ISSN: 0067-2904

Bacteriocin from *Streptococcus salivarius* optimized statistically by response surface methodology active against different clinical oral pathogenic Streptococci

Bayan Shakir Mahmood AL-Saeedi, Khalid Jaber Kadhum Luti*
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq.

Abstract

The aim of the present work was to study the possibility of producing an effective bacteriocin from bacteria naturally exists in the mouth. *Streptococcus salivarius* KA101 was selected among 120 oral isolates collected from healthy people based on its ability to produce an effective bactericidal bacteriocin. Modified tryptic soy agar with 2% yeast extract and 0.1% calcium carbonate was the best medium for the production of bacteriocin with an activity of 40 AU/ml. Response surface methodology (RSM) based on central composite design (CCD) was employed to build a model in order to evaluate the optimum effective factors for bacteriocin production using four factors: yeast extract, $CaCO_3$, pH and incubation time. Based on the optimization plot generated by RSM, the suggested optimal concentrations of yeast extract and $CaCO_3$, as well as pH and incubation time for maximum bacteriocin production (80.2 AU/ml), were 13 g/l, 1.4 g/l, 7 and 24 hrs respectively. The results showed that bacteriocin was active for 30min at 20, 30 and 40˚C while 50% of its activity was lost at 50˚C. In addition, maximum bacteriocin activity was observed at pH 6 and 7 for 30min and disappeared completely at pH 4 and 9. Moreover, bacteriocin activity was completely lost after 90min of incubation with trypsin and pepsin which confirm its protein nature. Antimicrobial activity of bacteriocin was tested against 47 clinical isolates collected from patients suffering from different dental diseases. The results showed that the bacteriocin had an inhibitory activity against different isolates of *S. mitis*, *S. mutans*, *S. oralis* and *Enterococcus*.

Keywords: Bacteriocin, *Streptococcus salivarius*, RSM.

البكتريوسين من *Streptococcus salivarius* المحدد الظروف المثلى لانتاجه احصائيا بواسطه منهجية استجابة السطح فعال ضد عزلات مرضيه سريريه لبكتريا Streptococci

بيان شاكر محمود السعيدي، خالد جابر كاظم لوتي*
قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصة

ان الهدف من هذا البحث هو دراسه إمكانية إنتاج بكتيريوسين فعال من الفلورا الطبيعيه للفم حيث تم اختيار *Streptococcus salivarius* KA101 من بين 120 عزلة تم جمعها من اشخاص اصحاء على أساس قدرتها على انتاج بكتريوسين فعال. وجد ان وسط الانتاج الامثل للبكتريوسين هو وسط Tryptic soy agar المُحور باضافه خلاصه الخميرة بنسبه 2 % وكاربونات الكالسيوم بنسبه 0.1% حيث اظهرت العزله اكثر انتاج للبكتريوسين وبمقدار 40 وحده /مل . ولغرض زياده انتاجيه البكتريوسين تم استخدام برنامج منهجيه

*Email: Khalidluti@yahoo.com

استجابه السطح RSM حيث ارتفعت الانتاجيه الى 80 وحده/ مل وتم تعين الظروف المثلى لاعلى انتاج عند درجة حرارة مثلى 37ْم بفترة حضن 24 ساعه ورقم هيدروجيني 7 ونسبه اضافه 13غم /مل و 1.4غم /مل لكل من خلاصه الخميره وكاربونات الكالسيوم على التوالي . وبينت نتائج التوصيف احتفاظ البكتريوسين بفاعليته لمدة 30 دقيقة في درجه حرارة 20 و30 و40 ْم بينما فقدت 50% من فعاليته نتيجة التعرض لدرجة حرارة 50°م. بالاضافة الى ذلك فقد احتفظ البكتريوسين بفعاليته عند الرقم الهيدروجيني 6 و7 لمدة 30 دقيقه واختفت كليا عند الرقم الهيدروجيني 4 و9.كما وفقد نشاط البكتيريوسين تماما بعد 90 دقيقه من الحضانة مع انزيم التريسين والببسين وهذا ما يؤكد طبيعته البروتينية. تم اختبار فعاليه البكتريوسين على 47 عزله مرضية تم عزلها من اشخاص يعانون من امراض اللثة والاسنان واظهرت النتائج الفعالية التثبيطية ضد عزلات مختلفه من *S. mutans S. oralis, Enterococcus* and *S. mitis* .

Introduction

Genus *Streptococcus* is the dominant microflora commonly found in the oral bacterial community. Their ability to establish in the oral cavity by outcompeting other bacteria has been attributed to the production of bacteriocin. Among all the dominant streptococci of the oral cavity, *S. salivarius* is numerically the most significant colonist, especially on the tongue. It forms an important part of the normal flora of the human oral cavity and upper respiratory tract. It is considered as one of the first microorganisms to be established in the mouth of infants. *Streptococcus salivarius* is Gram-positive bacteria, a species of lactic acid bacteria family produce at least two kinds of antimicrobial peptides that generally make this bacterium associated with health by keeping the oral cavity healthy and protected [1]. It was reported that some strains of *S. salivarius* can produce many antibiotics like bacteriocins [2].These bacteriocins have been reported to inhibit some oral pathogens such as *Corynebacterium diphtheriae*, *Streptococcus pneumonia* and *Streptococcus pyogenes* [3].

Several methods were used to enhance and optimize bacteriocin production which already developed due to the potential importance of bacteriocins producing strains in replacement therapy [4]. Optimization of bacteriocin production and enhancement of its activity are important to reduce the production cost [5].

Response surface methodology (RSM) is a statistical method that involves individual and interaction effects of variables to improve production and to build models to evaluate the effects of factors for a desirable response. RSM was successfully used in many areas of biotechnology, including some recent studies on optimization of bacteriocin production [6]. The aim of the present work was to study the possibility of producing an effective bacteriocin from bacteria naturally exists in the mouth. In fact, the aim was basically based on exploiting the interspecies interactions among oral microflora in order to produce active bacteriocin against some oral pathogens.

Materials and Methods

Isolation of oral microflora

One hundred oral samples were collected from healthy individuals who had refrained from eating, drinking and oral hygiene for 2 hours before collection. The total bacterial isolates obtained were 120 isolates which were identified based on the morphological identification and cultural characteristics of each isolate (data not shown). Brain heart infusion medium (BHI) and Mitis salivarius agar (MSA) were used in the isolation under aerobic and anaerobic conditions respectively.

Screening of isolates for bacteriocin production

All isolates were subjected to the screening process in order to select the higher bacteriocin producing isolate that can be used for further experiments. Agar plug diffusion assay was carried out among the isolates to investigate the antagonism activity and competitive behavior against each other. The assay was performed for 120 isolates in 12 experiments; each one involved testing 10 isolates against each other. Briefly, Plugs of 0.5 cm in diameter was made with a sterile cork borer from each isolate grown overnight on BHI agar at 37ºC under Microaerophilic conditions. The plugs were then placed on the plate surface of Mullar-Hinton agar streaked with 200 µl containing approximately 1×10^8 cells/ml of an overnight growth culture of another oral isolate. Plates were then incubated overnight under the same conditions. Isolates showed antimicrobial activity were selected for secondary screening by well diffusion assay which was used to evaluate the production of bacteriocin of each isolate [7].

Determination of the Optimal Production Medium

Seven culture media were tested in order to select the one that can support the maximum production of bacteriocin. These media were varied in their contents of carbon and nitrogen sources included: Nutrient broth (NB), Brian heart infusion broth (BHI), Tryptic soya broth (TSB), modified (TSB), Todd Hewitt broth (THB), MRS Broth, modified MRS.

Optimization Using Response Surface Methodology (RSM)

Response surface methodology (RSM) based on central composite design (CCD) was used with four independent parameters to optimize bacteriocin production by the selected isolate. The four important factors, which affect the response (bacteriocin production), involved yeast extract, $CaCO_3$, pH and incubation time. The factors and their range (low and high level) uploaded to the RSM program is presented in Table-1. The RSM was set with one replication for each factorial, and five replication of the center point. The data were generated by using the design expert 7 Software. The total numbers of experiments were 29 runs each one represented the interaction between the independent variables in one flask.

Table 1-Names and range of factors used to optimize bacteriocin production by RSM

Factors		Units	Low	High
A	Yeast extract	g/l	10	30
B	$CaCO_3$	g/l	1	2
C	pH		6	8
D	Incubation time	hrs	20	30

Partial purification of bacteriocin

Partial purified of bacteriocin was prepared via precipitation with ammonium sulfate. A cell-free supernatant (CFS) of the production isolate KA101 was prepared from 300ml of an overnight growth culture by centrifugation at 8000 rpm for 30 minutes at 4°C. Precipitation was performed by adding solid ammonium sulfate to the CFS with different saturation levels (20, 30, 40, 50, 60, 70, 80) % (w/v). Each saturation level was dialyzed separately in phosphate buffer using dialysis membrane tube (3000 kDa MW cutoff). Finally, the antibacterial activity (AU/ml) of the dialyzed protein was determined by agar well diffusion method.

Characterization of bacteriocin

Stability of bacteriocin

Bacteriocin was exposed to different temperatures (30, 40, 50, 60, 70, 80, 90) °C for 10 min followed by cooling in an ice-bath. The residual activity was then determined by agar –well diffusion technique. In addition, bacteriocin was treated with either 0.1 N HCI or 0.1 N NaOH to obtain the desired pH values between 4 and 9. The pH adjusted crude solutions were then incubated for 30 min. Then aliquots from each crude solution were neutralized and activity was measured by agar well diffusion technique.

Pepsin and trypsin were used for testing the sensitivity of partially purified bacteriocin. 10 mg of each enzyme was dissolved in 1ml of potassium phosphate buffer (0.1 M and pH 7.0), then 1 ml of proteolytic enzyme was added to test tube containing 9 ml bacteriocin with activity 80AU/ml to obtain a final concentration of 1mg enzyme/ml. 10ml bacteriocin with activity 80AU/ml without adding proteolytic enzyme was used as a control. All tubes were incubated at 37 ^{o}C and bacteriocin activity was measured at (zero, 30, 90) min by agar well diffusion method.

Mode of bacteriocin action

The mode of action of bacteriocin was investigated as follow: One ml of partially purified bacteriocin was added to 10 ml of an overnight culture of the indicator isolate KA109. Then, the optical density was measured at 600 nm at zero time and after 10, 30, 60 and 120 min of incubation at 37^{o}C in order to check if there is any bacterial growth in the bacteriocin containing culture. Control was prepared by adding 1 ml of distilled water instead of bacteriocin to the indicator culture.

Analytical methods

Determination of bacteriocin activity

Bacteriocin activity was detected using the critical dilution assay [7]. Briefly, a twofold dilution series of free cells culture supernatant of the producing isolate *Streptococcus salivarius* KA101were

prepared and bacteriocin activity was determined in each dilution against *Lactococcus lactis* KA109 using agar well diffusion assay. The highest dilution creating a detectable inhibition zone (DF) reflected the strength of bacteriocin activity. The bacteriocin activity which is known as arbitrary unites (AU) was determined using the following equation:

$$\text{AU/ml} = \ 1/DF \ \ 1000/(volums\ spotted\ in\ \mu l)$$

Determination of protein concentration

Protein concentration was determined following the procedure described by Bradford [8].

Assessment of in vitro antibacterial activity of bacteriocin

The Spectrum activity of bacteriocin produced by *Streptococcus salivarius* KA101 was determined against clinical bacterial isolate collected from patients from different private dental clinics in Baghdad city. The total number of clinical samples was 47 involved 12 *S. mitis*, 15 *S. mutans*, 10 *S. oralis* and 4 isolates of *Enterococcus* which were taken from patients suffering from dental caries, plaque and periodontal diseases. All isolates obtained were streaked on the selective medium Mitis Salivaris Agar, which then incubated at 37°C for 48h under anaerobic conditions in a candle jar. The assessment was performed by two methods: well diffusion method and agar plug diffusion method [9] [10].

Results and Discussion

In order to colonize the oral cavity, microflora undergoes extensive inter-species interactions which confer survival advantages in the environment of the oral cavity. Since the oral environment is very competitive, it is speculated that bacterial species isolated from such environment may produce inhibitory substances against each other. In fact, the main goal of this work was to investigate the production of these compounds particularly bacteriocins. Therefore, all isolates were subjected to the screening process in order to discover and select the higher bacteriocin producing isolate that can be used for further experiments in this study. The screening process was performed among the isolates to investigate the competitive activities against each other and to determine whether isolates could inhibit the growth of common oral bacterial species. As mentioned earlier, the assay was performed for 120 isolates in 12 experiments; each one involved testing 10 isolates against each other. Agar plug diffusion method was used to examine the antagonism among the isolates. Table-2 shows the antagonism inhibitory effect among the isolates KA1 to KA10; the rest of results for other isolates are not shown in this paper to avoid lengthening. Results showed that out of 120 isolates, 32 were able to produce bacteriocins with different size of inhibition zones and therefore were selected for the secondary screening.

Table 2-Primary screening of isolates (KA1 to KA10) for bacteriocin production by agar plug diffusion method.

Isolates	KA 1	KA 2	KA 3	KA 4	KA 5	KA 6	KA 7	KA 8	KA 9	KA 10
KA1	-	-	-	-	-	-	-	-	-	-
KA2	-	-	-	-	-	-	-	-	-	-
KA3	-	-	-	-	-	-	+	-	-	-
KA4	-	-	-	-	-	-	-	-	-	-
KA5	-	-	-	-	-	-	-	-	-	-
KA6	-	++	-	-	-	-	-	-	-	-
KA7	-	-	-	-	-	-	-	-	-	-
KA8	-	-	-	-	-	-	-	-	-	-
KA9	-	-	-	-	-	-	-	-	-	-
KA10	-	-	-	-	-	-	-	-	-	-

Secondary screening was achieved by well diffusion assay against the same isolates that showed sensitivity. Based on results, the bacteriocin produced by the isolate KA101was active against three isolates; KA102, KA104, and KA109 with an activity of 10, 10 and 20AU/ml respectively. Accordingly, the isolate KA101 was selected as the most active isolate for bacteriocin production and thereafter, was chosen for further experiments in this study. In addition, isolate KA109 was chosen to be used as an indicator. The two isolates were Gram positive cocci arranged in pairs and short chains,

catalase negative and did not induce hemolysis (Gamma hemolysis). Furthermore, results revealed that KA101 and KA109 were optochin and bacitracin resistant by disc diffusion method. These isolates were subjected to analytic profile index (API 20strep) which revealed that the isolate KA101 was *Streptococcus salivarius* and the isolates KA109 was *Lactococcus lactis*.

The selected isolate *S. salivarius* KA101 was subjected to different optimization experiments to determine the optimum conditions for bacteriocin production. These experiments included culture media, concentration of yeast extract and $CaCO_3$, pH and incubation time. Some physical parameters that were not investigated in this study have been adjusted according to the literature such as temperature [11].

Maximum production of bacteriocin by *S. salivarius* KA101was observed in the modified tryptic soy broth (modified TSB) with an activity of approximately 40 AU/ml Figure-1. The findings of the current study are consistent with those of Barbour (2013) who found that TSB medium containing yeast extract and calcium carbonate produced high titers of bacteriocin by *S. salivarius*.

Specific medium supplements showed the different effect on the production of bacteriocins by Gram-positive bacteria. Some of these supplements enhanced bacteriocins production such as yeast extract [12] [13] and calcium carbonate [14] [15].

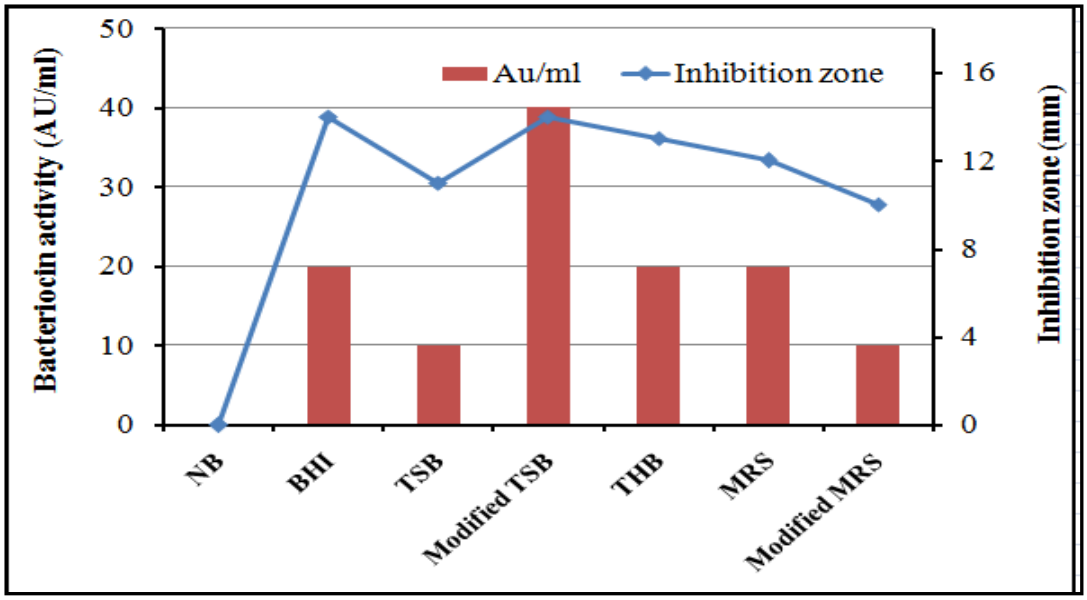

Figure 1-Bacteriocin production by *S. salivarius* KA101 in different media after 24hrs of incubation.

Response surface methodology (RSM) was employed to build a model in order to evaluate the optimum effective factors for bacteriocin production and study their interactions. Since it is an experimental strategy in which designed variables are varied together, instead of one factor at the time. The set of RSM based on central composite design (CCD) was achieved using design expert software. The statistical optimization set was included four factors: yeast extract, $CaCO_3$, pH and incubation time to obtain the highest response (bacteriocin concentration). The statistical optimization set was done with one replication for each factorial, and five replications of central point generating 29 runs for the prediction of response as shown in the Table-3 with the actual and predicted values of response.

Table 3-Experimental design and results of central composite design for bacteriocin production with actual and predicted value of response

Run	Factor 1	Factor 2	Factor 3	Factor 4	Bacteriocin AU/ml	
	yeast extract g/l	$CaCO_3$ g/l	pH	Incubation time Hr	Actual Response	Predicted Response
1	20	1	7	25	80	78.60
2	20	1	7	17.30	40	47.97
3	10	2.5	6	20	20	19.93926
4	10	1.5	8	30	40	36.24751
5	10	1	6	30	40	45.41418
6	30	2	7	25	20	21.31
7	30	2	7	35	10	7.91
8	30	1	8	20	40	41.69133
9	20	1	6	20	40	33.358
10	20	1	8	20	40	44.19133
11	10	1.5	6	20	20	22.43926
12	20	1.5	6	30	40	31.99544
13	40	1	8	30	20	27.82877
14	10	1.5	7	25	80	79.56
15	20	1.5	7	25	80	79.56
16	30	2	6	20	40	35.86
17	20	1.5	7	25	80	79.56
18	10	2	7	25	40	42.98
19	30	1.5	6	30	20	22.91
20	20	2	5	25	10	12.98
21	20	1	6	30	10	9.50
22	30	0.5	7	25	80	79.56
23	20	1.5	8	30	20	18.74751
24	30	2	8	20	40	33.27259
25	20	1.5	7	25	80	41.31
26	20	2	7	25	40	79.56
27	10	1	8	30	20	10.33
28	10	1	9	25	40	19.65
29	10	2.5	8	20	40	35.77

Experimental design and results of central composite design were analyzed by contour plot which describes the response over interactions among independent variables rang. The resulting graphics gave an excellent clarification for the effects of yeast extract, $CaCO_3$, pH and incubation time on bacteriocin production.

Contour plots in **F**igure-2A demonstrated the maximum bacteriocin concentration as a red area which illustrated the highest bacteriocin production 81.16 AU/ml with the combination of 13 g/l yeast extract and 1.4 g/l $CaCO_3$ when pH and incubation time hold on 7 and 24hrs respectively. Whereas maximum bacteriocin production reached to 80.585 AU/ml at a concentration of 13 g/l yeast extract with pH 7 when $CaCO_3$ and incubation time were hold at 1.4 g/l and 24hrs respectively Figure-2B. Moreover, the same trend was observed in the contour plot presented in Figure-2C which showed that

highest bacteriocin production at 80.585 AU /ml can be achieved where the concentration of yeast extract and incubation time were converging at 13 g/l and 24hrs when concentration of $CaCO_3$ was held at 1.4 g/l and pH at 7. In addition, the contour plot presented in Figure-2D revealed that maximum bacteriocin production of 80.7 AU/ml can be obtained at pH equal to 7 and the $CaCO_3$ at 1.4 g/l when the concentrations of yeast extract and incubation time were held at 13 g/l and 24hrs respectively. Contour plot in Figure-2E demonstrated that the highest production of bacteriocin 80.707 AU/ml can be attained with a combination of $CaCO_3$ at 1.4 g/l and 24hrs incubation time when yeast extract and pH hold at 13 g/l and 7 respectively. Finally, the result in figure, Figure-2F shows the corresponding contour and response surface plot for bacteriocin production with various pH values and different incubation time. The maximum bacteriocin production of 79.33 AU/ml can be achieved at pH 7 and 24hrs incubation time with yeast extract and $CaCO_3$ hold at 13 g/l and 1.4 g/l respectively.

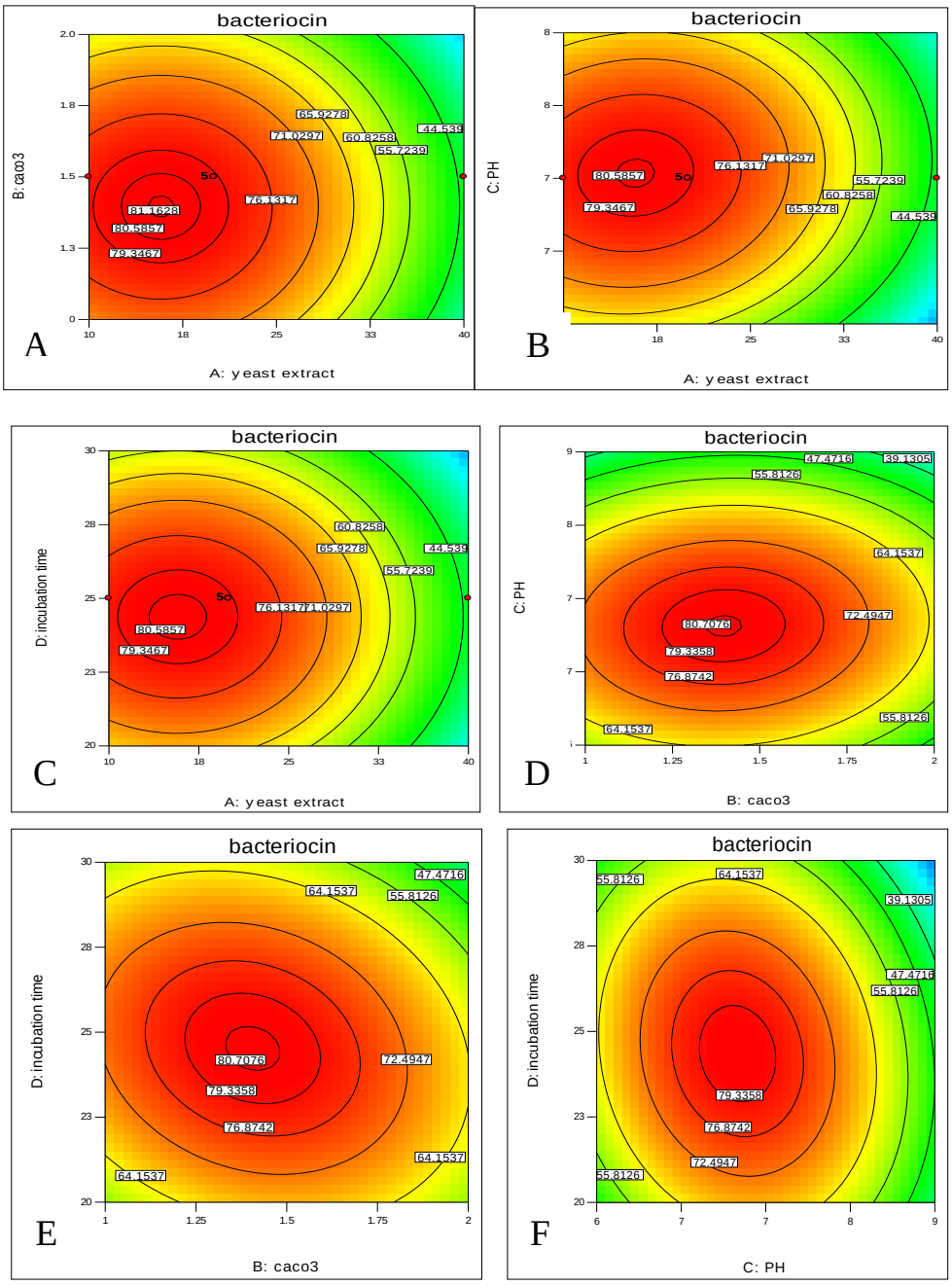

Figure 2-Effect of factors interactions on bacteriocin production by *S. salivarius* KA101. **A**: yeast extract and $CaCO_3$ concentrations, **B**: yeast extract and pH, **C**: yeast extract with incubation time, **D**: $CaCO_3$ with pH, **E**: $CaCO_3$ with incubation time, **F**: pH with incubation time.

Based on ANOVA analysis (Table- 4), all of the terms showed the significant effect on the response. ANOVA is an efficient method to compare the means of different statistical populations and to test the significance of a model [16]. The experimental data were analyzed by fitting to a second order polynomial model, which was statistically validated by performing analysis of variance equation obtained Full actual model on bacteriocin production.

Y = +79.56 -5.46 A -5.00 B +1.67 C -3.97 D +0.001 AB +1.25 AC +0.001 AD +1.25 BC -5.00 BD -3.75 CD -6.42 A2 -12.06 B2 - 15.81C2 -15.93D2

Where Y is bacteriocin activity AU/mL, A is yeast extract g/l, B is CaCO3 g/l, C is PH and D is incubation time hr.

The statistical significance of equation was checked by F-test and the analysis of variance (ANOVA) as given in Table-4. The F-value of 36.87 implies that the model is significant and the values of "Prob > F" which is less than 0.0500 also indicated that the model terms are significant. Furthermore, in this case A, B, C, BD, CD, A2, B2, C2, D2 are significant model terms. Values greater than 0.1000 indicate that the model terms are not significant since p-value is used as a tool to check the significance of each factor. The goodness of the model can be made by the determination coefficient (R2) and the correlation coefficient (R)[17]**.** The predicted R-Squared of 0.8428 is in reasonable agreement with the adjusted R-Squared of 0.9472. This result means that 94.72% of the total variation data of bacteriocin production can be described by the selected model.

Table 4-Analysis of variance (ANOVA) for the quadratic modal of bacteriocin production obtained from the experimental results

Source	Sum of Squares	Df	Mean of Square	f Value	p-value Prob > F
Model	15564.06	14	1111.718	36.8686	< 0.0001*
A-yeast extract	557.6264	1	557.6264	18.49291	0.0007
B-caco3	600	1	600	19.89817	0.0005
C-PH	66.66667	1	66.66667	2.210908	0.1592
D-incubation time	340.1878	1	340.1878	11.28186	0.0047
AB	0.1	1	0.1	0.01	1.0000
AC	25	1	25	0.82909	0.3779
AD	0.01	1	0.01	0.001	1.0000
BC	25	1	25	0.82909	0.3779
BD	400	1	400	13.26545	0.0027
CD	225	1	225	7.461813	0.0162
A^2	650.4749	1	650.4749	21.5721	0.0004
B^2	3934	1	3934	130.4657	< 0.0001
C^2	6760.526	1	6760.526	224.2035	< 0.0001
D^2	4844.797	1	4844.797	160.671	< 0.0001
Residual	422.1494	14	30.15353		
Lack of Fit	422.1494	10	42.21494		
Pure Error	0.0	4	0		
Cor Total	15986.21	28			

*Significant

The CCD was applied in the present study to determine the optimum process variables for bacteriocin production by *S. salivarius* KA101. Based on the regression model, an optimization plot can be generated using the Design expert 7 software to determine the optimum conditions for maximum response (bacteriocin). As can be seen from ramp charts presented in Figure-3, the suggested optimal concentrations of yeast extract and $CaCO_3$, as well as pH and incubation time for maximum bacteriocin production (80.2 AU/ml), were 13 g/l, 1.4 g/l, 7 and 24 hrs respectively.

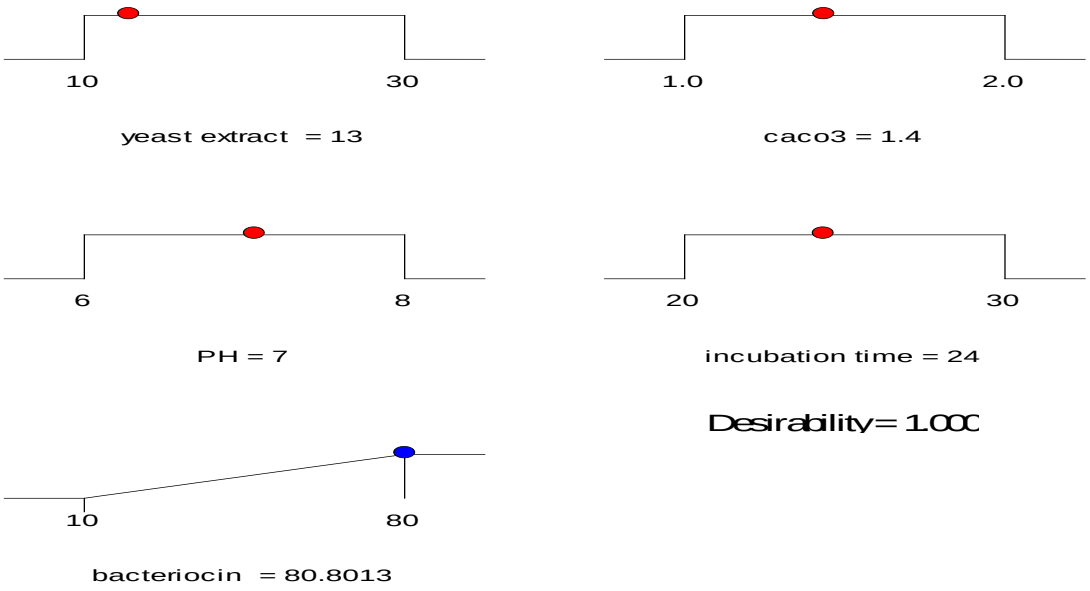

Figure 3-ramp charts of suggested optimal concentrations of yeast extract and $CaCO_3$, pH and incubation time for maximum bacteriocin production.

On the other hand, a bacteriocin produced by *S. salivarius* KA101 was precipitated from the culture supernatant saturated with different concentrations of ammonium. Maximum bacteriocin precipitation was obtained at 50% saturation level with an activity of 80 AU/ml and specific activity 131.14 AU/mg. Investigation of thermal stability revealed that bacteriocin was remained stable after 30min at 20 to 40°C however, approximately 50% of bacteriocin activity was lost after exposure to 50°C and only 20% of its activity was remained upon exposure to 60°C. In addition, bacteriocin activity showed stability at pH 6 and 7 in which bacteriocin kept its stability of 80 AU/ml for 30 min whereas, it decreased to approximately 50% at pH 5 and 8 and no activity was observed at pH 4 and 9 Figure-4.

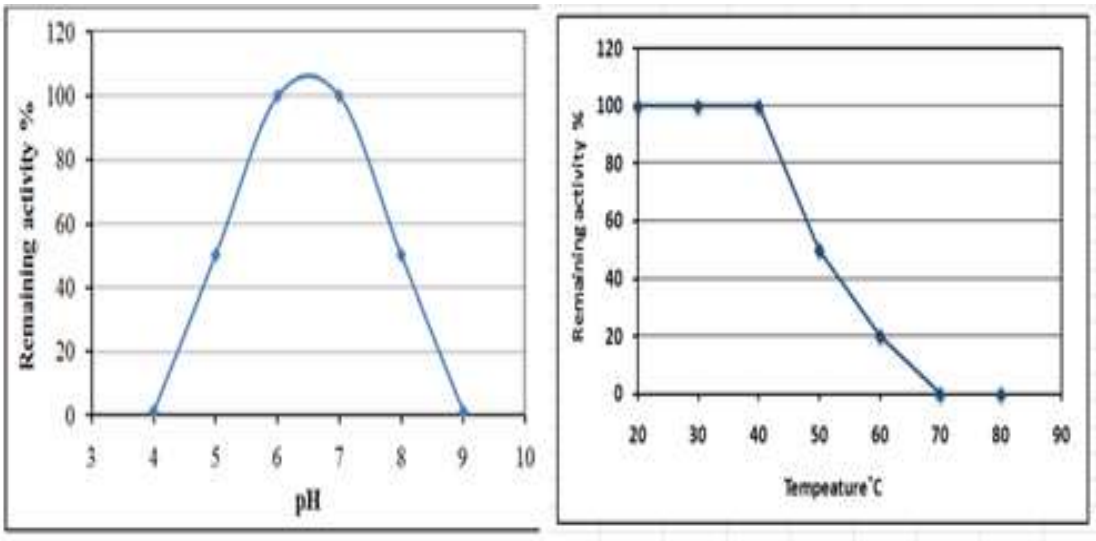

Figure 4-The activity of bacteriocin produced by *S. salivarius* KA101 isolate after exposed to different temperatures and pH.

Furthermore, the results in Figure-5 showed that bacteriocin activity was reduced to 45 and 60 % after 30 min of incubation with trypsin and pepsin respectively and the activity was completely lost after 90min which confirm the protein nature of bacteriocin.

The mode of action of the partial purified bacteriocin produced by *S. salivarius* KA101 was studied using *Lactococcus lactis* KA109 as an indicator strain. As can be seen in Figure-6, a significant decrease in the optical density at 600 nm was detected in the tube contained *Lactococcus lactis* KA109

culture with bacteriocin. Whereas, the absorbance was somewhat increased in the control (tube without bacteriocin). These results suggest that the bacteriocin has a bactericidal effect rather than bacteriostatic.

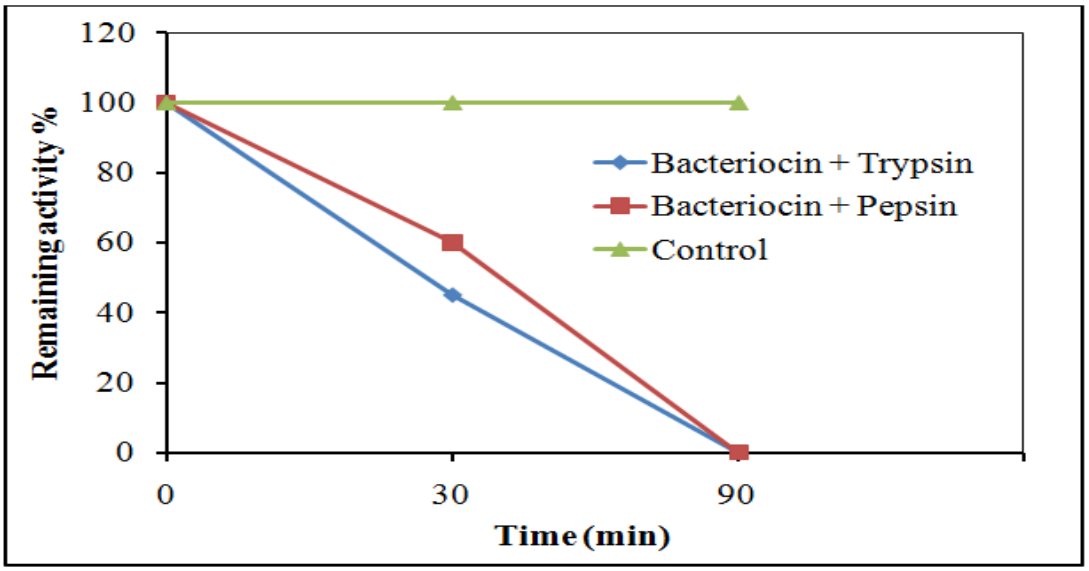

Figure 5-Effect of pepsin and trypsin enzymes on bacteriocin activity produced by *S. salivarius* KA101compared with control.

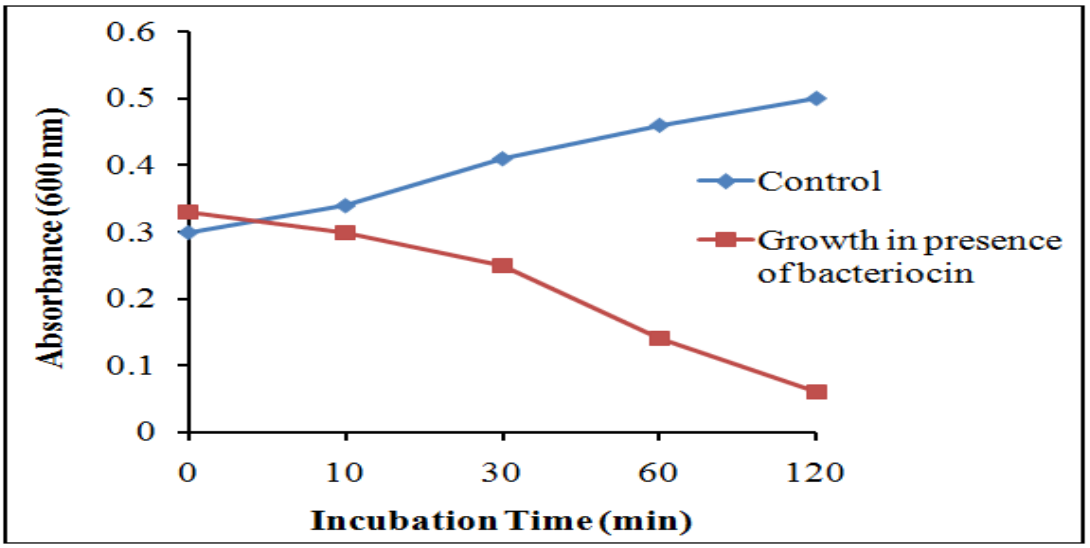

Figure 6-Growth of *Lactococcus lactis* KA109 in the absence (control) and presence of partial purified bacteriocin produced by *S. salivarius* KA101.

The partial purified bacteriocin produced by *S. salivarius* KA101 was tested against several clinical bacterial isolates collected from patients suffering from dental caries, supragingival dental plaque and periodontal disease including gingivitis microorganisms. Two methods were used to test the antimicrobial activity of bacteriocin: well diffusion and agar plug diffusion method. Well diffusion method is considered as the most sensitive assay than other screening methods to determine the antibacterial activity [18]. Whereas, agar plug diffusion method is often used to investigate the competitive behavior among microorganisms. Results showed that bacteriocin produced by *S. salivarius* KA101 was significantly active against 6, 9, 3 and 3 isolates out of 12 isolates of *S. mitis*, 15 *S. mutans*, 10 *S. oralis* and 4 isolates of *Enterococcus* respectively. According to results, it can be suggested that the bacteriocin could play a role in determining the composition of plaque caries, and gingivitis in vivo [19]. Furthermore, the results of agar plug assay revealed that isolate *S. salivarius* KA101 had a considerable antagonism capability against many isolates which reflect a competitive behavior for this isolate against other oral bacteria Table-5, [20].

Table 5- list of oral pathogenic isolates used to test the activity of partial purified bacteriocin produced by *S. salivarius* KA101.

Isolate	Case definition	Bacteriocin activity	
		Agar plug	Well diffusion
S. mitis	dental caries	+10	+12
S. mutans	dental caries	+12	+12
S. mutans	dental caries	+14	+11
S. mutans	periodontal disease	-	+11
S. mitis	periodontal disease	-	-
S. oralis	dental plaque	+14	+11
S. oralis	dental plaque	-	+10
S. mutans	periodontal disease	-	+12
S. salivarius	dental caries	-	-
S. mitis	dental caries	-	-
Enterococcus	dental plaque	+11	+15
S. mutans	dental caries	-	-
S. mutans	periodontal disease	+12	+13
S. mitis	dental plaque	-	-
S. salivarius	periodontal disease	-	+12
S. oralis	dental caries	+16	+18
S. mutans	periodontal disease	-	-
S. mitis	dental plaque	-	-
S. salivarius	periodontal disease	-	+11
S. oralis	periodontal disease	-	-
S. mitis	dental caries	-	+12
S. mutans	dental caries	+14	-
s.mutans	dental caries	-	-
S. oralis	dental caries	-	-
S. mitis	dental plaque	-	+11
Enterococcus	dental caries	-	+12
S. oralis	dental caries	-	-
S. oralis	periodontal disease	+11	+14
S. mitis	dental caries	-	-
S. mutans	dental caries	-	-
S. mutans	periodontal disease	-	-
Enterococcus	dental caries	-	+11
S. mutans	dental plaque	-	+12
S. mitis	dental caries	+11	+12
S. salivarius	dental caries	-	-
Enterococcus	dental caries	-	-
S. mutans	dental plaque	+12	+10
S. oralis	dental plaque	-	-
S. salivarius	periodontal disease	-	+11
S. mitis	periodontal disease	-	-
S. mitis	dental caries	-	-
S. mutans	dental plaque	+11	+11
S.mutans	dental caries	-	-
S. oralis	dental caries	+13	+14
S. oralis	dental caries	-	-
S. mitis	dental plaque	-	-
S. salivarius	periodontal disease	+11	+14

Streptococcus salivarus strains are safe and can be colonized and persist in the oral cavity [21]. Therefore, the results of this study, while preliminary, suggests that *S. salivarius* KA101 can be an attractive to investigate for potential oral probiotic because of antagonism behavior capability. The effectiveness of using probiotic for promoting health or treatment of infectious diseases via artificially utilizing commensally bacteria is in growing interest [22]. Many strains of *S. salivarius* such as strains K12 and M18 are now being used as probiotics worldwide due to their capability of producing different kinds of bacteriocins [23].

Teughels and co-workers [24] reported that inflammation and subgingival recolonization by periodontal bacteria were considerably decreased following treatment with oral *S. salivarius*. Furthermore, a strain of *S. salivarius* displaced *Streptococci mutans* in the oral cavity and consequently inhibited caries development [25]. Moreover, examination of the oral bacteria of volunteers who suffer from halitosis showed an absence of *S. salivarius.* However, colonization of bacteriocin producing *S. salivarius* in the oral cavity of those volunteers led to extensively reduce the level of oral malodor. Therefore, *S. salivarius* is used in combination with a chlorhexidine mouth rinse, a germicidal mouthwash, to reduce halitosis [26].

Conclusion

In disease control, bacteriocin can solve some of the most challenging problems of multi-drug resistant pathogens which became a serious problem. Some of the oral isolates can produce bacteriocins when tested against each other in both well diffusion method and agar plug diffusion method. *Streptococcus salivarius* which is oral microflora showed a significant inhibitory activity against several clinical oral pathogenic Streptococci. Using *Streptococcus salivarius* and/or its purified bacteriocin as probiotics in toothpaste can provide protection against bacterial infection. In fact, clinicians need to be aware of the beneficial properties of the resident microflora, and their treatment strategies should be focused on the control rather than the elimination of these organisms, especially in dental plaque, dental caries and periodontal disease.

References

1. Hyink,O., Wescombe, P.A., Upton, M., Ragland, N. and Burton, J. P. **2007**. Salivaricin A2 and the novel lantibiotic salivaricin B are encoded at adjacent loci on a 190-kilobase transmissible megaplasmid in the oral probiotic strain Streptococcus salivarius K12. *Appl Environ Microbiol*, **73**: 1107-1113.
2. Wescombe, P.A., Upton, M., Renault, P., Wirawan, R. E., Power, D., Burton, J. P., Chilcott, C. N. and Tagg, J.R. **2011**. Salivaricin 9, a new lantibiotic produced by Streptococcus salivarius. *Microbiol*.**157**: 1290-1299.
3. Bill, N. J. and Washington, J.A. **1975**. Bacterial interference by Streptococcus salivarius. *Am J Clin Pathol*. **64**(1): 116-120.
4. Cotter,P. D., Hill, I. C. and Ross, R. P. **2005**. Bacterial lantibiotics: strategies to improve therapeutic potential. *Curr Protein Pept Sci*. **6**(1): 61-75.
5. Mahrous, H., Mohamed, A., El-Mongy, M., El-Batal, A., Hamza, H. **2013.** Study bacteriocin production and optimization using new isolates of Lactobacillus spp. isolated from some dairy products under different culture conditions. *Food Nutri Sci*. **4**: 342-356.
6. Cassia, R. N. and Adriano, B. **2010**. Production of bacteriocinlike substances by lactic acid bacteria isolated from regional ovine cheese. *Braz J Microbiol*. **41**: 1009–1018.
7. Abd, F.N. and Luti **2017**.An exploitation of interspecies interaction for promoting bacteriocin production by local isolate of Bacillus SP. Msc thesis. department of biotechnology, College of science, University of Baghdad.
8. Bradford, M. **1976**. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal Biochem*. **72**: 248-254.
9. Valgas, C., DeSouza, S.M. and Smânia, E.F. **2007**. Screening methods to determine antibacterial activity of natural products. *Braz J Microbiol*.**38**: 369-380.
10. Balouiri, M., Sadiki, M. and KoraichiI, S. **2016.** Methods for in vitro evaluating antimicrobial activity. *J of Pharm Analysis*.**2**: 71-79.
11. Todorov, S. D. and Dicks, L. M. T. **2007.** Bacteriocin Production by Lactobacillus pentosus ST712BZ Isolated from Boza. *BrazJ of Microbiol*. **38**: 166-172.

12. Barbour, A. B. **2013**. Isolation, purification and antimicrobial action of peptides produced by Streptococcus salivarius isolate from Malaysian subjects. MSc Thesis, Institute of biological sciences ,University of Malaya, Kuala lumpur.
13. Jack, R.W. and Tagg, J. R. **1992.** Factors affecting production of the group A streptococcus bacteriocin SA-FF22. *J Med Microbiol*.**36**: 132-138.
14. Matsusaki, H., Sonomura, N., Nitisinprasert, S., Zendo,T., Sonomoto, K. **2006.** Biosynthesis of salivacin K21, a two- peptide bacteriocin produced by Lactobacillus salivarius AC21 development of thermotolerant microbial resources and their applications . *Appl Microbiol Biotechnol*.**5**: 106-120.
15. Yamamoto,Y., Yoshikazu, T., Shimosaka, M., Okazaki, M. **2003.** Purification and characterization of a novel bacteriocin produced by Enterococcus feacalis strain RJ-11. *Appl Environ Microbiol*.**69**: 5746-5753.
16. Myers, R. and Montgomery, RC. **2002.** Response surface methodology: Process and product optimization using designed experiments. Wiley, New York.
17. Mohanasrinivasan, V. and Suganthi, S. **2015.** Optimization studies for enhanced bacteriocin production. *J Food Sci Technol* .**2**: 212-222
18. Smânia-Jr., A., Valgas, C., de Souza, S. M.;Smânia, E. F. A. **2007.** Screening Methods to Determine Antibacterial Activity of Natural Products. *Braz J. of Microbiol*.**38**: 369-380.
19. Donoghue, H. D., and Tyler J. E. **1977.** Antagonisms amongst streptococci isolated from the human oral cavity. *Oral Biol*. **20**: 381-388.
20. Rahman,M., Nahidul Islam, Md., Nurul Islam, M. and Shahnoor, H. M. **2015.** Isolation and Identification of Oral Bacteria and Characterization for Bacteriocin Production and Antimicrobial Sensitivity. *Dhaka Univ J Pharm Sci*. **14**(1): 103-109.
21. Burton, J. P., Wescombe, P.A., Macklaim,J.M., Chai, M., Macdonald, K., Hale, J.D., Tagg, J., Reid, G., Gloor, G.B., Cadieux, P.A. **2013**. Persistence of the oral probiotic Streptococcus salivarius M18 is dose dependent and megaplasmid transfer can augment their bacteriocin production and adhesion characteristics. *PLoS One J* . **8**: 65980- 65991.
22. Weng, M.and Walker, W. A. **2006**. Bacterial colonization, probiotics, and clinical disease. *J Pediatr*.**149**: 107-114.
23. Eke, P. I., Dye, B. A., Wei, L. Thornton-Evans, G., Genco, R. J. **2012.** Prevalence of Periodontitis in Adults in the United States: 2009 and 2010. *J Dent Res*. **91**: 914- 920.
24. Teughels, W., VanEssche, M., Sliepen, I., Quirynen, M. **2008**. Probiotics and oral healthcare. *Periodontol*. **48**: 111-147.
25. Burton, J. P., Wescombe, P. A., Moore, C. J., Chilcott, C. N., Tagg, J.R. **2006.** Safety assessment of the oral cavity probiotic Streptococcus salivarius K12. *Appl Environ Microb*.**72**: 3050-3053.
26. Burton, J. P., Chilcott, C. andTagg, J. **2005.** The rationale and potential for the reduction of oral malodour using Streptococcus salivarius probiotics. *Oral Dis*.**11**: 29-31.