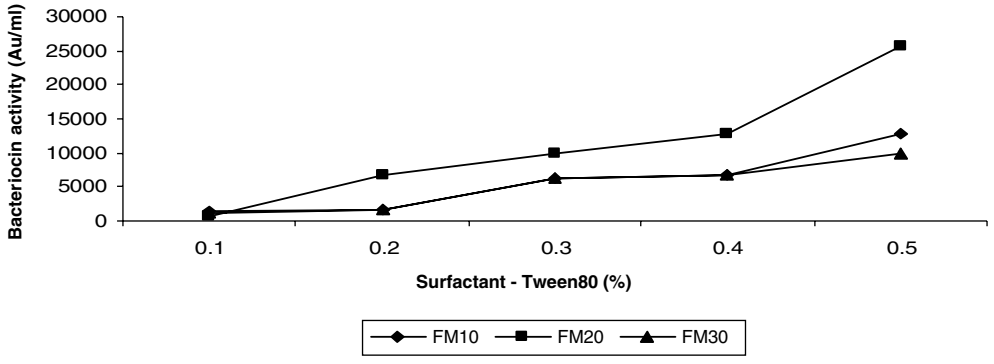

Figure 3. Bacteriocin activity (AU/ml) of *S. warneri* isolates (FM10, FM20 and FM30) during 12 h of incubation at 37 °C as a function of concentration of surfactant.

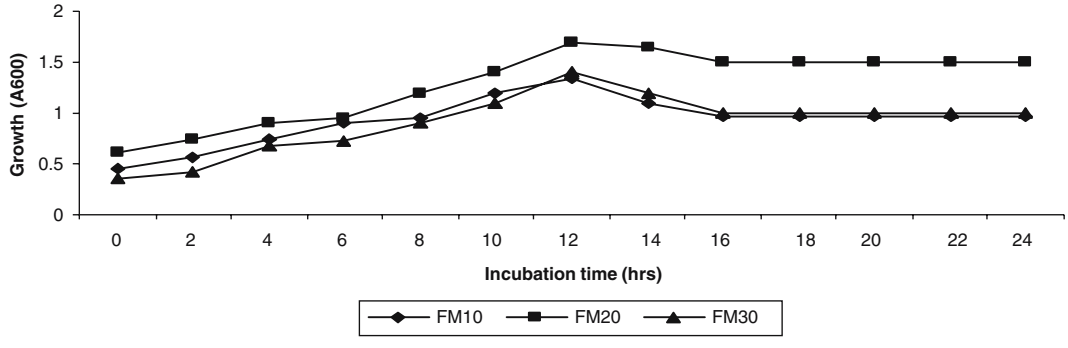

Figure 4. Growth (A_{600}) of *S. warneri* isolates (FM10, FM20 and FM30) in medium pH of 6.5 at 37 °C as a function of incubation time (h).

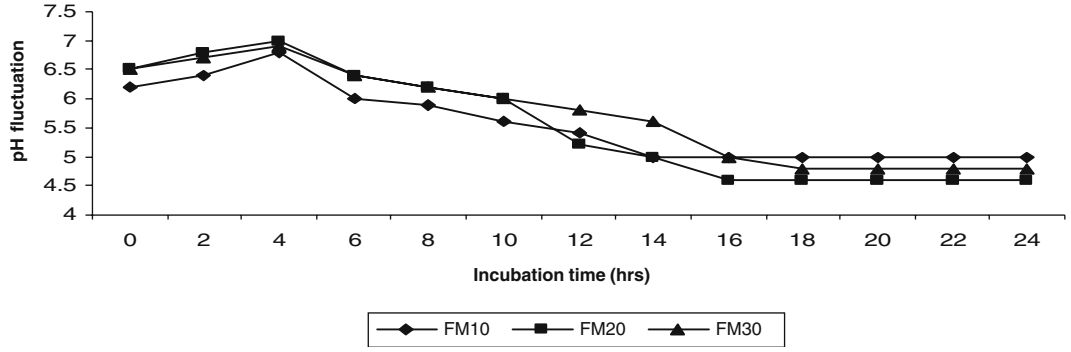

Figure 5. Fluctuation in medium pH of *S. warneri* isolates (FM10, FM20 and FM30) in medium pH of 6.5 at 37 °C as a function of incubation time (h).

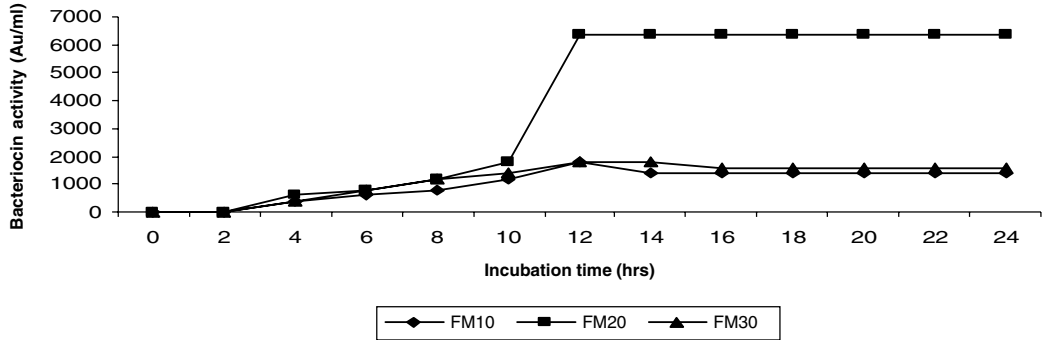

Figure 6. Bacteriocin activity (AU/ml) of *S. warneri* isolates (FM10, FM20 and FM30) in medium pH of 6.5 at 37 °C as a function of incubation time (h).

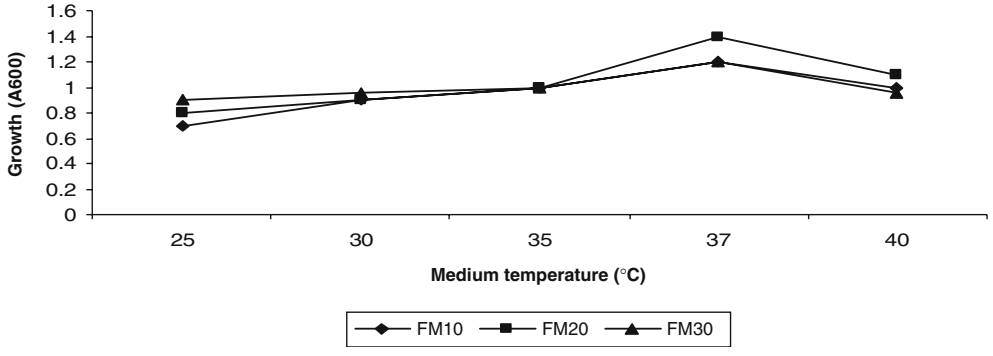

Figure 7. Growth (A_{600}) of *S. warneri* isolates (FM10, FM20 and FM30) during 12 h incubation at pH 6.5 as a function of medium temperature.

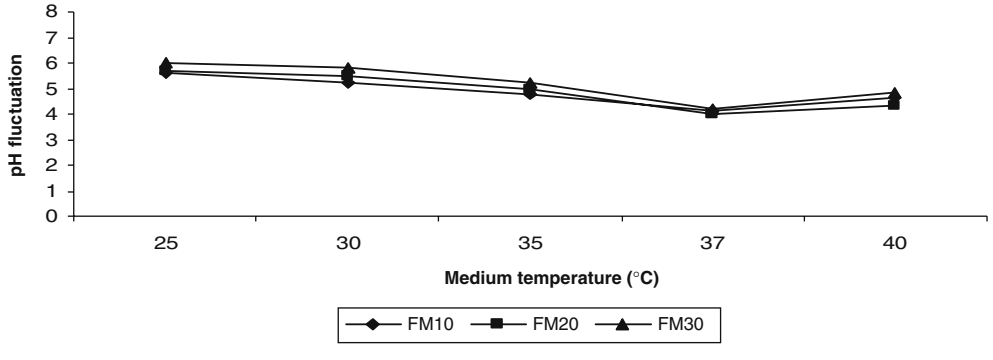

Figure 8. Fluctuation in medium pH of *S. warneri* isolates (FM10, FM20 and FM30) during 12 h of incubation at pH 6.5 as a function of medium temperature.

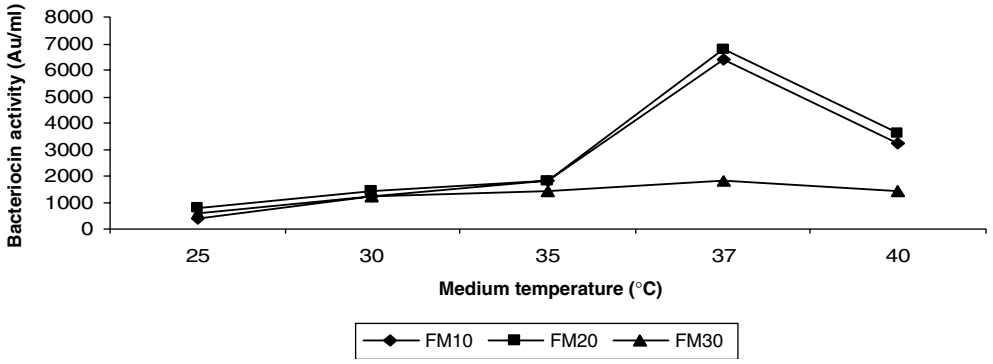

Figure 9. Bacteriocin activity (AU/ml) of *S. warneri* isolates (FM10, FM20 and FM30) during 12 h of incubation at pH 6.5 as a function of medium temperature.

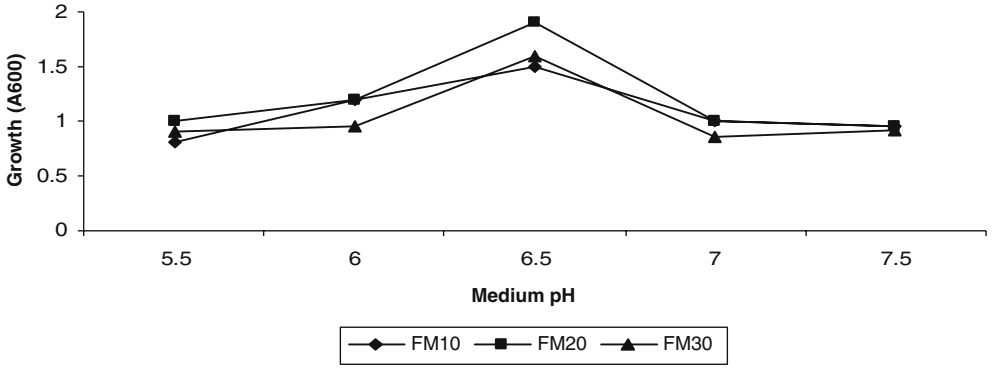

Figure 10. Growth (A_{600}) of *S. warneri* isolates (FM10, FM20 and FM30) during 12 h of incubation at 37 °C as a function of medium pH.

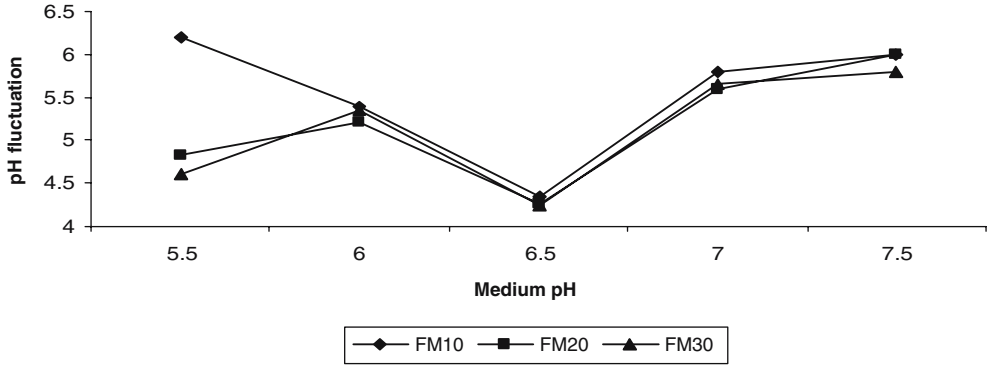

Figure 11. Fluctuation in medium pH of *S. warneri* isolates (FM10, FM20 and FM30) during 12 h of incubation at 37 °C as a function of medium pH.

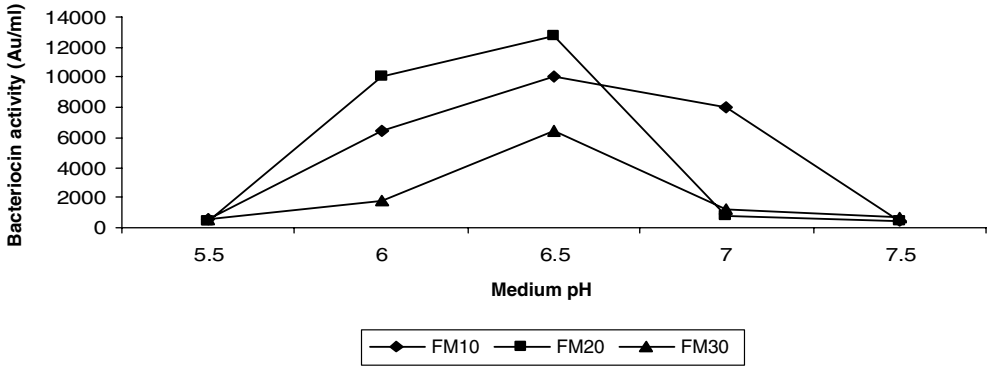

Figure 12. Bacteriocin activity (AU/ml) of *S. warneri* isolates (FM10, FM20 and FM30) during 12 h of incubation at 37 °C as a function of medium pH.

et al. (1990) reported that *Lactococcus lactis* produced bacteriocin not only during log phase but also throughout the growth phase. Kim *et al*. (2000) reported that the optimum production of bacteriocin was achieved at 37 °C with an initial pH of 7.0. In the present study, the bacteriocin activity was high in the initial medium pH of 6.5 and final pH of 4–5. Similarly, Ogunbanwo *et al*. (2003) reported that the highest antibacterial activity was exhibited in an acidic pH range of 2–6, while inactivation occurred at pH 8–12.

Characterization of bacteriocin

The proteinaceous nature of bacteriocin, warnerin was determined by treatment with various enzymes. The antibacterial activity of partially purified warnerin was not affected by the treatment of lysozyme. However, the activity was completely lost after treatment with pepsin suggesting a proteinaceous nature for the warnerin; while using α-chymotrypsin as enzyme source, it showed very less activity (Table 5). In accordance with these, Martirani *et al*. (2002) reported that the bacteriocin, bacillocin 490 was completely inactivated by pronase E and proteinase K. Chin *et al*. (2001) reported that the antibacterial activity was lost after treatment with proteases such as pronase E, α-chymotrypsin, trypsin, and pepsin. But lipase, catalase, α-amylase and lysozyme did not affect the antibacterial activity.

Antibacterial activity of bacteriocin

Table 6 shows the antibacterial effect of warnerin against selected Gram-positive and Gram-negative organisms. Bacteriocin synthesized by the test isolates exhibited higher antibacterial activity against *S. aureus*. This indicated that the bacteriocin is active against phylogenetically related species. This is consistent with the earlier reports of Martirani *et al*. (2002) for the bacillocin 490/5 produced by *Bacillus licheniformis*. Based on the inhibitory action, the strains such as *M. luteus*, *L. monocytogenes*, *K. pneumoniae*, *S. typhi*, and *V. cholerae* were sensitive to the strain FM20. But the bacteriocin produced by the other two strains, FM10 and FM30 had very low inhibitory action against these strains. The bacteriocins produced by the test isolates have no inhibitory action against *S. flexneri* and the species itself. Graciela *et al*. (1995) reported that bacteriocin from the producer organism had no inhibitory effect on the organism producing it and in a mixed fermentation environment production of bacteriocins may prove advantageous for a producer organism to dominate the microbial population.

Table 5. Effect of various enzyme treatments on the production of warnerin by *S. warneri* isolates.

Microbial strains	Enzyme source (mg/ml)	Growth (600 nm)	Final pH	Activity of warnerin (AU/ml)
S. warneri	Pepsin	0.4	6.5	–
FM10	α-Chymotrypsin	0.8	6.2	–
	Lysozyme	1.2	5.25	6400
S. warneri	Pepsin	0.6	6.0	–
FM20	α-Chymotrypsin	0.9	5.6	400
	Lysozyme	1.8	4.2	12,800
S. warneri	Pepsin	0.4	6.2	–
FM30	α-Chymotrypsin	0.7	5.95	200
	Lysozyme	1.4	4.46	6800

Warnerin (bacteriocin) activity after 12 h incubation at 37 °C in the MRS was determined by well diffusion assay.

Table 6. Antibacterial effectiveness of warnerin against several Gram-positive and Gram-negative bacteria.

Indicator stains	Activity of *S. warneri* isolates		
	FM10	FM20	FM30
Staphylococcus aureus, MTCC 902	+ +	+ + +	+ +
Micrococcus luteus, MTCC 1541	+	+ +	+
Listeria monocytogenes, MTCC 1143	+	+ +	+
Klebsiella pneumoniae, MTCC 2405	–	+ +	–
Salmonella typhi, MTCC 734	+	+ +	+
Shigella flexneri, MTCC 1457	–	–	–
Vibrio cholerae, MTCC 1738	–	+ +	–
Pseudomonas aeruginosa, MTCC 2905	–	+	–
Staphylococcus warneri, FM10	–	–	–
Staphylococcus warneri, FM20	–	–	–
Staphylococcus warneri, FM30	–	–	–

+ + + Strong suppression.
+ + Moderate suppression.
+ Weak suppression.
– No suppression.

Molecular mass determination

Among the test isolates, *S. warneri* FM20 produced relatively higher amount of warnerin. The molecular mass of warnerin seemed to be low, probably 2500 Da. Kim *et al*. (2000) reported that the molecular weight of the bacteriocin was estimated to be about 4200 Da. Nakamura *et al*. (1998) reported bacteriocin with 3500 Da from *Staphylococcus epidermis*. Kurniasih & Ray (2000) showed that the molecular mass of bacteriocin ranging from 6000 to 11,600 Da. The molecular mass of warnerin seemed to be low probably ranging from 3000 to 8500 Da. There was no distinctly visible band of this molecular mass in the stained gel and this may be attributed to the very low concentration of bacteriocin compared to other proteins precipitated from the cell-free supernatant. This is also holds good for the present study.

To conclude, the produced warnerin has a wide range of antibacterial spectrum against Gram-positive and Gram-negative pathogens. Among the three isolated strains of *S. warneri* (FM10, FM20, and FM30), FM20 isolates produced the highest amount of bacteriocin (warnerin 20), which exhibited bactericidal activity against several pathogens associated with skin infections and hence could be used as a therapeutic agent to combat these infections.

Acknowledgements

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