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Optimization, Purification, and Characterization of Enterocin SH1 Produced By *E. Faecium* SH1 and Enterocin SH2 Produced by *E. Faecalis* SH2 Isolated from Dental Caries

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Abstract
The current study was aimed to producing, optimization, purification and characterization of bacteriocins using *E. faecium* SH1, and *E. faecalis* SH2 isolated from dental caries. The optimum conditions for bacteriocin production were in Todd Hewitt broth supplemented with 1% glucose at 37°C for 24 hr. under anaerobic conditions and inoculum size of 4 McFarland standard (1.2×10^9 CFU/ml) and pH 7 for enterocin SH2 while for enterocin SH1, the optimum pH was 5.5. At these conditions, the CFS of enterocin SH1 gives 33 mm inhibition zone diameter against vancomycin resistant *E. faecalis* while the enterocin SH2 gives 27 mm against the same isolate. Purification of bacteriocin was made partially by ammonium sulphate at (50 and 95)% saturation levels and the best level for precipitation of bacteriocins was 95%, followed by gel filtration chromatography using Sephadex G-50 column and the activity of purified enterocin SH1, and enterocin SH2 was increased to 39 and 36 mm, respectively against *E. faecalis*. The results showed that enterocin SH1 was broad spectrum activity against G+v and G-v pathogenic bacteria such as *Salmonella entirica*, *Staphylococcus aureus*, *Pseudomonas aeroginosa*, and *Escherichia coli* while enterocin SH2 was had narrow activity against G+v such as *Streptococcus pyogenes* and *E. faecalis*. The molecular weights of bacteriocins were determined by Tris- Tricine SDS-PAGE, enterocin SH1 was 8kD in weight while enterocin SH1 was 3 kD. Physical characteristics were also studied and the results showed that enterocin SH1 was stable at wide range of pH values (3-12) and after 121ºC for 20 min. Enterocin SH2 keep its effectiveness within the pH range 3-10 but lost of activity at pH 2, 11 and 12. Enterocin SH2 also stable at temperature until 100º C while lost of activity after autoclaving.
Keywords: Bacteriocin. *Enterococcus faecium*. *Enterococcus faecalis*. enterocin.

Introduction

The emergence and current dissemination of antimicrobial resistance among microorganism's area unit important health and economic issue, resulting in accumulated rates of morbidity and mortality associated with microorganism infections. Research and development for brand new antimicrobial agents is presently required to beat this downside. Among the various approaches studied, bacteriocins appear to be a promising chance (Simon *et al*., 2020). Bacteriocins are ribosomally synthesized proteins and protein complexes with a broad spectrum of antibacterial activities against many food-borne pathogens and closely related species (Negash *et al*., 2020). They represent a potential solution to this worldwide threat due to their broad- or narrow-spectrum activity against antibiotic-resistant bacteria (Soltani *et al*.,

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2021). *Enterococcus* is a large genus of LAB, are Gram positive, catalase-negative, facultative anaerobic, and non-spore forming (Moraes *et al*., 2013) and several species from this genus have been used as probiotic for humans or animals (Franz *et al*., 2011). In addition, some *Enterococcus* spp. act as protective agents against food spoilage and pathogenic bacteria, such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Clostridium perfringens* (Huang *et al*., 2016).

Lactic acid bacteria produce antimicrobial substances that include organic acids, hydrogen peroxide, and bacteriocins (Reis *et al*., 2012). Bacteriocins have potent inhibitory activity against sensitive strains of bacteria. Enterocin is a bacteriocin obtained from the *Enterococcus* species. Numerous enterocin-producing enterococci and enterocins have been reported. They include enterocin A from *E. faecium* CTC492 (Aymerich *et al*., 1996), and enterocin Q from *E. faecium* L50 (Cintas *et al*., 2000), bacteriocin BacC1 produced by *E. faecium* C1 (Goh *et al*., 2014), enterocin E23 produced by *Enterococcus faecium* E23 (Niederhäusern *et al*., 2020), and enterocin M produced by environmental isolate *Enterococcus faecium* AL41 (Mareková *et al*., 2007)

Bacteriocins have many positive properties that have made them particularly interesting for various applications. LAB bacteriocins are inherently tolerant of high thermal stress and are known for their activity over a wide pH range. These antimicrobial peptides are also colorless, odorless, and tasteless, which further improves their potential stability. They are also easily degraded by proteolytic enzymes due to their proteinaceous nature. Consequently, bacteriocin fragments do not live long in the human body or in the environment, which minimizes the chance of target strains interacting with degraded antibiotic fragments (Perez *et al*., 2014) .

In the present study, the antimicrobial activity of the bacteriocins produced by *E. faecium* SH1 and *E. faecalis* SH2 against medical pathogens including VRE was examined. Additionally results of optimization, purification and characterization studies of bacteriocin reported.

Materials and Methods

Bacteriocin producer

The producer isolates *E. faecium* SH1 and *E. faecalis* SH2 were isolated from dental caries patients at Al-Shaheed Al- Sader Hospital, Baghdad, and identified by many tests including catalase test, growth at 10, and 45 ºC and 6.5% NaCl medium, furthermore identified by vitek 2 system. These isolates were chosen as the best bacteriocin producer

Screening for bacteriocin production

For bacteriocin production screening, used the Agar Well Diffusion (AWD) method according to (Hashim *et al*., 2017**)**. The producer isolate was cultured in Todd Hewitt broth (10 ml) and incubated for 24 hr. at 37º C under anaerobic conditions. After incubation, the whole broth was centrifuged at 7,000 rpm for 15 min and cell-free supernatant was collected. To avoid the inhibitory effects of organic acids, the pH of the CFS was adjusted to 7 with 0.5N NaOH. The CFS was then filtered through a 0.2 µm pore size (Millipore, Merck).

Optimization of bacteriocin production

Many parameters were tested to chosen the best conditions for bacteriocin production at highly level and these are:

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1- Determination of the optimum aeretion for bacteriocin production

The effects of aeration on bacteriocin producing was examined by incubation of 10 ml of modified THB by adding 1% glucose inoculated with 4 McFarland of selected bacteria under aerobic condition, microaerophilic CO_2 (5%) condition using candle jar and anaerobic condition using anaerobic jar.

2- Determination of the optimum pH for bacteriocin production

The effect of pH on the growth and on production of bacteriocin of isolates were tested. The modified THB was preparing in 10 ml tubes at different values of pH as in following 4, 5.5, 7, 8.5, and 10 with 0.5 N HCl or 0.5 N NaOH after autoclaved. Tubes after that were inoculated with selected strains and then incubated at 37 °C under anaerobic conditions for 24 h. After change the culture of pH, the bacterial growth, and the bacteriocin production were measured.

3 -Determination of optimum inoculum size for bacteriocin production

The effect of inoculum volume of producer strain was examined by using 1(3×10^8 CFU/ml), 2(6×10^8 CFU/ml), 3(9×10^8 CFU/ml), and 4(1.5×10^9 CFU/ml) McFarland standard under anaerobic conditions for 24 hr. at 37º C.

4- Determination of the optimum medium for bacteriocin production

Four 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: Brian heart infusion broth (BHI), modified BHIB , Tryptic soya broth (TSB), modified TSB, Todd Hewitt broth (THB), modified THB. Active culture of producer strain was inoculated into above selective media and incubation was carried at 37 ºC under anaerobic conditions for 24hr. then the activity of CFS was measured by AWD method against indicator strain.

5- Determination of the optimum incubation period for bacteriocin production

The effect of incubation period was testing in modified THB medium at different incubation time (16, 24, 48 and 72) hr. 10 ml of selected strain (1.5×10^9 CFU/ml) was incubation at 37ºC under anaerobic conditions.

6- Determination of the optimum temperature for bacteriocin production

To study the effect of incubation temperature on bacteriocin production, the bacterial isolates grown in optimal medium for production at different incubation temperatures (20, 25, 30, 37, and 40) ºC. Producer strain was inoculated (1.5×10^9 CFU/ml) to 10 ml of modified THB and incubated for 24 hr. under anaerobic conditions at 37˚ C.

Partial purification of bacteriocin

Partial purification of bacteriocin was prepared via precipitation with ammonium sulfate at different levels. A CFS of the production isolate was prepared from 750 ml of an overnight growth culture by centrifugation at 10000 rpm for 30 min. at 4°C. 720 ml of CFS was Precipitation by adding solid ammonium sulfate at saturation level (50) % after neutralized the pH by using 0.5N NaOH to CFS and stay for 1 hr. incubation to confirm as bacteriocin. . In the 50 % level, 209.52 g of ammonium sulfate added gradually to the CFS placed in an ice bath with continuous mixing for 1hr. at stirrer. After overnight with continuous mixing at 4°C, the mixture was centrifuged at 10000 rpm for 20 min at 4°C. The obtained precipitate was collected and dissolved in phosphate buffer saline (0.1 M, pH 7) . The remaining solution was assayed to next levels 95% by adding 221.76 g of ammonium sulfate and also

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the obtained precipitate was collected and dissolved in 10 ml of potassium phosphate buffer and dialysis overnight at 4˚C by dialysis membrane (molecular weight cut off (MWCO) 1000 Da) against the same buffer with frequent changes of buffer to remove ammonium sulfate from bacteriocin which may hinder further purification experiments. After completion of dialysis an aliquot was assayed for protein concentration at A_{600}.

Protein estimation

The protein focus was resolved according to (Waterborg., 2009) . Concentration of protein in samples was calculated using a standard curve prepared using a standard series of bovine serum albumin (BSA).

Antimicrobial and spectrum activity of purified bacteriocin

Antimicrobial activity of purified enterocin SH1 and enterocin SH2 and mix bacteriocins was measured by AWD method against *E. faecalis* and *E. coli*, *Salmonella entirica*, *Enterobacter cloacae*, *pseudomonas aeroginosa*, *Proteus mirabilis* ,*Staphylococcus aureus*, *Staphylococcus hemolyticus*, *Leuconostic mesenteroides*, and *Streptococcus pyogenes* as indicator strains. The purified enterocin SH1 (100µl) and enterocin SH2 was added in the 5 mm wells on MHA plates previously spreader with 0.1 ml suspension of indicator strain (0.5 McFarland standard). The diameter of inhibition zone determined after incubation for 24 hr. at 37 ºC.

Determination of the molecular weight of Enterocin SH1

Tricin SDS-PAGE was carried out according to the method of Schagger and Jagow (1987). The gel was prepared with the following composition: the separating gel (15%) was overlaid by the spacer gel (10%) and the stacking gel (4%) was poured on top of the spacer gel. 20 µl aliquot of bacteriocin sample was mixed with 20 µl twofold concentrated sample buffer and heated for 5 min at 60 º C. Poly molecular mass standards were included in each gel. Electrophoresis was run at constant voltage (30 V in the stacking gel and at 150 V during the rest of separation). At the end of electrophoresis, the gel was dyed for 30 min and then put into the decolorizing solution. The decolorizing solution was changed every 1 hr until clear electrophoretic bands appeared. Finally, the electrophoretic gel was photographed and analyzed.

Characterization of enterocin SH1, and enterocin SH2

- **Thermal stability of bacteriocin**

In order to test the thermo stability of bacteriocin, samples were exposed to different temperatures (40, 60, 80, 100,and 121) °C for 20 min. The residual activity was then determined by AWD method against indicator strain.

- **pH stability of bacteriocin**

Bacteriocin preparation was treated with either 0.5N HCI or 0.5N NaOH to achieve the desired pH values between (2,3,4, 5,6, 7,8, 9,10, 11 and 12). The pH adjusted crude extracts were then incubated for 30 min. After incubation, aliquots were neutralized and activity was measured by AWD method against indicator strain.

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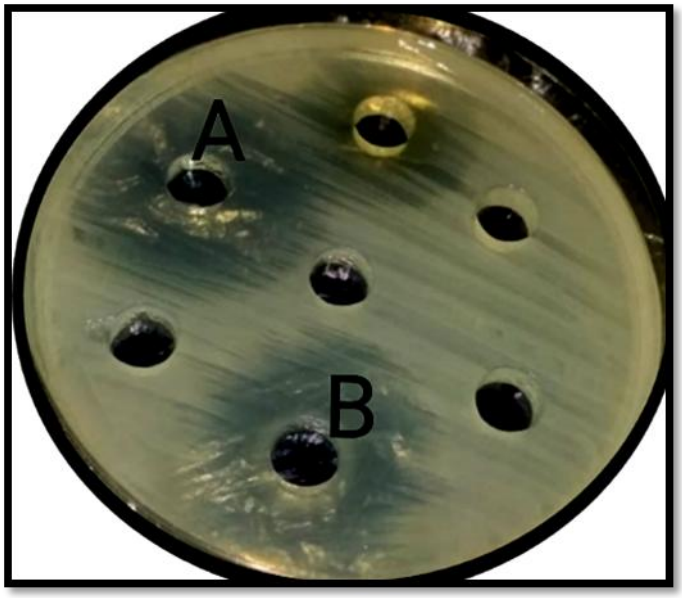
Statistics analysis

All experiments were performed in triplicate. Results were analyzed by analysis of variance (ANOVA) and Duncan's test with the SPSS 23.0 software. All results were presented as mean ± standard errors (SEs). A P-value < 0.05 was considered statistically significant.

Results and Discussion

Screening on bacteriocin production

The CFS of *E. faecium* SH1 was obtained from THB modified by adding 1% glucose and incubated under anaerobic conditions at 37˚C and pH 7 for 24 hr. give 30 mm inhibition zone diameter by AWD method, while *E. faecalis* SH2 gives 27 mm under the same conditions against Multidrug resistant *E. faecalis* as in figure (1).



Figure(1): Effect of crude bacteriocins on *E. faecalis*. A, crude enterocin SH1. B, enterocin SH2.

Optimum conditions for bacteriocin production

1- Optimum aeretion

The maximum inhibition zones were (30, and 27) mm for enterocin SH1, and enterocin SH2, respectively obtain from culture under anaerobic condition against *E. faecalis*. The inhibition zone gradually decreases with increased oxygen concentration and decreasing the concentration of CO_2 where reduced to (17, and 21) , (12, and 12) under 5-15% CO_2 and aerobic respectively as in table (1).

Table (1): Effect aeration on bacteriocin production.

Atmospheric CO_2	**Inhibition zone diameter (Mean ± SE)**	
	Enterocin SH1	Enterocin SH2
Aerobic	12 ± 0.23 a	12 ± 0.58 a
Under CO_2	17± 0.52 b	21 ± 0.35 b
Anaerobic	30 ± 0.35 c	27 ± 0.29 c

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Values with different letters have significant differences and values with similar letters mean have no significant differences . **(p<0.01)

There is no study to determine the effect of anaerobic or aerobic conditions on bacteriocin production in the studied bacterial species but there is a study by (Barbosa *et al*.,2019) those results agreement with the current result as he showed that the highest productivity of bacteriocin in *S. bovis* HC5 was recovered from anaerobic culture.

2- Optimum pH

The results show that pH 7 was the best value for enterocin SH2 production when the diameter of inhibition zone reached 27 mm. While for enterocin SH1, the pH 5.5 give the highest inhibtion zone 33 mm. The lowest activity recorded at basic pH that reached (5) mm for enterocinSH1 and no inhibition zone obtain by enterocin SH2 as in table (2).

Table (2): Effect of pH on bacteriocin production.

pH of Medium	**Inhibition zone diameter (Mean ± SE)**	
	Enterocin SH1	**Enterocin SH2**
4	9 ± 0.58 a	7.67 ± 0.77 a
5.5	33 ± 0.58 b	14 ± 0.58 b
7	30 ± 0.35 c	27 ± 0.58 c
8.5	30 ± 0.0 c	19.07 ± 0.12 d
10	5 ± 0.58 d	0 ± 0.0b
Values with different letters have significant differences and values with similar letters mean have no significant differences . **(p<0.01)		

The current result showed that the acidic conditions were the optimum for production of enterocin SH1, while (Louiza *et al*., 2015)) and (Qiao *et al.*, 2019) found that neutral conditions were the best for bacteriocin production form *E. faecium* as the current result for enterocin SH2.

3- Optimum inoculum volume.

The results showed that the optimum inoculum volume for highest enterocin SH1, and enterocin SH2 production was 4 McFarland standard (1.5×10^9 CFU/ml) as in table (3). There as a gradual increase in the production of bacteriocin with increased in cells number in the inoculum that added to cultivation media, where the greatest zone of inhibition (33, and 27) mm for enterocin SH1, and enterocin SH2, respectively that have yielded with the 4 McFarland standard, then the bacteriocin activity was decreasing gradually with decreased cells number in the inoculum (2 McFarland) reached (8.5, and 5.17) mm for enterocin SH1, and enterocin SH2 respectively, while there are no bacteriocin activity at 1 McFarland .

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Table (3): Effect of initial inoculum size on bacteriocin production.

Inoculum size (McFarland)	**Inhibition zone diameter (Mean ± SE)**	
	Enterocin SH1	**Enterocin SH2**
1 (3×10^8 CFU/ml)	0 ± 0.0 a	0 ± 0.0 a
2 (6×10^8 CFU/ml)	8.5 ± 0.76 b	5.17 ± 0.17 b
3 (9×10^8 CFU/ml)	15 ± 0.17 c	9.33 ± 0.33 c
4 (1.5×10^9 CFU/ml)	33 ± 0.58 d	27 ± 0.29 d
Values with different letters have significant differences and values with similar letters mean have no significant differences . **(p<0.01)		

The present results show that the bacteriocin production by a producer isolate in generally, if the cell density is bigger in the lag and exponential phases of growth, the extracellular bacteriocin concentration can be higher and this agrees with (Tabasco *et al.*, 2009) and (Abbasilias *et al.*, 2016)

4- Optimum production medium

Results show that the optimum production medium for production of crude enterocin SH1, and enterocin SH2 was modified THB by adding 1% Glucose under anaerobic condition and optimized pH at 37ºC for 24h as in table (5). The lowest enterocin SH1, and enterocin SH2 production was in the Nutrient Broth medium where the inhibition zone diameters were (9, and 2) mm respectively against *E. faecalis* as in table (4) . This result may due to differences in medium components , where the THB and BHB give the higher activity and this is the result of the similarity in some of the components such as dextrose, and disodium phosphate .

Table (4): Effect of culture medium on bacteriocin production.

Culture media	**Inhibition zone diameter (Mean ± SE)**	
	Enterocin SH1	**Enterocin SH2**
Todd Hewitt Broth (THB)	28.33 ± 0.67a	21.67 ± 0.67 a
modified Todd Hewitt Broth	33 ± 0.58 b	27 ± 0.29 b
Brain Heart Broth (BHB)	25 ± 0.58 c	14.5 ± 0.29 c
modified Brain Heart Broth	25.33 ± 0.67c	18.67± 0.88 d
Tryptic Soy Broth (TSB)	12.57± 0.51d	7.33 ± 0.33 e
modified Tryptic Soy Broth	16.93 ± 0.67 e	9 ± 0.0 f
Nutrient Broth (NB)	9.07 ± 0.12 f	2 ± 0.0 g
modified Nutrient Broth	12.5± 0.29 d	4.83 ± 0.17 h
Values with different letters have significant differences and values with similar letters mean have no significant differences . **(p<0.01)		

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Biomass amounts increase when the initial glucose concentration was between (0.5 – 2 %) during fermentation of *E. faecium* DPC1146 (Raafat *et al*., 2016) . Callewaret and De Vuyst (2000) reported that the inhibition of the bacteriocin production is due to higher concentrations of the nitrogen source used. Tulini *et al* (2011), show that *E. faecium* 130 produced more bacteriocin in MRS (6400 AU ml^{-1}) broth in comparison to the TSB (3200 AU . ml^{-1}) and BHIB (800 AU.ml^{-1}).

5- Optimum incubation time

The evolution of the enterocin SH1, and enterocin SH2 production was followed up at different incubation periods. Maximum production was observed after 24 hr. of incubation with the greatest inhibition zone of (33,and 27) mm respectively . The activity decreased after (48, and 72) hr. and the lowest inhibition zone was recorded after 72 hr. and no activity for obtain for enterocin SH2. Table (5).

Table (23): Effect of incubation time on bacteriocin production.

Incubation period	**Inhibition zone diameter (Mean ± SE)**	
	Enterocin SH1	**Enterocin SH2**
16 hr.	31 ± 0.29 a	24.33 ± 0.33 a
24 hr.	33 ± 0.58 b	27 ± 0.29 b
48 hr.	12 ± 0.58 c	11.33 ± 0.67 c
72 hr.	7.17 ± 0.17 d	0 ± 0.0 d
Values with different letters have significant differences and values with similar letters mean have no significant differences . **(p<0.01)		

The bacteriocin production increased during the exponential growth phase, reached a maximum activity at 24 hr. and began to decrease after this time and the pH also decreased. This suggested that the production of bacteriocin depends on the cell density. Similar results have also been observed in some bacteriocins produced by Lactic acid bacteria, such as bacteriocin FGC-12 (Lu *et al*,. 2017) . At the end of growth, the bacteriocin activity decreased due to the decrease in the number of viable cells, the decrease in pH (Whang *et al*,. 2018).. The results agree with (Phumisantiphong *et al*,. 2017) who found that the highest activity from *E. faecalis* 478 was after 24 hr.

6- Optimum temperature

The optimum temperature for production of enterocin SH1, and enterocin SH2 was 37°C with maximum inhibition zone reached (33, 27) mm respectively ,while at (20 and 40)°C, the antimicrobial activity of enterocin SH1 reduced to (8, and 11) mm respectively and there is no effective obtain by enterocin SH2 at these temperature. There is no inhibition zone obtain after cultured at 10 ˚C . Table (6).

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Table (6): Effect of temperature on bacteriocin production.

Temperature	Inhibition zone diameter (Mean ± SE)	
	Enterocin SH1	Enterocin SH2
10 ºC	0 ± 0.0a	0 ± 0.0a
15 º C	6.9 ± 0.21 b	0 ± 0.0a
20 º C	8 ± 0.12 b	0 ± 0.0 a
25 º C	10.73± 0.55 c	5± 0.29 b
30 º C	15.83 ± 0.52 d	8.83 ± 0.17 c
37 º C	33 ± 0.58 e	27 ± 0.29 d
40 º C	11.67 ± 0.57 c	7 ± 0.0e
Values with different letters have significant differences and values with similar letters mean have no significant differences . **(p<0.01)		

The reduction in the bacteriocin production at higher and lower temperature referred to slow growth that led to retardation of bacteriocin production. This result agree with (Tulini *et al*,. 2011) who study on bacteriocin produced *E. faecium* and show that a reduction of the bacteriocin production at 4˚, 10˚ , 15˚, and 30 ˚ was observed when compared to the control incubated at 37˚ C .

3.7 Ammonium sulfate precipitation and desalting by dialysis

Cell free supernatant from *E. faecium* SH1, and *E. faecalis* SH2 was subjected to ammonium sulphate saturation from 50% and 95%. The inhibition zone about 3,and 5 mm for enterocin SH1, and enterocin SH2, respectively at the 50% level and this indicate that the producer isolates may produce more than one bacteriocin. Antimicrobial increased with increasing saturation to 95% with maximum antimicrobial activity.

The results agree with many studies for example (Cheukuri *et al*,. 2019), and (Gaaloul *et al*,. 2015) where precipitated bacteriocin produced by *E. faecium* within the range.

Purification of bacteriocins

After the first stage bacteriocin purification by precipitation and dialysis, the partial purified bacteriocin was applied directly to next step gel filtration chromatography. The sample was loaded over Sephadex G-50 column. The curve was plotted between the absorbance fraction numbers which gave one protein peaks. Each eluted 5 ml fraction read at 280 nm and the curve was plotted between the absorbance and fraction number which gave protein peak of enterocin SH1 was (15, 16, and 17) ,and (12, 13, and 14) for enterocin SH2. Figure (2).

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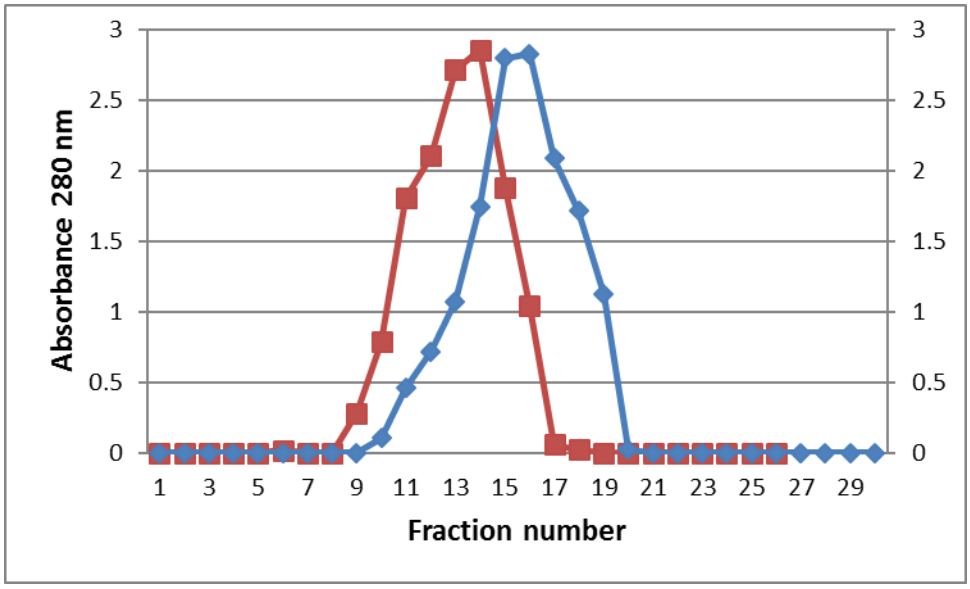
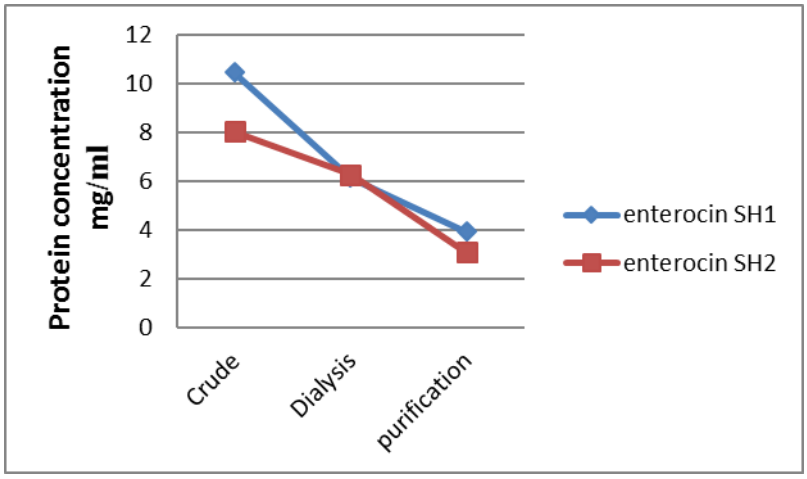


Figure(2): Purification of enterocin SH1(blue), and enterocin SH2 (red) by gel filtration chromatography, using Sephadex G-50 column with dimensions (1.5x46) and (1.5x43) cm that equilibrated and eluted by 0.02 M of sodium buffer (pH7), flow rate was 1ml/min, with 5ml for each fraction.

Peaks of bacteriocin were appeared maximum activity of enterocin SH1, and enterocin SH2 when assayed against *E. faecalis*. They were observed in the fractions 16 for enterocin SH1 and in the fraction 13 for enterocin SH2.

Estimation of protein concentration

The concentration of protein in crude , dialysis, and purified enterocin SH1, and enterocin SH2 were determined by Lowry method . The protein concentration gradually less where in crude bacteriocin were 10.461, and 8.033 and after dialysis were 6.143, and 6.276 while after purification by gel chromatography the concentration were 3.912, and 3.087 mg\ ml for enterocin SH1 and enterocin SH2 respectively. The activity after purification increased for all bactericoin with decreased in the concentration. Figure (3).



Figure(3): Protein concentration of crude, dialysis, and purified enterocin SH1, and enterocin SH2 (mg/ml).

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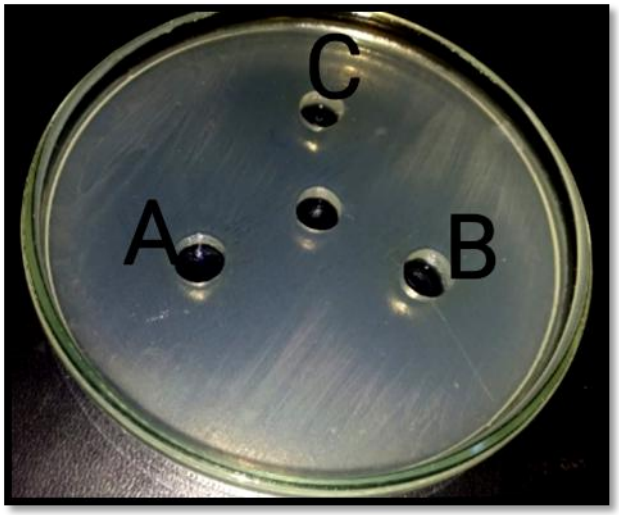
Spectrum activity

Purified enterocin SH1 has broad spectrum activity against G+ve and G-ve bacteria which appear MDR such as *S. aureus* (22 mm) isolated from UTI , Enteropathogenic *E. coli* (19 mm) isolated from diarrhea, *Pseudomonas aeroginosa* (13 mm) isolated from burn, *S. haemolyticus* (26 mm) isolated from dental caries, *Salmonella entirica* (12 mm) isolated from blood, *Leuconostic mesenteroides* (30 mm) isolated from caries with highest spectrum activity about 39 against *E. faecalis* isolated from dental caries. While enterocin SH2 has activity only against gram positive bacteria as *E. faecalis* and *Streptococcus pyogenes* with the inhibition zone diameter 34 mm and 36 mm for enterocin SH1 and 28 mm for mixed between them as in figure (4).



Figure(4): Effect of purified bacteriocins on *S. pyogenes*. A, enterocin SH1. B, enterocin SH2. C, mixed between enterocin SH1 and enterocin SH2.

The result of enterocin SH1 agree with (Cheukuri *et al*,. 2019) and with (Bayoub *et al*,. 2011) where they found that the bacteriocin produced from *E. faecium* has activity against G+ve and G-ve bacteria. For enterocin SH2, difference result showed by (Abanoz *et al*,. 2018) where the bacteriocin produced by *E. faecalis* KT11 exhibited antimicrobial activity against *S. aureus*, *B. subtilis*, and *L. monocytogenes* but enterocin SH2 was had activity only against related speciesand this differences may due to difference in the source of producer strain where in the current study the sample was isolated from caries and in that study isolated from traditional cheese and thus the difference in the type of bacteriocin produced where the enterococcal spp. produced more than one bacteriocin. Another reason, the indicator isolates that used in this study may be resistant to bacteriocin .

Molecular mass determination

The purified enterocin SH1, and enterocin SH2 that collected from different steps of purification were analyzed by Tricine-SDSPAGE. A single target band appeared, indicating that the resultant bacteriocin is a single active peptide in nature. The molecular weight of the enterocin SH1 was about 8 kDa while the molecular weight of enterocin SH2 was about 3 kDa. Figure (5).

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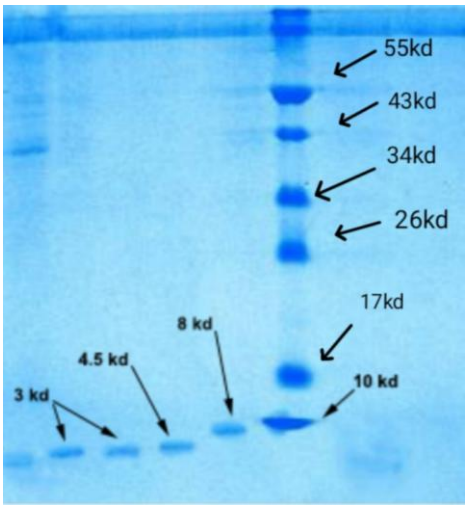

Figure(5): Molecular weight of purified bacteriocins. Enterocin SH1 8kDa, enterocin SH2 3kDa.

It is consistent with many reports that the molecular weight of enterocin is in the range of 2.7–7.9 kDa. within this range of molecular weights, some bacteriocins such as bacteriocin produced by *E. faecalis* RJ-11 (5 kDa) isolated from rice bran (Yamamoto *et al*,. 2003) and partially purified bacteriocins produced by *E. faecalis* isolated from mangrove forests in southern Thailand (Aran *et al*,. 2015), bacteriocin produced by *E. faecium* TJUQ1 with high bacteriocin-producing ability was isolated from pickled Chinese celery (Qiao *et al*,. 2020) They are all small molecule proteins in nature. To the best of our knowledge, this is the first report to screen for bacteriocins produced by *E. faecalis* , *E. faecium* from human dental caries, suggesting that these bacteriocin are also novel.

Characterization of enterocin SH1, streptoocin SH3, and enterocin SH2
Temperature stability

Enterocin SH1, and enterocin SH2 were subjected to different temperatures (60℃, 80℃, 100℃, and 121℃) for 20 min, and the residual antimicrobial activity against *E. faecalis* was assessed by an AWD method. Enterocin SH1 and enterocin SH2 were stable at different temperature range from 40 - 80 º C for 20 min but the activity reduced at 100 and 121ºC for enterocin SH1 but the activity of enterocin SH2 was completely lost at 121ºC for 20 min. Figure (6)

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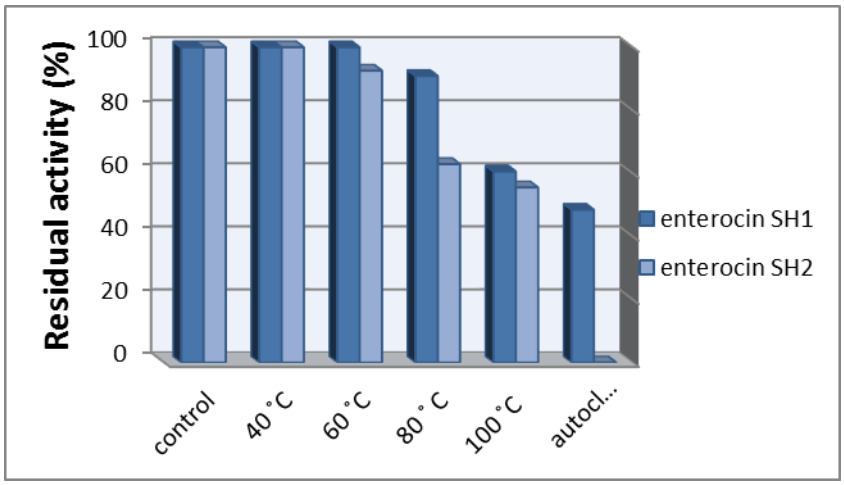

Figure (6): Thermal stability of enterocin SH1, and enterocinSH2.

Some scholars pointed out that bacteriocins produced by *Enterococcus* have high temperature tolerance and high temperature resistance. For example, the good thermal stability of the bacteriocin produced by *E. faecium* SH1 is in agreement with the bacteriocins from *E. faecium* JCM 5804 T (Park *et al*,. 2003) , *E. faecium* LM-2 (Liu et al,. 2011) also bacteriocin from *E. faecium* HY11240 was stable at 100ºC but the activity decreased by two fold after 121ºC for 20 min (Abanoz and Kunduhoglu ,.2018). The effect of temperature on bacteriocin activity showed that enterococcin was heat-resistant (Vaicikauskaite *et al*,. 2019). For enterocin SH2, similar result obtain by (Belguesmia *et al*,. 2010) and different result obtain by (Tosoni *et al*,. 2019) where the bacteriocin produced by *E. faecalis* KT11 was thermostable even at 121 C for 20 min.

pH stability

Effect of pH on the stability of enterocin SH1, and enterocin SH2 at different pH levels was studied between pH 2 and 11 for 30 hr. The bacteriostatic activity of the enterocin SH1 was not affected by pH in the range of pH 4 to 9. At pH 3, 10, 11, 12 the bacteriocin activity decreased, and completely lost at pH 2. At pH 3–10, enterocin SH2 was active but the activity lost at pH 2, 11, and 12 as in figure (7). The results showed that the bacteriocin produced by *Enterococcus* had good acid and alkali resistance .

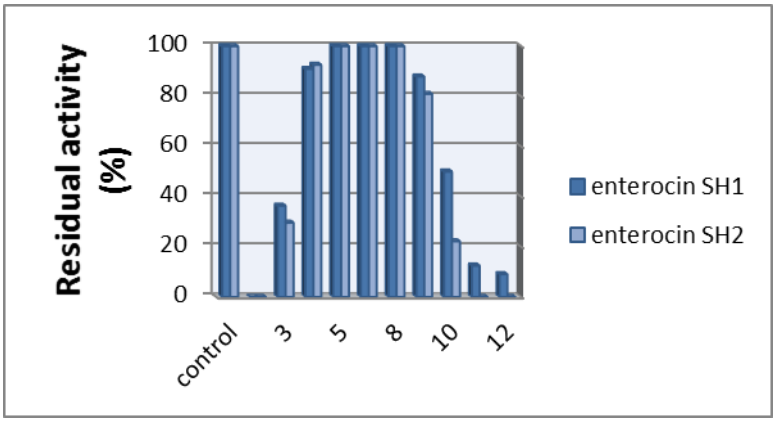

Figure (7): pH stability of enterocin SH1, and enterocinSH2.

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Abanoz *et al.*,(2018) reported that bacteriocin produced from *E. faecium* KY11 was lost their activity at pH 2 and 10 but the activity at pH range 5 to 7 was stale and decreased at pH 3, 4, and 9 after 2 hr. of treated and this differences may to difference in the period of treated where in current study the time was 30 min.

Conclusions

The results of this study has shown that production of enterocin SH1, and enterocin SH2 increased after culturing in the modified THB with 1% Glucose at 37 ºC for 24 hr. under anaerobic conditions with initial pH 7 for enterocin SH2, and 5.5 for enterocin SH1.enterocin SH1 was more stability at pH range from 3 to 12 and temperature until autoclaved for 20 min. The acivity of enterocin SH2, lost at pH 2, 11, 12 and autoclaving. Enterocin SH1 have broad spectrum activity against G+ve and G-ve bacteria while enterocin SH2 has specific inhibitory effect against related species. One peptide band with a 8 kDa for enterocin SH1, and 3kDa for enterocin SH2 molecular weight, were identified after tricine-SDS-PAGE analysis. In conclusion, due to its broad antimicrobial spectrum of enterocin SH1 and stability at high temperatures and over a wide pH range, enterocin SH1 is thought to be a potential efficient bio-preservative in various food products. Furthermore, the fact that enterocin SH1, enterocin SH2 are effective against the drug-resistant pathogens used in this study makes it a promising antimicrobial agent in combating multidrug-resistant pathogens.

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