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Research paper

Potential use of bacteriocin producing lactic acid bacterial strain isolated from milk products and its application as the fish feed

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Abstract

Bacteriocins are gene-encoded inhibitory proteins and those produced by Gram-positive Lactic acid bacteria. Some bacteriocins even display antagonistic activity towards Gram-positive food borne pathogens and spoilage organisms. This present study involves isolation of Bacteriocin-producing lactic acid bacteria from a variety of milk and milk products. The physico-chemical properties of the isolated bacteriocin producing bacterial strains were screened. The isolated bacteriocin bacterial strains were biochemically characterized and identified. Further, the isolated effective bacterial strain was used as a fish feed and its effect on their growth was evaluated. The evaluated data continue to demonstrate that the bacteriocin producing bacterial strains will have greater potential in the food products industry.

Keywords: *Bacteriocin; Lactic acid Bacteria; Pathogens; Antagonism; Antimicrobial property.*

1. Introduction

Probiotics are live, non-pathogenic microorganisms, which when ingested reaches the gut to confer positive health benefits to the host as reported by De Vrese and Schrezenmeir [1]. The importance of colonic bacteria and the need to study probiotics has gained great attention in the last few decades in accordance with Brooks and Kalmokoff [2]. Vouloumanou et al. [3] reported beneficial effects of such Probiotics include prevention and reduced severity of respiratory infections as solved by, diminished and manageable symptoms of irritable bowel disease of the lower gastrointestinal tract as reported by Hungin et al.[4] and curing acute infectious diarrhoea [5].

Lactic acid bacteria produce a variety of substances with antimicrobial activity, including antimicrobial peptides collectively known as bacteriocin[6]. Bacteriocins are known for their low toxicity, high potency in situ production as reported by Cotter et al. [7] and for their greater specificity of in their antoagonistic activities aiding the producer in accordance to the study of Sahoo et al. [8]. Frequent use of antibiotics has led to the emergence of antibiotic resistance in bacteria. Lantibiotics are lanthionine-containing antibiotic peptides as studied by Sahl and Bierbaum [9]. Lantibiotic compounds are ribosomally synthesized antimicrobial peptides against which bacteria are not able to produce resistance, hence making them a good alternative to antibiotics according to the findings of Singh and Sareen [10]. Hence the present study was used for isolation of bacteriocin producing Lactic Acid Bacteria from milk and milk products. The physico-chemical properties of the bacteriocin producing bacterial strains were screened. Further, the effective isolated bacterial strain was prepared as a fish feed and its effect to enhance the growth of the fish was evaluated.

2. Materials and methods

2.1. Samples and enrichment of the bacterial strains

The samples were collected from both natural sources (milk, curd, etc) and artificial sources (Yoghurt, cheese and butter samples). Serial tenfold dilutions of the samples were made with sterile distilled water, of which 0.1ml of the sample was plated on to sterile de-Mann, Rogosa and Sharpe (MRS) agar from 10^{-5} to 10^{-7} dilutions. The medium used was autoclaved at 121°C, 15 psi pressure. The milk samples were serially diluted in peptonated saline solution and plated out onto MRS (deMan Rogasa Sharpe) agar plates, supplemented with 50mg/litre Natamycin to avoid yeast contamination. The plates were incubated at 37ºC for 48 hours. The pure colonies were maintained in the same agar as slants and supplemented with 15% glycerol.

2.2. Standard indicator bacterial strains

The following are the indicator organisms used for study were purchased from NCIM.
Bacillus cereus (NCIM 2106), *Staphylococcus aureus* (NCIM 2127), *Escherichia coli* (NCIM 2068), *Proteus mirabilis* (80CC 29906) and *Klebsiella pneumoniae* (NCIM 2883).

2.3. Antagonistic activity

Cultures incubated for 37°C at 24hr were swabbed on the MRS agar plates. Wells were created in the MRS agar plates and 50µl of test pathogenic organisms (*Escherichia coli, Staphylococcus aureus, Bacillus cereus, Klebsiella pneumoniae and Proteus mirabilis*) were added into the wells and incubated at 37^0 C for 24 hrs.

2.4. Production and purification of bacteriocin

Isolated Bacterial strains were propagated in 100 ml MRS broth for 72 hours at 37ºC. For extraction of bacteriocin, a cell free solution was obtained by centrifuging the culture at 10,000 rpm for 20 minutes at 4ºC. It was adjusted to pH 7.0 by means of 1M NaOH to rule out the antimicrobial effect of organic acid, followed by filtration of the supernatant. Ammonium sulphate competes with the protein present in the solution against the water gradient and gradually, precipitates the protein. The crude bacteriocin was treated with different concentration of ammonium sulphate; 10,20,30,40,50,60,70 and 80% respectively. After 3 hrs, the suspension was centrifuged at 10,000 rpm for 30 mins. The supernatant from each concentration was dialyzed against deionized water with four changes over 3 days and tested for antimicrobial activity with the method of Elizete et al. [11].

2.5. Agar well diffusion method

Pre-poured brain heart infusion (BHI) agar plates were overlaid with 3.0 ml BHI soft agar containing 0.1 ml (28 x 10^6) sensitive culture. Wells (5mm in diameter) were cut into these agar plates and 50 µl of the culture supernatants was placed into each well, and kept at 4^0 C for 10 to 12 h to allow the bacteriocins to diffuse into the agar. The plates were then incubated at 37^0 C for 24 h and zone of inhibition were measured in mm diameter. Preparation of cell free neutralized supernatant (CFNS): producer strain was grown overnight at 37 ^{0} C. Supernatant fluid was obtained by centrifuging at 10,000 g for min. pH of the supernatant was adjusted to 7.0 with 1NaOH (Merck, Darmstadt, Germany) (filter sterilized through a filter membrane of 0.22µm (Millipore corp., Bedford, Mass)). Cell free neutralized supernatant (CFNS) is widely used for final characterization of bacteriocin or bacteriocin-like inhibitory substances from indigenous lactic acid bacteria.

2.6. Physical characterization of bacteriocin

2. 6.1. Effect of pH and temperature

Bacteriocin preparations were adjusted to different pH levels between 1.0 to 10 with 1N NaOH or 10mM HCl (pH-meter: EDT Instruments, UK). Samples were maintained for 1h at 37^0 C. All the samples were then re-adjusted to neutral pH (pH 7.0) and assayed for activity by agar well diffusion assay using the method of Bhunia et al. [12].

The thermo stability of the bacteriocin preparations were determined by heating 2.0 ml of the preparations at 40^0 C, 60^0 C, 80^0 C and 100^0 C for 30 min. Samples were removed at intervals of 5 minutes, cooled and assayed for remaining activity as done by Rasool et al. [13].

2.7. Chemical characterization of bacteriocin

2.7.1. Effect of NaCl on the production of bacteriocin

A set of fresh tryptone soya broth (TSB: tryptone1.5%, soya peptone 1.0%) containing NaCl at a final concentrations of 0.5, 1.0, 2.5and 5% was inoculated with a fixed volume of inoculums of KS1 culture. Broth without NaCl solution was taken as control. The optical density and bacteriocins activity was determined after day 0, 1, 2, 3 and 4 as solved by Bhunia et al. [12].

2.7.2. Effect of organic solvents on the production of bacteriocin

Equal volumes of CFNS were mixed with different organic solvents like methanol, ethanol, formaldehyde, chloroform and isopropanol in the final concentration of 1.0% (pre-cooled at 4^0 C). All the organic solvents were obtained from Sigma except chloroform which was obtained from BDH. Samples were stirred and incubated at 37^0 C for 30 min and evaporated in a rotary evaporator (Rotavapor, Buchi-461, Sibata, Switzarland). Dried samples were dissolved in 50mM sodium phosphate buffer, pH 7.0 and assayed for antimicrobial activity using the method of Bhunia et al. [12].

2.7.3. Effect of surfactants and bile salt concentration

Bacteriocin preparations were treated with different detergents: triton X-100, sodium dodecyl sulphate (SDS), ethylene diamine tetra acetic acid (EDTA), tween 20 and tween 80 at a final concentration of 1.0%. Control consists of either bacteriocin preparation or detergent in 50mM sodium phosphate buffer, pH 7.0. All the samples and controls were incubated at 37^0 C for 6h and titers for bacteriocins activity were determined as done by Muriana and Klaenhammer [14].The zone of inhibition was measured in BHI agar plate and AU/mL was noted. The effect of bile salt concentration was determined in various bile salt concentrations (0.5% to 2%).

2.8. Critical dilution assay

A series of two-fold dilutions of crude bacteriocin preparation was made in the same medium (MRS broth) used for the growth of producer as well as indicator strain as done by Hardy [15]. Antagonistic activity of each dilution was assayed by dropping 10µl of each dilution onto the MRS agar plate pre-incubated with Bacillus cereus (as sensitive culture). The plates were incubated at 37^0 C for 18-24 h and zone of inhibition were measured in mm. The bacteriocin titre (inhibitory activity) was expressed as arbitrary units/ml. Arbitrary units (AU) is defined as 10µl of the highest dilution of crude preparation yielding a defined zone of inhibition on the lawn of cells of Bacillus cereus as solved by Pucci et al. [16], as was calculated as : reciprocal of the highest dilution giving definite zone of inhibition divided by the volume of CFNS dropped on the pre-poured sensitive culture plate (10µl) multiplied by 1000 for ml calculations according to the method of Bhunia et al. [17].

2.9. Minimal inhibitory concentration (MIC)

2.9.1. Tube dilution test

Two-fold dilutions of bacterial preparation culture isolates were prepared in 50mM sodium phosphate buffer (pH 7.0). A standard inoculum (2 x10^8cell) of the test organism (Bacillus cereus) was added and was incubated at 37^0C for 18 h. The lowest dilution of the bacteriocin preparation that prevents visible growth of sensitive culture was noted as the minimal inhibitory concentration (MIC).

2.10. Detection of the bacteriocin activity

Bacteriocin activity of the cell free- supernatant of KS1 was checked by spot-on-the lawn method. Activity was measured as the reciprocal of the highest two-fold dilution showing antimicrobial activity. According to the study, 1/8 was the highest dilution showing antimicrobial activity against the indicator strain Bacillus cereus which was used in all measurements for bacteriocins activity.

Bacteriocin activity of cell-free supernatant of KS1 (crude extract) was calculated as:

8× 200 = 1600 AU/ml

AU/ml = Arbitrary Unit;

8 = reciprocal of the highest dilution (1/4 ;) 200 = 1000 µl / 5 µl (conversion factor)

An example to the titration of the bacteriocin activity of the fraction obtained by ammonium sulphate precipitation of the supernatant of KS1, the highest dilution which showed a clear zone on the

lawn was 1/8. According to the above formula, the corresponding bacteriocin activity was calculated as 8 × 200 = 1600 AU / ml.

2.11. Morphological identification of the isolated bacterial strains

The isolated LAB were first set to Gram's staining and motility test after which biochemical characterization such as the test for Catalase, Urease, IMViC, Indole production were investigated along with Methyl Red-Voges Proskauer (MRVP) Test, Citrate utilization test, Triple Sugar –Iron Agar Test. In order to test the antibiotic resistance 24h old MRS broth culture (taken from single colonies on MRS agar) was swabbed on Mueller Hinton agar and incubated at 37°C for 24 h. Resistance was assessed against gentamicin, trimoxazole, azithromycin, tetracycline and amoxicillin. Basal Nutrient agar supplemented with Sheep Blood (collected from the jugular vein in sterile 3.80% Trisodium citrate (1 mL citrate solution for 10 mL blood))was used to test the haemolytic activity of the isolated bacteria.10 mL of the blood was aseptically added to 90 mL of the base. It is mixed well and poured in the plate.

2.12. Influence of bacteriocin produced by KS1 on the fish feed

Commercial fish feed and puffed rice were coated with bacterial isolate and two fish (1 and 2) were fed separately in different container. Fish 1 was fed with uncoated commercial fish food and served as control. Fish 2 was fed with bacteriocin coated fish feed served as test.

2.12.1. Procedure of coating bacterial strain to fish foods

Bacterial isolates were grown in MRS broth in a shaking incubator at 37^0 C overnight. After incubation, the cells were harvested by centrifugation (2000 rpm), washed twice with PBS buffer and resuspended in the same buffer. The absorbance at 600 nm was adjusted to standardize the number of bacteriocin (10^5-10^6 CFU/mL). Probiotic diets were prepared with cells resuspended in 5mL of PBS to 10^6 CFU and mixed.

Fishes were fed twice everyday with the parameters of 5% body weight per day with 80% water change every day, during 15 days feeding trials and survival was estimated visually each morning and body weights were measured in every 5 days as done by Vijayabaskar and Somasundaram [18].

3. Results

Milk samples were used from both natural and artificial sources, out of which, four strains named KS1, KS2, KS3, KS4 isolated from artificial sources were able to produce bacteriocin. When the isolated four strains where compared for bacteriocin production KS1 was able to shown effective bacteriocin production. pH experiment results showed that there were no activity at pH 1 and 2, and bacteriocin activity increased from pH 3 to 5. The bacteriocin produced by the KS1 was stable till 10 min in every temperature used, but was labile at 100^0 C after 30 min. The maximum activity of bacteriocin was found at 80^0 C. Effect of NaCl played an important role on the bacteriocin activity, hence in this study the NaCl concentrations were studied from 0.5% to 25 %. It was noted that zone of inhibition (20 mm) was maximum in 2.5% of NaCl concentration on 2nd day. NaCl concentration of 0.5% and 1% reached its maximum zone (6 mm) of inhibition in 1st and 2nd day. 5% NaCl concentration reached maximum zone (6 mm) of inhibition in 1st day. In control, maximum zone (10 mm) was shown in 1st day. The maximum activity of bacteriocin was achieved of about 160 AU/mL with 2.5% NaCl. This was co-related with growth of KS1. To study the effect of organic solvents on CFNS of KS1 isolate by using B. cereus as indicator strain, different organic solvents were treated with CFNS. Formaldehyde and Chloroform showed zone of inhibition of 10 mm whereas Methanol showed 6 mm. In formaldehyde bacteriocin activity was about 80 AU/mL, whereas with Chloroform and Methanol as the organic solvents, bacteriocins showed maximum activity of about 160 AU/mL. Chloroform showed maximum zone (10 mm) of inhibition and arbitory unit (160 AU/mL) .Effect of surfactants on bacteriocin showed a varied activity. Tween 20 and SDS showed less zone of inhibition (10 mm) whereas EDTA had greater zone (26 mm). But all the surfactants showed the maximum activity up to 160 AU/mL. This proved that bacteriocin produced by the bacterial strain KS1 showed maximum activity with all the surfactants used in the study.

The KS1 isolate can survive in the presence of bile salt, but growth decreases with the increase of bile salt concentration. In MRS agar plate, production of bacteriocin studied by agar well diffusion method showed KS1 inhibits Escherichia coli (34 mm) more strongly than *Bacillus cereus* (8 mm). There was no inhibition against *Staphylococcus aureus, Proteus mirabilis and Klebsiella pneumoniae.*

Antimicrobial activity of cell free neutralized supernatant of all bacteriocin producing strains were checked against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Proteus mirabilis and Klebsiella pneumoniae*. The result showed that there is no inhibition against *Staphylococcus aureus, Proteus mirabilis and Klebsiella pneumoniae* while the antimicrobial activity was against Escherichia coli and Bacillus cereus. Maximum inhibition was shown against Bacillus cereus by CFNS of KS1, KS2, KS3 and KS4. KS1 showed the highest zone of inhibition (40 mm) followed by KS2 (20 mm), KS3 (18 mm) and KS4 (18 mm). Minimum inhibitory concentration of bacteriocin produced by KS1 showed up to 1/8 dilution of the bacteriocin against Bacillus cereus.

3.1. Identification of the bacterial strains

Table 6 summarizes the results of the biochemical tests of the isolated bacterial strains. The isolated strains KS1, KS2, KS3 and KS4 were rod like and showed small round white colonies. Morphological test shows that all the isolated bacterial strains were gram positive. By comparing Bergey's manual of Determinative Bacteriology it was found that all the isolates were Lactobacillus sp. as the TSI agar results showed only acid production for all the isolates.

3.2. Influence of bacteriocin produced on fish feed

It was noted that both fish 1 and 2 had increased in weight (5.2g to 5.71g and 5.3g to 5.83g). When compared to commercial and natural (puffed rice) fish feed, it was observed that fish 2 fed with bacteriocin produced by KS1 exhibited greater increase in weight while fish 1 fed with commercial food showed slight increase in weight. From this result, it is predicted that the bacterial isolate which could be non-toxic, could prevent fish pathogen by producing potential bacteriocin and other antimicrobial substances.

4. Discussion

Bacteriocins are bactericidal or bacteriostatic peptides that are mostly active against closely related to the producer. Among the lactic acid bacteria (LAB) a high diversity of bacteriocins are produced of which several have been patented for applications in food. They have considerable attention as food preservatives and as potential replacement of antibiotics according to the finding of Veera et al. [19].

Sumathi and Reetha [20] isolated probiotic strains of Lactobacillus spp. which were isolated from different commercially prepared milk products such as milk, curd, yogurt, cheese and butter. The spectrum of inhibitory activity of the bacteriocin producers against Gram positive and Gram negative pathogens were tested and observed that activity was restricted to Gram positive organisms, but Lactobacillus and Streptococcus were active against both Gram positive and Gram negative organisms. 22 isolates were observed

as the positive for bacteriocin production against food *pathogen L. monocytogenes.*

The present study was carried out to isolate bacteriocin producing bacterial strains from milk products such as milk, curd, butter, cheese and yogurt. The inhibitory activity of the isolated bacterial strains was checked against gram positive and gram negative pathogenic bacteria. Only four bacterial strains were able to produce "bacteriocin" KS1, KS2, KS3 and KS4 which were isolated from butter, cheese and yogurt and were observed as positive for *Bacillus cereus* and *Escherichia coli*. KS1 bacterial strain showed higher production of bacteriocin and as well as antibacterial activity (40 mm) against the pathogens.

Natthida et al. [21] checked for thermostability of bacteriocin producing bacterial isolates isolated from pickled Garcinia schomburgkiana Pierre. The crude bacteriocin was thermostable since its activity was still found at 60°c to 100°c and 60°C to 90°C for 15 and 30 min, respectively, while any activity did not appear at 100°C for 30 min. Thus, the crude bacteriocin was stable on heat treatment up to 80°C for 15 min.

In the present study, bacteriocin produced by KS1 was effective in a wide range of temperature for 10 min and activity was maximum upto 160 AU/mL at 80^0 C. There was no activity found by the bacteriocin produced by KS1 when the temperature was increased to 100^0 C for 30 min. This results correlates with the results of the above mentioned author.

Veera et al. [19] studied lactic acid bacteria isolated from curd samples and they checked for tolerance to bile salt and NaCl concentration. Both the two isolates were tolerated to bile salt. Among the two strains, one strain showed comparatively better tolerance at 1% and 2% concentrations for 8 h. The NaCl effect on growth of both the strains in the medium was studied. Both the organisms (0.815 and 0.926) were tolerated at 1% NaCl and lowered the growth (0.083 and 0.085) at 5%. In the present study KS1 tolerated to bile salt concentrations from 0.5 % to 2 % till 48 h of incubation. Bacteriocin activity was studied from 0.5% to 5% NaCl concentration and growth of 2.5% and 5% were noted to 1.692 and 0.825 respectively.

Aguilar et al. [22] showed antimicrobial bacteriocin produced in supplemented MRS broth showed a broad antimicrobial spectrum determined by well diffusion assay against all strains of *Listeria monocytogenes* and *Lactobacillus sakei* ATCC 53103, *Enterococcus faecium* CHUM, *L. delbrueckii* subsp.*lactis* ATCC 12315, L. acidophilus ATCC 43121. Inhibition zones obtained in this trial measured 2 mm to 5 mm. The present study showed the antibacterial activity produced by bacteriocin producing Lactobacillus sp. against Bacillus cereus by agar well diffusion method, it was upto a maximum zone of inhibition of 40 mm which was maximum compared to other literatures.

Musikasang et al. [23] checked antibiotic sensitivity of their isolated LABs against various antibiotics. To penicillin G, all LAB strains showed high susceptibility to erythromycin (MIC < 0.25 μg/ml) and tetracycline. All selected LAB strains showed a moderate resistance to chloramphenicol (MBC 8 μg/ml) and most of them exhibited high MBC values (64–128 μg/ml). In the current study, bacteriocin produced by KS1 was checked for antibiotic sensitivity. It was sensitive to Gentamicin, Co-trimoxazole, Azithromycin, Tetracycline, and resistant to Amoxicillin (6 mm).

Lacticacid bacteria contributes to the taste and texture of fermented products and inhibits food spoilage bacteria by producing growth-inhibiting substances and large amounts of lactic acid. As agents of fermentation, Lacticacid bacteria are involved in making yogurt, cheese, cultured butter, sour cream, sausage, cucumber pickles, olives and sauerkraut. *Lactobacillus* and *Bifidobacterium* spp. are prominent members of the commensal intestinal flora and are the commonly studied probiotics bacteria as shown by Mahsa et al. [24].

In the present study, *Lactobacillus* sp. (KS1) which was producing bacteriocin was very effective in using it for both commercial fish feed and natural fish feed increase in the weight of the fishes proved that such a bacterial strain was found to be non-toxic and could prevent other pathogenic bacteria affecting the fish growth. Hence isolation of such bacteriocin producing LAB could be of great use in food industry which especially helps in food industry in feed. Barbour et al. [24] studied the variable Characteristics of Bacteriocin-Producing *Streptococcus salivarius* strains (Lactic acid bacteria) isolated from Malaysian Subjects.

5. Conclusion

Bacteriocins are produced mostly a by lactic acid bacterium which play a major role as a probiotics and confers a great health benefits for host. In the present study we isolated bacteriocin producing bacterial strains (KS1, KS2, KS3 and KS4) from butter, cheese and yogurt studied their antimicrobial activity against human pathogenic organisms which caused gastrointestinal tract infections, including Bacillus cereus and Escherichia coli (opportunistic human pathogens). Heat, pH and bile salt stability of bacteriocin produced by KS1 strain shows its capability to survive in gastro intestinal tract of animals which can inhibit other opportunistic bacteria in the gastrointestinal tract. The research work also revealed non-toxic nature of the bacterial isolate which can be used as probiotic food to increase the height and weight of the fish. The antagonistic activity of the isolated bacterial strain could keep the health of the fish and protected them from pathogenic strains. This could be of great use to increase the breeding of fishes and helps to maintain the quality of the fishes in the fish industry.

Acknowledgement

We express my sincere thanks to Department of Biotechnology and Management of Stella Maris College for providing all the facilities for successful completion of the project.

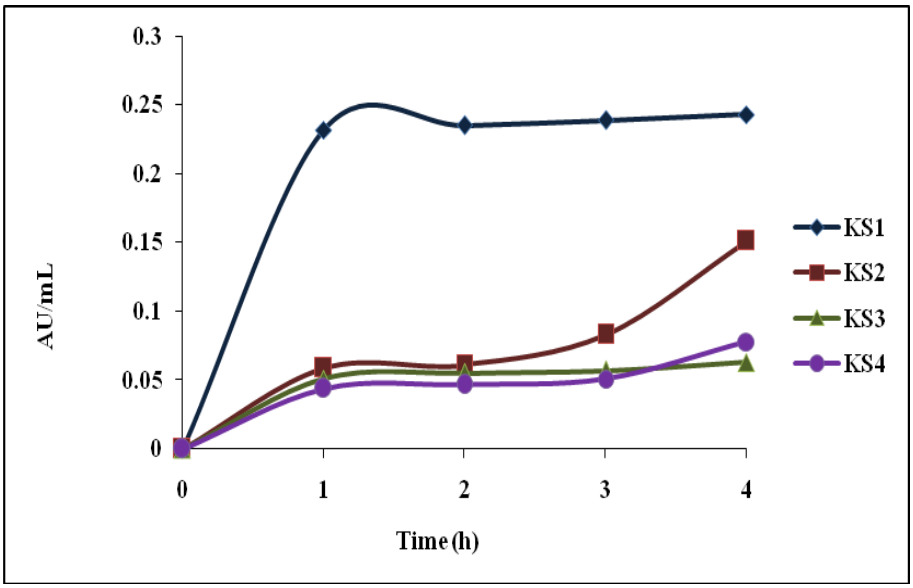

Fig. 1: Growth Pattern and Bacteriocin Production by the Isolated Bacterial Strains.

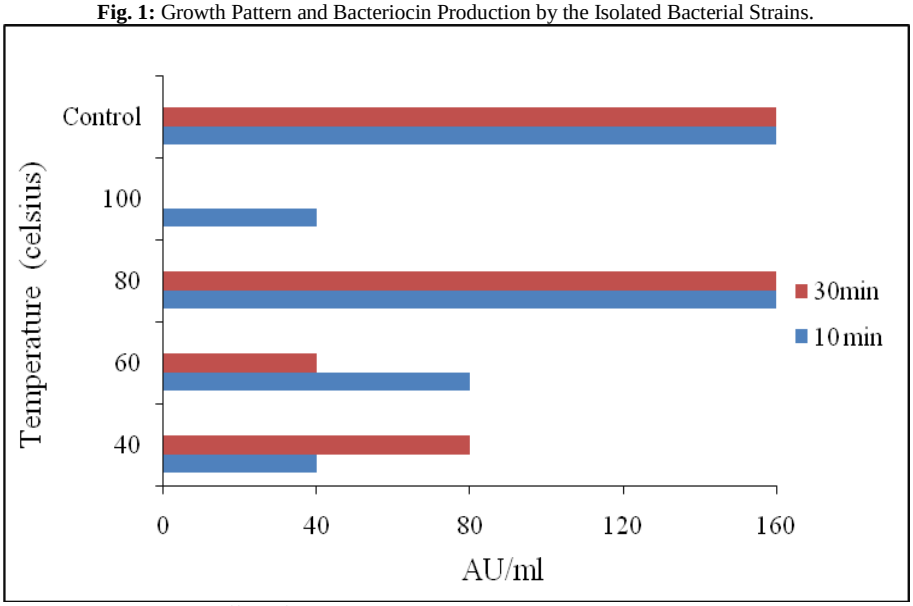

Fig. 2: Effect of Temperature on Bacteriocin production by KS1 Strain.

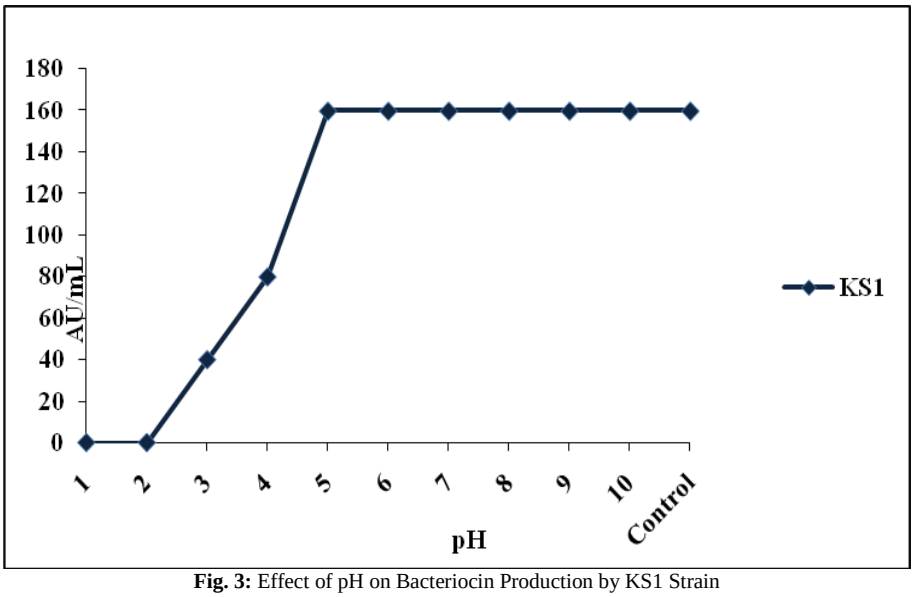

Fig. 3: Effect of pH on Bacteriocin Production by KS1 Strain

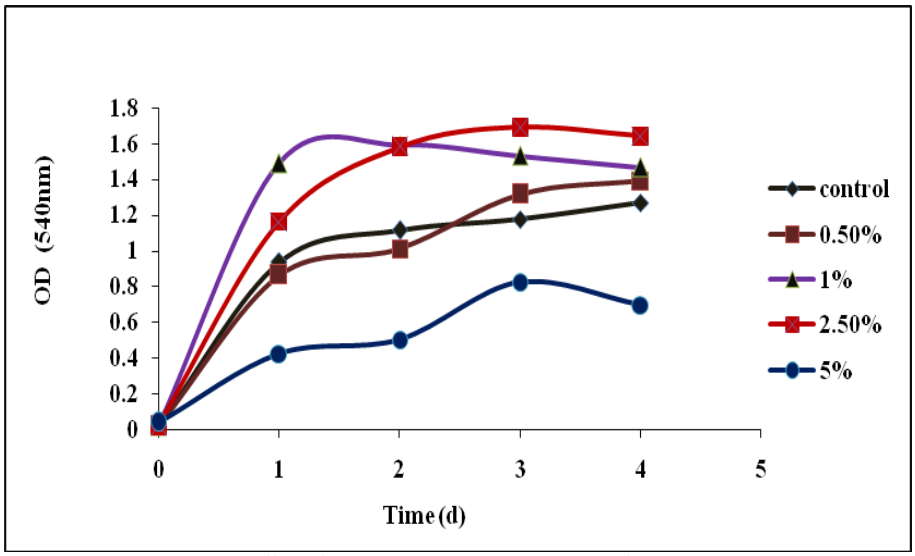

Fig. 4: Effect of NaCl Concentrations on Growth of KS1 Strain

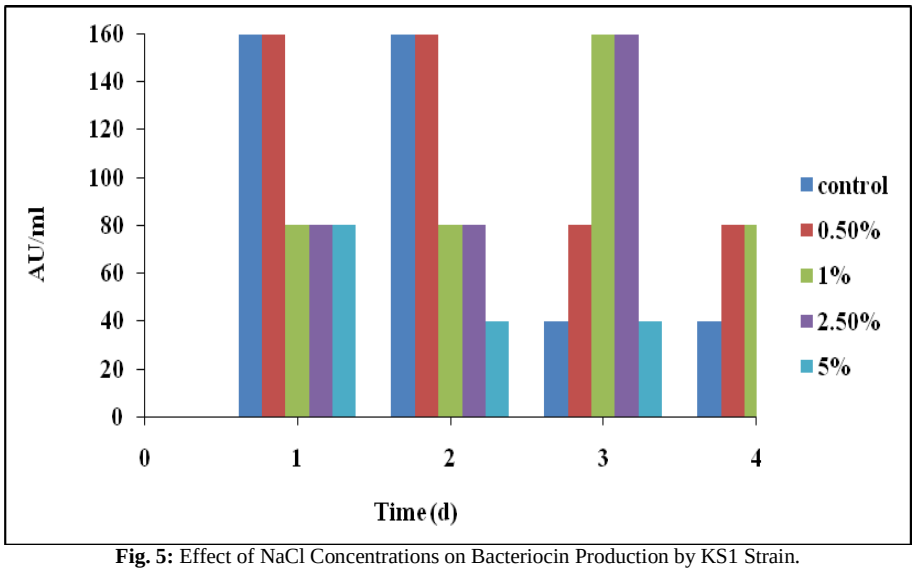

Fig. 5: Effect of NaCl Concentrations on Bacteriocin Production by KS1 Strain.

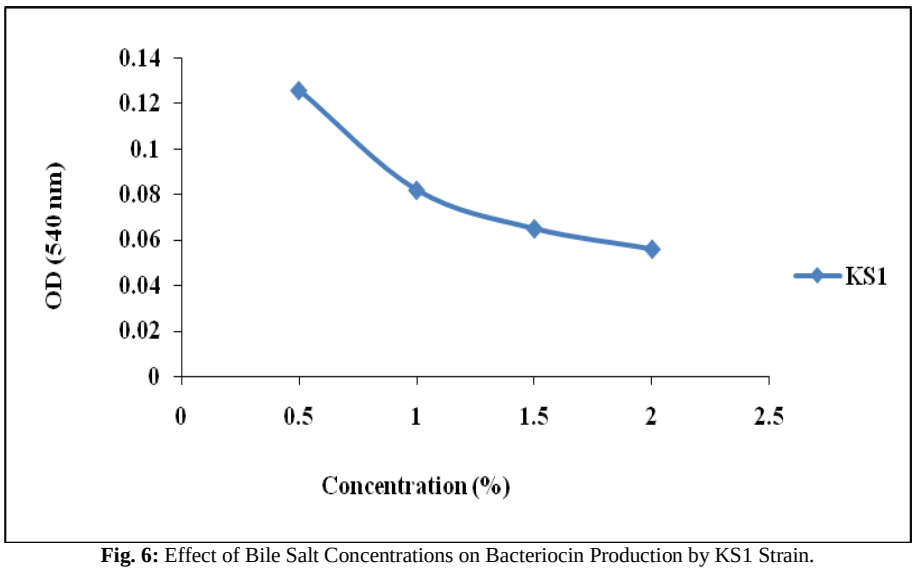

Fig. 6: Effect of Bile Salt Concentrations on Bacteriocin Production by KS1 Strain.

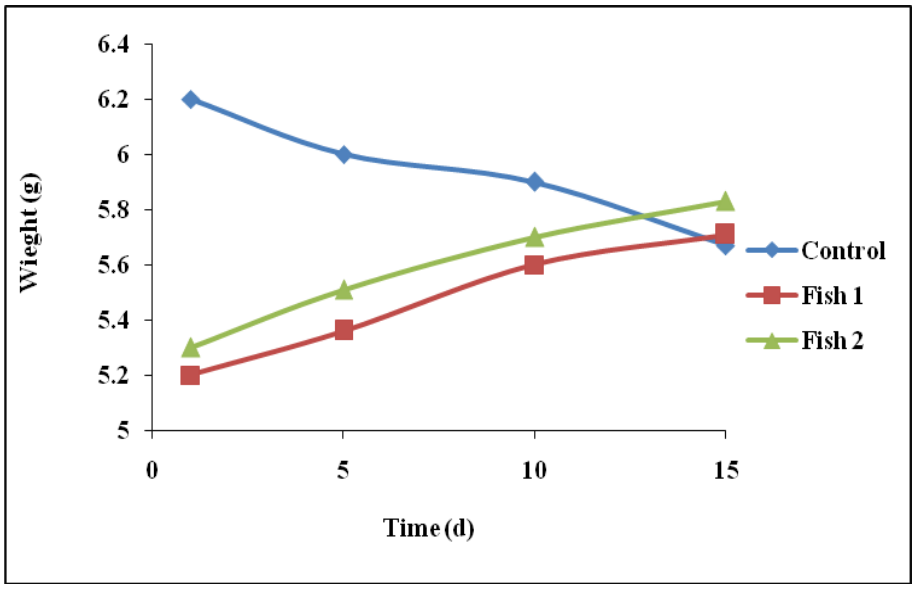

Fig. 7: Influence of Bacteriocin on Fish Feed (Fish 1-Commercial Food and Fish 2- Puffed Rice.

Table 1: Effect of Temperature on Bacteriocin Production by KS1 Strain

Temperature (^{0}C)	Activity in AU/mL	
	10 min	30 min
40	40	80
60	80	40
80	160	160
100	80	0
Control	160	

Table 2: Effect of pH on Bacteriocin Activity in AU/M

pH	Zone of inhibition in diameter (mm)	Activity in AU/mL
1	0	0
2	0	0
3	10	40
4	4	80
5	36	160
6	4	160
7	28	160
8	2	160
9	8	160
10	20	160
Control	40	160

Table 3: Effect of NaCl Concentrations on Bacteriocin Production by KS1

NaCl	Zone of inhibition in diameter (mm)			
Concentration	D-1	D-2	D-3	D-4
Control	10	4	2	2
0.5%	6	6	2	2
1%	6	6	2	2
2.5%	6	20	2	2
5%	6	4	2	2

Table 4: Effect of Organic Solvents on Bacteriocin Activity Produced by KS1

Organic solvents	Zone of inhibition in diameter (mm)	AU/mL
Formaldehyde	10	80
Chloroform	10	160
Methanol	6	160
Control	10	160

Table 5: Effect of Surfactant on Bacteriocin Activity

Surfactants	Zone of inhibition in diameter (mm)	AU/mL
Tween 20	10	160
EDTA	26	160
SDS	10	160

Table 6: Biochemical Tests for the Isolated Bacterial Strains

BIOCHEMICAL TEST	KS1	KS2	KS3	KS4
Morphology	Rod	Rod	Rod	Rod
Gram stain	+ve	+ve	+ve	+ve
Indole	–	–	–	–
Methyl red	+	+	–	+
Vogues	–	–	–	–
Citrate	+	+	+	+
Catalase	–	–	–	–
Urease	–	–	–	–
TSI	+	+	+	+
Milk agar	+	+	+	+
Tributyrin agar	+	+	+	+

Table 7: Antibiotic Sensitivity Test of KS1Strain

Antibiotic disc		Diameter (mm)	Inference
1	Gentamicin	16.0	Sensitive
2	Co-trimoxazole	30.0	Sensitive
3	Azithromycin	22.0	Sensitive
4	Tetracycline	22.0	Sensitive
5	Amoxicillin	6.0	Resistant

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