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Research Article

Evaluation of Streptocin SH3, a Bacteriocin produced by Streptococcus sanguinis isolated from Human Dental Plaque

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ABSTRACT

***Background*:** Bacteriocin is a peptidic toxin has many advantages to bacteria in their ecological niche and has strong antibacterial activity**.** Objective: The aim of this study was to evaluation of bacteriocin using *Streptococcus sanguinis* isolated from human dental caries**.**
***Subjects and Methods*:** Thirty five streptococcus isolates were diagnosed and tested for their production of bacteriocin, and then the optimal conditions for production of bacteriocin were determined. After that, the purification of bacteriocin was made partially by ammonium sulfate at 95% saturation levels, followed by and gel filtration chromatography using Sephadex G-50 column. Finally, physical characteristics were also studied and determined the bacteriocin stability.
Results: Among all streptococcal isolates, the *S. sanguinis* SH3 isolate with batter antimicrobial activity was selected, and used in further experiments. The optimum conditions for bacteriocin production were in Todd Hewitt broth (THB) supplemented with 1% glucose at 37°C for 24h under anaerobic conditions and inoculum size of (1.2×10^9CFU/ml) and pH 7, where the inhibition zone diameter reached to 29 mm against *Enterococcus faecalis*. The purified bacteriocin had high inhibitory activity against *S. pneumoniae, S. pyogenes*, *E. faecalis*, *E. faecium* and *Leuconostoc mesenteroides*. Streptocin SH3 keeps its effectiveness within the pH range 3-10, and temperature until 80ºC for 20 min.
Conclusion: Bacteriocin produced from *S. sanguinis* has high activity against many pathogens and has high stability over wide ranges of temperature and pH, which makes it a good alternative in the medical and food preservation fields.

Introduction

Bacteriocin is an antimicrobial peptidic toxin produced by Gram-positive, Gram-negative bacteria, and members of the archaea (1,2). Bacterial species produce these peptides as an ecological feature in their niches for self-protection and competitive advantage. Bacteriocin may has function as a killing peptide to eliminate or inhibit the competing species (3), and facilitate the dominance of a producer strain into the niche (4). Alternatively, bacterial strains may produce bacteriocins as a signal peptides for cell to cell communication within microbial communities (5). Bacteriocin exhibits significant potency against antibiotic resistant pathogenic bacteria, a major problem in the world due to excessive use of

antibiotics in humans and animals(6,7). As a result of the increase in antibiotic resistant strains, resistance to cephalosporins, a broad-spectrum antibiotic used in the management of infections caused by Pseudomonas areuginosa. Also, vancomycin resistance in enterococci, and methicillin resistance in Staphylococcus aureus that caused problems in hospitals. So, there is an urgent need to develop new antibiotics to eliminate multi-drug resistant pathogens (6). Like that, to control the misuse of antibiotics in food, bacteriocins are considered as a safe alternative due to their high therapeutic and nontoxic features. In food preservatives, bacteriocin has received exciting interest as a preservative and for control of spoilage and pathogenic bacteria (8). Moreover, bacteriocin has potential activities on human health, such as anticancer and antiviral agents, in recent years (9,10). Streptococcus sanguinis (S. sanguinis) plays an important role in promoting oral health, but may come to be pathogenic, including dental plague and endocarditis (11). It is lactic acid, gram-positive bacteria, nonspore-forming, nonmotile, catalase and oxidase negative, facultative anaerobic arrangement in chains or pairs of cocci (12). So a bacterium can produce bacteriocin, it requires suitable growth medium. Similarly, different factors affecting the production of bacteriocin are the type of bacterial species, fermentation conditions, and enough presence of nutrition (13). The production of bacteriocin is growth associated due to the fact that production takes place for the duration of the mid-exponential phase and will increase to attain a maximal level at the end of the exponential phase or the start of the early-stationary phase (14, 15). Specifically, many lactic acid bacteria (LAB) bacteriocins have been tested with high safety and tolerance to acids, heat, and bases (16). This study at pursuits optimization, purification, and characterization of bacteriocin produced with the aid of S. sanguinis and observes its effectiveness in opposition to pathogenic bacteria.

Subjects and Methods

Isolation of producer isolate and screening for bateriocin production

The producer isolates *S. sanguinis* SH3 was isolated from a patient with dental plaque and identified by morphological and biochemical tests, then confirmed by vitek 2 system. It was selected from among 35 isolate of streptococci (*S. mutans, S. parasanguinis, S. mitis, S. sobrinus*) isolated in this study for having the highest activity against indicator isolates.

The Agar Well Diffusion (AWD) method was used to evaluate the activity of the isolate according to (14) as follows: Tubes contained 10 ml of THB were inoculated with 1.2×10^9 CFU/ml of an overnight culture of the producer isolate. Then, tubes were incubated for 24 h and 37°C under CO_2 (5%) using candle jar . After incubation, the cultured broth was centrifuged at 6000 rpm for 15 min and the cell-free supernatant (CFS) was collected. Then, 0.1 ml of an overnight growth (24 h) culture of the indicator bacterium (1.5 X 10^8 CFU/ml)) was spread on the surface of MHA. In the plate, circular well 5mm in diameter was cut by using a cork borer after that 100 µl of CFS were put in wells, then plate was incubated for 18 h. After that, the activity was measured in mm.

Determination of the optimal conditions for bacteriocin production

To determine the medium and culture conditions that support the maximal production of bacteriocin, several optimization experiments were performed. Different culture media such as Todd Hewitt Broth (THB), Tryptic Soy Broth (TSB), Brain Heart Infusion Broth (BHIB), Nutrient Broth (NB), supplemented with 1% Glucose were inoculated with the isolate ($1.2 * 10^9$cells/ml) inoculum size. They were further incubated at 10, 15, 25,25, 30, 37, and 40°C for 24 h and to check the effect of culture medium as well as temperature on bacteriocin production. Also, to determine the effect of initial pH on bacteriocin production, the culture medium adjusted at different pH ranges from 4–10 and incubated at 37ºC for 24 h. Subsequently, the effect of the incubation period on the production of bacteriocin was also determined, where the isolate was incubated for 18 , 24, 48, 72 h at 37◦C. The effect of initial inoculum size on bacteriocin production also measured at different sizes (3.0×10^8, 6×10^8, 9×10^8, and 1.2×10^9). Finally, the effect of aeration on production of bacteriocin was determined and the producer isolate was cultured under aerobic, anaerobic (gas pack) and co_2 (5%) conditions. The activity was measured in mm in terms of inhibition zone diameter .

Production and purification of bacteriocin

According to (17), the producer isolate was grown in 750 ml THB supplemented with 1% Glucose and incubated at 37°C under anaerobic conditions, pH 7, and inoculum size $1.2 * 10^9$ CU/ ml. After 24h of incubation, it was centrifuged at 10,000 rpm for 15 min at 4◦C. After that, the supernatant was collected and the pH was adjusted to 6.5 with 1 M sodium hydroxide (NaOH), then, the antimicrobial activity was determined by agar well diffusion method. To precipitated bacteriocin, ammonium sulfate was added at 50% and 95% saturation levels to crude supernatant and held overnight at 4◦C then, centrifuged at 10,000 rpm at 4° C for 30 min. The precipitate was dissolved in phosphate buffer saline (0.1 M; pH 7). The remaining solution was assayed next level 95% saturation and also the precipitate was collected and dissolved in 5 ml of buffer and bacteriocin activity was measured at each saturation level against *E. faecalis* by AWD method.

After that, the precipitates were dialyzed overnight against the same buffer by using dialysis membrane (SIGMA) of molecular weight cut-off 1000 DA . The precipitate was further purified using Sephadex G-50 column (2x46 cm). Column was loaded with 15 ml of dialyzed partially purified samples. After the complete entry of the sample into the column, the peptides were eluted by using potassium buffer pH 7 with a flow rate of 1 ml/ min. 5ml for each fraction was collected, the absorbance of each fraction was read at 280 nm by spectrophotometer, and the plot was drawn between fraction number and its absorbance . All the collected fractions were examined for bacteriocin activity against *E. faecalis*. Active fractions were pooled, assayed for a specific activity, and protein concentration.

Measuring protein concentration

Protein concentrations of crude, precipitated and purified bacteriocin were determined according to Lowry (18).

Determined the activity of purified bacteriocin and activity unit

Activity of bacteriocin was measured by AWD **method** on MHA against *E. faecalis, S. pyogenes, S. enterica, S. pneumoniae, E. faecium, Staphylococcus aureus, and L. mesenteroides* as indicator strains. The activity was determined by measuring of inhibition zone diameter around the wells and activity unit was expressed as AU/ml according to (19) with the following equation:

$$AU = \frac{Inhibition\ area\ (mm^2) - Well\ area\ (mm^2)}{Volume\ sample\ (ml)}$$

Temperature and pH stability of streptocin SH3

To determine the effect of pH, and temperature, Streptocin SH3 was incubated at 40,60, 80, 100◦C for 20 min and at 121˚C using autoclave for 15 min, and then cooled to room temperature. To test pH stability, Streptocin SH3 was treated with either 1N HCI or 1N NaOH to obtain the desired pH between (2- 12) then, incubated for 30 min. After that, the aliquots were neutralized to pH 6.5 and the residual activity was determined by the AWD method against indicator isolates (20) .

Statistics analysis

Data analysis was carried out using SPSS-V.16 software. The mean, standard error, and significant differences between values were determined by ANOVA and Duncan test.

Results

Screening and optimal conditions for bacteriocin production

The cell-free supernatant of producer isolate was obtained from Todd Hewitt Broth (THB) inoculated with 1.2 * 10^9 cells/ml and incubated at 37˚C for 24h under 5% co_2 conditions give 18 mm inhibition zone diameter by agar well diffusion method against *E. faecalis* isolated from urine as in figure (1). The results showed that the production of bacteriocin is affected by the culture conditions. The activity of the bacteriocin disappeared under aerobic conditions and at a temperature between 10˚-25˚ C, also when using tryptone soya broth medium and pH of 8.5-10. At temperature 30, pH 5.5-7, incubation time 18-48h, inoculum size from 3×10^8 - 9×10^8, and using nutrient broth, and brain heart infusion broth, the activity ranged between 7.5 ± 0.5- 21 ± 0.52 mm but, the maximum activity was 29 mm obtained from culture under anaerobic condition at 37 ˚C for 24 h and pH 7 in THB supplemented with 1% glucose against *E. faecalis.*

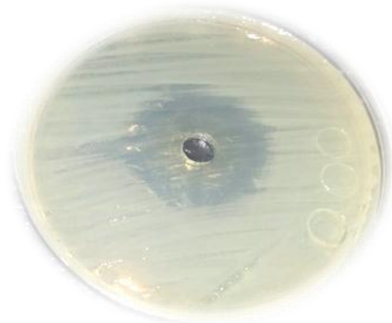


Figure 1: Activity of Cell- Free Supernatant of *S. sanguinis* SH3 against *Enterococcus faecalis*

Purification of streptocin SH3

The curve of purified bacteriocin was plotted between the absorbance and fraction numbers of protein eluted by gel as shown in figure (2). Each eluted 5ml fraction read at 280nm. When measuring the effectiveness of bacteriocin in each fraction , highest activity obtained from fraction 13. During the purification procedures, each step resulted in a considerable loss of protein concentration while the activity increased.

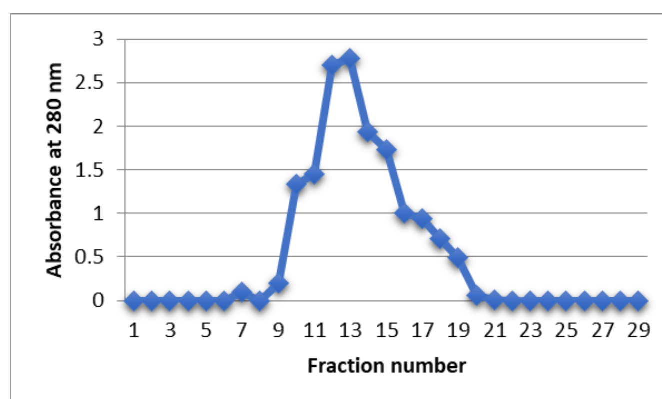


Figure. 2: Purification of streptocin SH3 by gel filtration chromatography using Sephadex G-50 column with dimensions (2x46) cm that equilibrated and eluted by 0.02 M of sodium buffer saline (pH7) and flow rate was 1ml/min with 5ml for each fraction.

Estimation of protein concentration

The protein concentration of crude, dialysis, and purified streptocin SH3was determined by Lowry method. There was gradually decrease in the protein concentration. In crude bacteriocin, the protein concentration was 10.461 mg/ml after dialysis, the concentration decreased to 6.201 mg/ml while after purification, the concentration reached 3.21 mg/ml. The activity after purification increased for streptocin SH3, the concentrations of streptocin SH3 shown in figure (3).

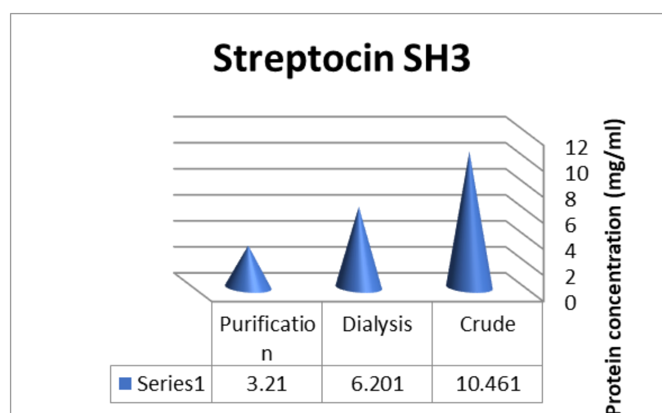


Figure 3: Protien concentration measured by Lowry method

Spectrum activity of streptocin SH3

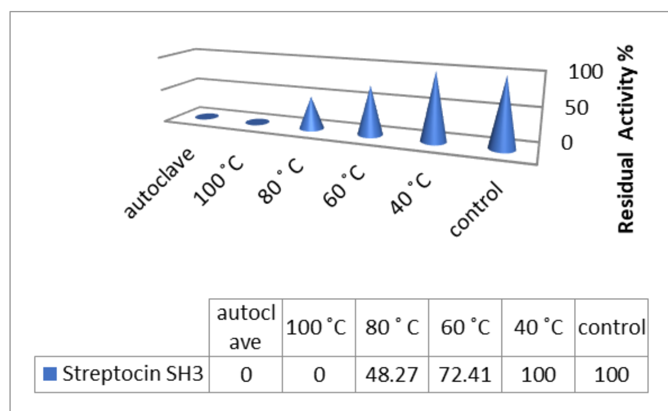
Streptocin SH3 was active only against some pathogenic closely related gram positive bacteria as in table (1).

Table 1: Spectrum activity of streptocin SH3 against some pathogenic bacteria.

Bacterial isolates	Inhibition zone (mm)	Activity Unit/ ml
S. aureus	0 ± 0.0	-
S.pyogenes	19 ± 0.53	3610
S. pneumoniae	15 ± 0.27	2250
E. faecalis	35 ± 0.17	12250
Salmonella enterica	0 ± 0.0	-
pseudomonas aeruginosa	0 ± 0.0	-
E. faecium	39± 0.12	15210
Leuconostoc mesenteroides	9 ± 0.07	810

Thermal stability of streptocin SH3

The streptocin SH3 was remained active until 80˚C for 20 minutes , but the activity lost after exposed to 100˚ C and autoclaved as in figure (4).

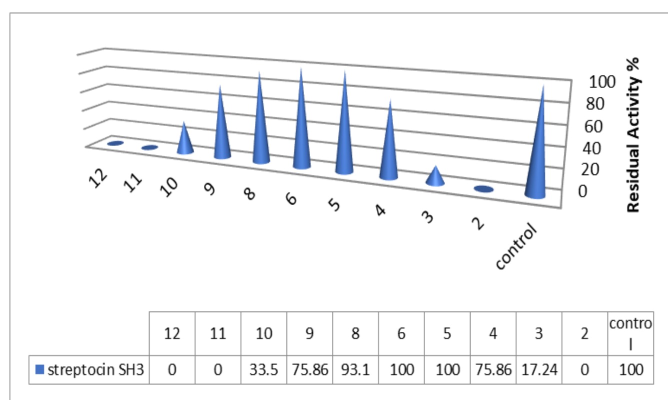


	autoclave	100 ˚C	80 ˚ C	60 ˚C	40 ˚C	control
Streptocin SH3	0	0	48.27	72.41	100	100

Figure. 4: Thermal stability of streptocin SH3

3.7 pH stability

The bacteriocin activity of the crude streptocin SH3 at different pH levels is shown in figure (5). streptocin SH3 was active in a wide pH range (4-8) , at pH 3, 9, and 10 the bacteriocin activity decreased, and did not inhibit the indicator bacteria and completely lost at pH 2, 11, and 12.



	12	11	10	9	8	6	5	4	3	2	control
streptocin SH3	0	0	33.5	75.86	93.1	100	100	75.86	17.24	0	100

Figure.5: pH stability of streptocin SH3

Discussion

The use of bacteriocin to eliminate of disease-associated bacteria as an alternative to antibiotics is gain more attention. In this study, *S. sanguinis* SH3 produced bacteriocin with high activity against gram positive bacteria. Bacteriocin was produced in large quantities under anaerobic conditions more than aerobic and this result may due to increase of the bacterial growth rate as observed. As mentioned by (21) that the anaerobic growth condition is favored in *S. intermedius* with a 24 % faster growth rate than aerobic, and associated with the up-regulation of the central carbon metabolism also, its adaptation to anaerobic conditions and accelerated growth allows *S. intermedius* to multiply in complex polymicrobial environments , both as a pathogen and a commensal. The current results also agree with obtained by other researchs (22,23) who found that always anaerobic conditions favor bacteriocin production by *streptococcus* spp. include Mutacin by *S. mutans* and Bovicin HC5*S by S. bovis* HC5 in simple media, respectively. Also the present results showed that the neutral medium was the best for bacteriocin production and this agree with the results of other studies (24, 25) on optimize of bacteriocin of *E. faecalis*. In general, if the density of bacteria is bigger in the lag and exponential phases of growth, the bacteriocin concentration will be higher, and in this point is agree with (26, 27). The present results show an increase in bacteriocin production when added glucose to the culture medium and this agree with (28) who found the biomass amounts increased the initial glucose concentration was between (0.5–2%) during fermentation of *E. faecium* DPC1146. For optimization of bacteriocin by *S. sanguinis* in this study using culture media TSB with 2% yeast extract, which appear the best compared with THB medium and this results coincide with (29) who used TSB with 2% yeast extract for bacteriocin production by *S. salivarius*. The bacteriocin production increased with increasing bacterial growth rate to reach the highest production at incubation time 24h. After that, the activity decreased. These results agree with (30) who found that the high activity of bacteriocin from by *E. faecalis* 478 was obtained after 24h. This suggested that bacteriocin production depending on the cell density. Similar results have been obtained in some bacteriocins of Lactic acid bacteria, for example, bacteriocin FGC-12 (31). At the end of stationary phase, due to decrease in the number of viable cells and pH, the bacteriocin activity decreased (7). Also decreasing of activity after prolonged incubation of the producer strain has been reported to occur as a result of extracellular proteases, protein aggregation or readsorption to the producer cell surface (32).

The reduction in the bacteriocin production at high and low temperatures referred to slow growth of producer bacteria. This result agrees with (33) who study the effect of temperature on bacteriocin production by *E. faecium* and show that the bacteriocin production reduced at (4, 10 , 15, and 30) ˚C compared to the control incubated at 37˚C. Some of bacteriocins produced by streptococci and enterococci purified from culture incubated at 37˚C such as enterococcin produced by *E. faecalis* CG-9 (34) and streptocin STH_1 produced by *S. sanguis* (35).

Some of bacteriocin from *Streptococcus* precipitate by ammonium sulfate such as sanguicin produced from *S. sanguinis* isolated from dental caries and the highest activity of sanguicin was at a saturation level 70% of ammonium sulfate (36).

The streptocin SH3 active against gram positive bacteria and this agree with the results of many researchers on the spectrum activity of some lactic acid bacteria such as the bacteriocin produced from *E. faecium* (37, 38), and benterocin Gr17 produced by *E. faecalis* (39).

The Streptocin SH3 was remained active until exposed to 80˚ C. There were some different results obtained by (36) where the activity of sanguicin produced by *S. sanguinis* was lost after heating at 60˚C for 10 min and by Skilton (40) , whose showed that the streptoccin san-K11 produced by *S. sanguinis* was heat stable until at 100˚C for 10 minutes, but in current study, streptocin SH3 was active at 60 ˚ C and lost its activity after 100 ˚ C , this differences may due to the difference in the period of exposure of the bacteriocin to heat, as in the previous studies it was 10 min, but in the current study 20 min.

The effect of pH on bacteriocin production was studied, some of bacteriocins lost their activity at pH 2, 11, and 12 such as bacteriocin produced from *E. faecium* KY11240 lost their activity at pH 2 and 10 but the activity in pH range 5 to 7 was stable and decreased at pH 3, 4, and 9 after 2h of treated (37), and bacteriocin produced from *S. sanguis* (41) where the bacteriocin was stable at pH range (3-10).

Conclusion

The mouth is a reservoir for many bacteriocin-producing bacteria; including Streptococcus spp. *S. sanguinis* SH3 produces streptocin SH3 in greater quantities under anaerobic conditions, which raises a broad topic for physiological study about the relationship between growth conditions and bacteriocin production. The produced bacteriocin is highly stable within wide temperature and pH ranges , which can be used to eliminate bacterial resistance to antibiotics and in the field of food preservation.

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Conflict of Interest

No conflict of interest

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