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ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF 4 KDA BACTERIOCIN, PRODUCED BY *Enterococcus faecalis* AK71, WITH ACTIVITY AGAINST GRAM- POSITIVE AND GRAM-NEGATIVE BACTERIA

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ABSTRACT

Strain AK71, isolated from Egyptian fermented milk named Zabady, and identified as *Enterococcus faecalis*, produces a 4000 Da bacteriocin-like compound with inhibitory activity against *Staphylococcus aureus* 91161, *Enterococcus faecalis* 90519 and *Serratia marcescens* 98027. Bacteriocin AK71-like compound is inactivated by proteinase K but not when treated with catalase and trypsin. No change in activity was recorded after either 30 min or 2 h at pH values between 3.0 and 8.0, and after treatment at 100°C for 30 min. Activity was lost after treatment at 121°C for 20 min. The mode of action is bacteriostatic. The highest level of bacteriocin activity (51200 AU/ml) was recorded when cells were grown in MRS broth at 30°C for 12 h. Bacteriocin AK 71 shows activity towards Gram-negative bacteria making it different from most bacteriocins described for *Enterococcus spp.*

Keywords: Bacteriocin AK71, *Enterococcus faecalis*.

INTRODUCTION

Lactic acid bacteria are well known for their roduction of ribosomally synthesized antimicrobial proteins or peptides, collectively know as bacteriocins. In general these protein complexes have an antagonistic activity against bacteria genetically closely related to the producer strain (Green *et al* 1997, cf De Kwaadsteniet et al., 2005)

The bacteriocins of lactic acid bacteria were classified by Klaenhamer (1993) into four classes. Class II: bacteriocins which were named Lantibiotics. They are small(<5 kDa) membrane-active peptides which contain posttranslationally modified amino acids residues. Nisin is the best example for lantibiotics (Bella et al., 2000). Class II of bacteriocins are unmodified, heat stable, low molecular mass(<10 kDa) and membrane active peptides. This class is divided into three subgroups; subgroup a comprises peptides mainely affect *Listeria monocytogenes*, e.g., pediocin PA-1 (Marugg *et al*., 1992) and sakacin A (Holck *et al* 1992). Subgroup b comprises a two peptide bacteriocins,e.g., lactococcin G (Nissen- Meyer et al., 1992) and brochocin-C (McCormick *et al*., 1998). Subgroup c comprises thiol-activated peptides,

which require reduced cystein residues for activity e.g., lactococcin B(Venema *et al* 1993). Class III is a large heat labile proteins which are larger than 10 kDa such as helveticin J, lactacins A and B (Joerger and Klaenhammer, 1986).
Class IV is a Complex bacteriocins for which proteins require another chemically different moiety (lipid, carbohydrate) for activity such as plantaricin S, lactocin 27, and leuconocins.

Enterococcus faecium and *E. faecalis* are indigenous species in the gastrointestinal tracts of humans and animals. Great attention has been focused on the development of these strains as probiotics due to their benifcial health effects on the host, which include the inhibition of tumour cell line growth, improvement of the intestinal immune sysem, treatment of diarrhea, and reduction of hypercholestrolaemia (Reuter 1997, Agerholm-Larsen *et al*., 2000).

Enterococcus faecalis and *faecium* produce bacteriocins inhibiting Gram-positive , food borne and intestinal pathogens, enabling their use as biopreservatives in fermented dairy products(Kang and Lee, 2005)

Bacteriocins produced by *Enterococci* are called enterocins and belong to class II. The enterocins are generally active against other enterocci as well as strains of *Listeria monocytogenes* (Giraffa, 1995). Some enterocins are active against other LAB as well as *Clostredium spp*. (Franz *et al*., 1999).

Only few strains of *Enterococcus spp*. were reported to produce bacteriocins with inhibitory activity against Gram- negative bacteria. For example, enterocin 4 is produced from *Ent. faecalis* INIA4 (Joosten *et al*., 1996) and bacteriocin ST15 is produced by *Ent. mundtii* (De Kwaadsteniet *et al*., 2005).

The aim of this work was to isolate an Enterococuus spp. strain fromEgyptian local fermented milk that produces bacteriocin active against both against Gram-negative and Gram-positive bacteria.

MATERIALS AND METHODS

Gram-positive and negative bacteria that are used as indicators for testing the effect of bacteriocin were obtained from BFEL collection (BFEL, Institute for Microbiology, Kiel, Germany).

The G(+) *Enterococcus faecalis* 90519, *Staphylococcus aureus* 91161, *Staphylococcus epidermidis* 91112, and *Listeria monocytogenes* were grown in BHI broth (Biolab, Merck) at 37°C while Gram-negative *Escherichia coli* 98082, *Enterobacter aerogenes* 9805*Serratia marcescens* 98027 , and *Salmonella typhimurium* were propagated in LB broth (Biolab, Merck) and incubated at 37°C.

Bacterial strains were isolated from the Egyptian fermented milk. Number of samples were of Laban rayeb (natural fermented milk) and Yoghurt made my large factories or small dairy shops.

Two ml of each sample were inoculated into 8 ml of MRS broth and incubated at 37° under anaerobic conditions using a Gas Generation Kit (Oxoid) for lactobacilli isolation. Another 2 ml of the sample were inculated

into M17 broth which incubated aerobically at 30°C for 18-24h to isolate lactic acid cocci. Streaking was done onto respective agar media and selected single colonies were collected and grown in MRS or M17 broths tubes under the same conditions mentioned above, then repurified again by streaking. A total of 177 isolates were isolated from the samples and the purity of each was ascertained by microscopic examination. Bacterial isolates were kept as stock cultures frozen at -80°C in their respective broths supplemented with 30% of sterilized 87% glycerol. Each isolate was twice before further studies**.**

Lactobacilli and lactic acid cocci isolates were grown for overnight under conditions described above and was cooled at 4°C for 15 min before centrifugation. Cells were collected by centrifugation at 16000 rpm for one minute at room temperature using eppendorf microcentrifuge, Germany. The supernatant was adjusted to pH 6.5 using 1M NaOH to eliminate the effect of lactic acid if present on the test organisms. Then, CFCS was treated with catalase enzyme(5mg/ml) to avoid the inhibition due hydrogen peroxide production.Treated CFCS was incubated at 37°C for 1h and then sterilized by passing through 0.45 µm membrane at room temperature (Bondage *et al.*, 2001).

Disc diffusion method of Bhunia *et al*, 1988 and Zamfir *et al*, 1999 with slight modification was used to detect the inhibition zone. A 5 ml of soft agar (0.7% w/v agar) was mixed with 200µl of the prepared suspension cells of the bacterial indicator (OD_{620nm} 0.45-0.5) and was poured on the surface of a solidified BHI agar plate. Fiter paper discs of 6 mm were laid on the top of the soft agar lawn layer. Ten or 20 µl of sterilized CFCS were spotted on the discs. Plates were incubated overnight at 37°C and the inhibition zone was measured in mm. A clear zone of inhibition of at least 2 mm in diameter was recorded as positive (Cadirci and Citak, 2005).

Bacteriocin-poducing strain was identified on the basis of its microscopic morphology, bicameral and genetic properties. The strain was grown in MRS broth at 30°C for overnight and was tested for gram staining.

Catalase reaction and cell morphology according to Marshall, 1993. Growth at 10 and 45°C in MRS broth for overnight was done as described by Batdorj *et al*., 2006.

The bacteriocin-producer strain was identified genetically using PCR-16S rRNA gene amplification of chromosomal-DNA (extracted according Leenhouts *et al*, 1990) by universal primers namely, 16SF (Carroll *et at*., 2000) and DG74 (Greisen *et al*., 1994) (MWG-biotech company,Germany). Polymerase chain reaction process was performed using a Thermocycler (Mastercycler 5333) Eppendorf AG- Germany (Reaction was carried out at 93°C/5 min for initial denaturation, 92°C/ 1 min for denaturation, 55°C/1min for annealing and 72°C/ 2 min for elongation).

The PCR amplified product was purified by PCR clean-up protocol(Nucleospin Extract ll, Macherey-Nagel, Germany) before ligated with pBluescrpt II SK Vecctor and transformed into chemical competent cells of *E. coli* (Nova blue) for cloning. White and blue colonies were picked up from Lb agar medium containing Ampicillin, X- Gal and IPTG and were applied for plasmid DNA isolation using standard protocol of Nucleospin plasmid quick Pure (Macherey & Nagel, Germany). After growing in LB broth containing

ampicillin, analysed by agarose gel electropherisis and DNA bands were visualized and photographed under UV light using a Gel Doc™ XR system and quantified by Biorad software. DNA Sequence was performed with the Thermo Sequence Cycle Sequencing kit (Amersham) using T7, T3 promoter primers at MWG-Biotech, Germany. Sequence analysis was performed using Molecular Biology software as DNASIS, DS gene. Data base searches and sequence comparisons through gene bank were made using Blast N, Blast P, and Blast X programs. All molecular biology techniques including ligation, cloning, restriction enzymes digestions, purification of PCR products and plasmid miniprep were performed accoding to instructions of manufacturers or protocols given by Sambrook *et al*, 2001

Arbitrary units (AU ml^{-1}) of bacteriocin activities were calculated according to Yamamoto et al, 2003. CFCS or partially purified samples were twofold serially diluted with deionised water or phosphate buffer (pH 6.5) and then 10 or 20 µl were spotted onto the discs laid over the overlaid lawn of *Enterococcus faecalis* 90519 as a sensitive indicator. The plates were incubated at 37°C overnight, and titre was defined as the reciprocal of the highest dilution (2^n) that resulted in inhibition of indicator lawn.Thus, the AU of antibacterial activity per millilitre was defined as 2^n x 1000 µl 10 or $20µl^{-1}$.

GMRS, GM17, LMRS, LM17, and BHI broths media were tested for their ability to support bacteriocin production. An overnight culture of Bacteriocin-producer strain was inoculated at a 2%, v/v rate (OD_{620nm} = 1.784) into 100ml of each medium and incubated at 30°C and 37°C respectively without agitation for 24h without controlling the pH to determine the time course of bacteriocin production. Samples were taken at appropriate intervals (0, 1, 2, 4, 6, 8, 10, 12, 14, and 24h) and the antimicrobial activity of neutralized CFCS was assayed either by determination of inhibition zone diameter (Cadirci and Citak, 2005) or by serial dilutions and calculate AU/ ml as described previously after measuring both pH and OD_{620nm} (Yamamoto *et al*, 2003). The GMRS broth proved to be the best medium for bacteriocin production; therefore it was used throughout the study.

The effect of enzymes (proteinase K, catale and trypsin), medium pH and temperature on the bacteriocin antibacterial activity was studied.

Tow ml of CFCS were incubated with 1 mg ml^{-1} and 0.1 mg ml^{-1} proteinase K, with 5 mg ml^{-1} of catalase and with a mixture of 0.1 mg ml^{-1} proteise K and 5 mg ml^{-1} catalase at at 37°C for 24h. The antibacterial activity was determined by disc diffusion method (Bhunia *et al*., (1988) and Cadirci and Citak, (2005).

The partially purified bacteriocin was tested by incubating 500 µl of the bacteriocin with either 1 mg ml^{-1} of proteise K and 1 mg ml^{-1} of trypsin at 37°C for 2h. The residual activity was determined as AU/ml using disc diffusion method (Bhunia *et al*, 1988 and Yamamoto *et al*, 2003).

The effect of pH on the bacteriocin activity was determined by adjusting 3ml of the CFCS to specific pH (from 2.0 up to 12.0) with sterile 1M NaOH. The tubes were kept for either 30 min or 2 h at 37°C, then their pH was adjusted to 6.5 (De Kwaadsteniet et al, 2005) before the antibacterial activity was determined (Cadirci and Citak, 2005).

The effect of temperatures on bacteriocin activity was tested by heating 500 µl of CFCS at 60°, 70° and 100°C and were kept at each temperature for 15, 30 and 90 min. Sterlization of CFCS at 121°C for 20 min was also detemined.

The effect of temperature on partially purified bacteriocin activity was determined by exposing 500µl of the bacteriocin to different heat temperatures. Heating at 70°C for 30 min, 100°C for 15 and 30 min and 121°C for 20 min were used and the activity units (AU/ml) were determined (Bhunia *et al*, 1988 ; Zamfir *et al*, 1999 and Yamamoto *et al*, 2003).

An 14-h-old culture of *E. faecalis* AK71 was inoculated (2%, v/v, OD_{620nm} = 1.864) into 5L MRS broth and incubated at 30°C for 12-14h, without agitation. The supernatant which was obtained by centrifugation at 10000 rpm at 4°C for 15 min (Beckman centrifuge, J.A20.1, USA) was adjusted to pH 6.5 using 40% NaOH and kept cold in an ice bath.

The peptide was precipitated from one litre of neutralized CFCS with the slow addition of a 50 % ammonium sulphate (Sambrook et at, 1989) while stirring at 4°C for 12h. The suspensions were centrifuged at 15000 rpm for 1h at 4°C (Beckman centrifuge, JLA-16, USA). The salted-out proteins were dissolved in 30 mM sodium phosphate buffer (pH 6.5) to 1.5% of the initial volume (Maisnier-Patin et al, 1996) and then dialysed against either distilled water or the same buffer for 24h at 4°C with gentle stirring using Spectra/Por tubing membrane with MWCO 6-8 kD cut off (Ohmomo et al, 2000).

The dialysate was concentrated by dialysis against 50% PEG 20,000 solution (pH was adjusted to 6.5 by NaOH pellets) with gentle stirring at 4°C for overnight using the Spectra/Por tubing membrane with MWCO 6-8 kD cut off. The samples were stored at -20°C until use. Both dialysed and concentrated samples were tested for bacteriocin contents and its antibacterial activity (AU/ml).Their protein concentration was determined according to Bradford (1976).

The samples after dialysis and concentration were applied for fractionation by Chromabond C18 column (Water Millipore, Germany) (De Kwaadsteniet *et al*, 2005) using 20% (v/v) isopropanol in 25 mM ammonium acetate (pH 6.5) as washing buffer. The column was washed with the same buffer before loading 1 ml of concentrated sample. Elution was carried out gradually with increasing concentrations of isopropanol in 25 mM ammonium acetate (pH 6.5). Fractions of 2 ml were collected into test tubes and bacteriocin activity was tested by disc diffusion method and represented in mm diameter of clear inhibition zone or AU/ml with serial twofold dilutions.The fractions that showed activity were dried under vaccuum using Vaccuum Concentrator (Bachofer, Germany). Dried residues were resuspended in 5 µl of 30 mM sodium phosphate buffer (pH 6) for activity determination.

Tricine-SDS-PAGE was used to monitor the purification steps of bacteriocin and to determine the molecular mass according to Schägger and Von Jagow (1987). The dialysed samples of CFCS were applied for fractionation by SDS-PAGE. A low molecular mass protein marker with size ranging from 1.060 to 26.600 kDa (Ultra- Low Range Color Marker, Sigma, Germany) was used as standard. After electrophoresis, the gel was fixed and cut ino two vertical parts. The section of the gel containing the sample and

the marker was stained with Comassie blue R- 250. The remaining part, containing only the sample, was washed three times with sterile distilled water containing 0.1 % Tween 80 at room temperature to prevent the effect of SDS on bacteriocin (Yamamoto *et al*, 2003).
The piece of gel containing the bacteriocin spot was tested for its inhibitory effect by placing the piece on the top of a solid BHI agar plate and covered with a layer of the the soft agar containing the bacterial indicator (*Ent. faecalis* 90519). Plates were incubated at 37°C overnight. The inhibitory zone was detemined.

To study the effect of the antibacterial compound on *Ent. faecalis* 90519 at different stages of growth. A 100, 200 , 500 µl, and 2 ml of neutralized and dialysed CFCS (6400 AU/ml) were separately added to 10 ml growing cells in the early exponential phase(OD_{620nm} = 0.45-0.5) at the onset of the growth and after 1, 2, 3 h of growth. The OD (620 nm) of the culture and cell counts were determined at appropriate intervals. Colony forming units were enumerated on BHI agar plates (De Kwaadsteniet *et al*., 2005 and Ivanova *et al*., 1998).

Adsorption of bacteriocin to the producer strain was studied according to Yang *et al*, (1992). After growth of The bacteriocin producer strain (*Ent. faecalis* AK71) in GMRS broth for 18 h at 30°C, 300 ml of the culture was adjusted to pH 6.0, centrifuged at 15000 rpm for 15 min at 4°C and washed in sodium phosphate buffer(pH 6.5). The cells were resuspended into 10 ml of 100 mM NaCl at pH 2.0 and stirred for 1 h at 4°C. Cell suspensions were centrifuged under the same conditions and the supernatant were tested for bacteriocin activity after re-neutralization to pH 6.5-7.0.The CFCS that resulted from the centrifugation of the culture in the first step was tested for activity units (AU/ml) as a control.

RESULTS AND DISCUSSION

Isolation, screening and identification of the bacteriocinogenic strain AK71

One hundred and seventy seven cultures were isolated from the Egyptian fermented milks and their morphology was examined under the microscope. They were identified as 104 lactobacill and 73 lactic acid cocci.

All isolates were screened for bacteriocin production against pathogenic indicators using disk diffusion method (Bhunia *et al*., 1988 and Zamfir *et al*., 1999)**.** Fifteen of them were able to inhibit the growth of number of the indicators. However, strain AK71 was the one selected as the bacteriocin producer strain because it formed a large and clear inhibition zone (table 1)**.**

Table (1): Screening of lactic acid cocci isolates for bacterocin production against pathogenic indicators

Isolates number	Indicators							
	G(+)				G(-)			
	S. aureus	*S. epiderm*	*Ent. Faecalis*	*Lis. Mono*	*E. aerogenes*	*E. coli*	*Salm. typh*	*Ser. marcescens*
51	+ (8mm)	-	-	-	-	-	-	+ (8mm)
52	+ (8mm)	-	-	-	-	-	-	+ (8.5mm)
60	+ (9mm)	-	-	-	+ (8mm)	-	-	-
101	-	-	-	-	+ (9mm)	-	+ (8.5mm)	-
43(AK K71)	+ (18mm)	-	+ (18mm)	-	-	-	-	+ (18mm)

- **The isolates were grown into M17 broth in the presence of the buffer of M17 (Na-ß-glycerophosphate.5H_2O) and incubated at 30°C for 18-20h (filter paper disc diameter is 6mm, volume of toxin solution is 10 μ l)**
- **(+) symbol means inhibition zone was formed and (-) symbol means there was no inhibition zone.**

The selected strain (fig 1) was catalase-negative, Gram-positive diplococci able to grow at 10 and 45°C. For proper identification, the PCR-16S rRNA gene amplification of chromosomal DNA using universal primers, 16SF and 74DG, was carried out. The PCR amplified product was ligated with pBluescipt II vector and transformed into chemical competent cells of E. coli (Nova blue) for cloning. The resultant clones were apllied for plasmid DNA isolation (fig 2). DNA sequence was carried out and the data were analysed and the resulting sequence was 99% identical to those of various *Ent. faecalis* strains and in particular to *Ent. faecalis* V583. Thus, the bacteriocin-producer strain was identified ambiguously as to the species *Ent. faecalis*.

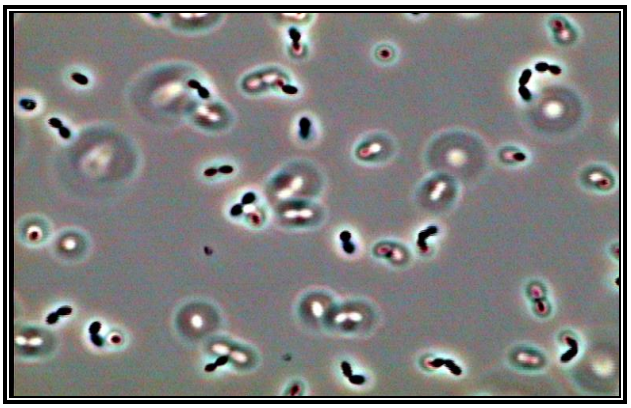

Fig.1: Morphology of Ent. faecalis AK71, Bacteriocin-producer strain isolated From Egypt- tian Yoghurt-like product (Zabady).

Fig. (2): Isolation of Clones pDNA of 16S rDNA fragment of bacteriocin-producer isolate from *E. coli* (Nova blue) transformants. Lane 1: λSty1; lane 2 and 8 represent the control (blue colonies which represent the pBluescrpt II SK Vecctor); lane 3, 4, 5, 6, and 7 represent the clones pDNA of successful transformation (white colonies, in which pBluescrpt II SK Vecctor ligated with 16S rDNA fragment were transformed into chemical competent cells, *E. coli* (Nova blue). 0.8 % agarose gel was used for electrophoresis analysis.

Figure (3) illustrates bacteriocin production during bacterial growth stages. The excretion of enterocin-like compound AK71 into the growth medium started during log phase (after 4 h of incubation) and reached a maximum titre of 1600 AU ml^{-1} afer 12 h of incubation at the stationary phase in MRS broth at 30°C. The activity was stable at the 14^{th} h but decreased after 24 h of incubation reach to 800 AU ml-1. During the 24 h of growth, the pH of the GMRS broth decreased from 6.62 to 5.0 at maximum toxin production during the stationary phase, then the pH decreased slightly to 4.58 at the end of this stage. The optical density at 600 nm of the culture increased from 0.123 to 2.022 (fig 3). In different media, low levels of bacteriocin AK71 activity were recorded. The activity was 200, 100, 200, 100, 400, 200, 200, and 0 AU ml^{-1}, in LM17/ 30°C , LM17/37°C , GM17/ 30°C , GM17/37°C, LMRS/ 30°C, LMRS/ 37°C, GMRS and BHI/ 37°C respectively (table 5).

The antimicrobial activity (AU ml^{-1}) towards *Ent. faecalis* 90519, protein concentration (mg ml^{-1}), and the specific activity (AUmg^{-1}) of bacteriocin AK71 upon partial purification are shown in (table 2) The activity detected in the crude bacteriocen was 400 au ml^{-1}.

After purification a specific activity of 65863 AUml^{-1} was obtained which represents 17.8 times the activity in the crude extract. The protein concentration was reduced to 8.9% of the crude extract contents.

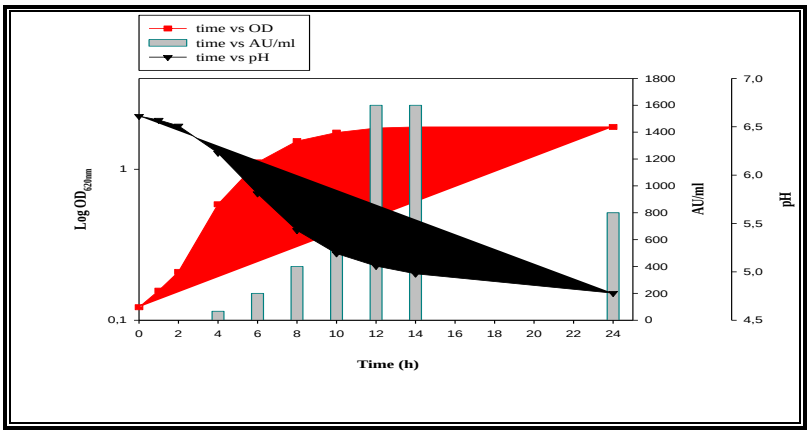

Fig. 3: Production of bacteriocin Ak71 produced by *Ent. faecalis* in GMRS Broth at 30°C.

Table (2): Partial purification of bacteriocin-like compound produced by *Ent. faecalis* AK71

Purification stage	Volume ml	Bacteriocin activity, AU/ml	Activity purification ratio	Protein conc. mg/ml	Specific activity AU/mg protein	Purification ratio of protein	Recouv-ery of protein, %
Crude extract (CFCS)	35400	400	1	0.170	2344.66	1	100
Dialysed 50% amm.sulphate-CFCS	1114	12800	32	2.538	5043.342	2.2	47
concentra-ted sample by PEG 50%	179	51200	128	14.755	3471.18	1.5	44
Fractionation by Chromabond C18	358	62577.8	156.4	1.5	41718.5	17.8	8.9
Concentration Under vacuum	4.5	52690.5	131.7	0.8	65863.1	28.1	0.047

- **The protein concentration of the samples were calculated after applying the samples to photometer to determine the absorbance at 595 nm according to Bradford 1971.**
- **Au/ ml was considered as the reciprocal of the highest twofold dilution of the bacteriocin samples that showed clear inhibition zone against the pathogenic indicator(Ent. faecalis 90519) after overnight incubation of plates at 37°C**
- **Total Au was calculated by multiple AU/ ml by the volume**
- **Specific activity was determined by dividing total Au on total protein conc.**
- **Concentration ration was calculated by dividing the specific activity of purification steps on the specific activity of the first stag of purification**
- **Fractionation using Chromabond C18 carried out by 20%(v/v) isopropanol in 25mM ammonium acetate(pH 6.5) as a washing buffer and the bacteriocin was eluted with 40% or 60% isopropanol in 25 mM ammoniun acetate(pH 6.5)**
- **Concentration and evaporation of isopropanol from samples was done under vacuum for 5-6 h using 1.5 ml eppendor.**

Table (3) presents the effect of various treatments on the bacteriocin activity in the crude extract measured as diameter of the inhibition zone against Ent. Faecalis 90519 and *St. aureus* 91161. The activity of bacteriocin AK71 was not affected on heating of CFCS at temperatures of 60° or 70°C for 90min. (the inhibition zone occurred was ca 18 mm by either control or tested samples). Bacteriocin activity started to be affected at 100°C, to lose about 40% of it at 15min, and then completely deactivated byond this 15min period. Heating treatments above the 100°C deactivated the bacteriocin.

No change in activity was observed after incubation of CFCS for 30 min on 2h within a pH range between 3 and 8 (the inhibition zone was ca 18 mm either by control or tested samples against *Ent. Faecalis* and *St. aureus*).While bacteriocin was completely inactivated at pHs lower higher than the above range.

Catalase-treatments at (1 and 5 mg/ml) did not affect bactrerocin activity. Complete inactivation of antimicrobial activity was observed after treatment of CFCS with proteinase K (0.1 and 1 mg/ml) (figure 5) or a mixture of catalase and proteinase K (5mg/ml of and 1mg/ml of respectively). The same factors were studied with partially purified bacterocin and the effect was determined by measuring unit of bacterocin activity and is reported in table (4).

The measure of the effect on bacterocin activity of course was more accurate and the inhibition zone values. Maximum inhibition zone was obtained at 12800 Au/m but the zone was decreased from 18mm into 12mm by decreas the activity into half of its concentrate (6400Au/ml). while 3200Au/ml concentrate did not show any inhibition zone.

Table (5) presents minimum bacterocin concentration that produce the inhibition against number of bacterial indicators. CFCS or partially purified (dialysis after ammonium sulphate precipitation) samples containing Bacteriocin AK71 were active against *Ent. faecalis* 90519, *St. aureus* 91161, and *Serr. Marscens* 98027. However, *Staphylococcus epidermidis* 91112, *Listeria monocytogenes, Enterobacter aerogenes* 9805, *Escherichia coli* 98082, *Salmonella typhimurium* were resistant (table 5). The most sensitive indicator was *Ent. faecalis* 90519. The producer strain was completely resistant to bacteriocin AK71 it produced. The most sensitive indicator (*Serr. Marscens* 98027) was inhibited with 800Au/ml, this was followed by *St. aureus* 91161 (3200Au/ml) and the least affected was *Ent. Faecalis* 90519 (51200Au/ml).

Separation of diylised CFCS after ammonium sulphate precipitation by tricine-SDS-PAGE against different molecular weight markers clearly indicated that bacteriocin AK71 is a peptide with a approximate 4 kDa molecular weight (fig 4)**.**

Figure (6) illustrates the effect of various bateriocin concentrations on the growth of *Ent. faecalis* 90519. The bacteriocin decreased the growth rate of the indicator. The effect increased with bacteriocin concentration (100, 200, 500, and 2000 μl of 6400Au/ml). Addition of 2 ml bacteriocin AK71 caused a half-log reduction in the count compared to the control. The (log CFU ml^{-1} of the control was reduced from 8.3 in the control into 7.8 in the sample treated

with 2ml. The optical density of the culture somewhat decreased from 1.585 for the control to 0.901 for the treated sample. Therefore, bacteriocin AK71 exerts a bacteriostatic action on the growth of *Ent. faecalis* 90519. the inhibition effect increased with the bacteriocin concentration.

No antibacterial activity was found after treatment of the *Ent. faecalis* AK71 cells, bacteriocin-producer strain, with 100 mM NaCL at pH 2.0 (Yang et al, 1992). This means that *Ent. faecalis* AK71 produces the bacteriocin extracellularly.

Table (3): Factors affecting *Ent. faecalis* AK71 bacteriocin-like compound activity in the crude extract

Treatment	**inhibition diameter zone, mm**	
Heat treatment	untreated sample	treated sample
60°C		
15min	18 mm	18mm
30 min	18mm	18mm
90 min	18mm	18mm
70°C		
15 min	18mm	18mm
30 min	18mm	18mm
90 min	18mm	18 mm
100°C		
15 min	18mm	12mm
30 min	18mm	0mm
90 min	18mm	0mm
121°C/ 20 min	18mm	0mm
pH		
2	18mm	3mm
3	18 mm	18 mm
4	18mm	18mm
5	18mm	18mm
6	18 mm	18mm
7	18mm	18mm
8	18mm	18mm
9, 10, 11 and 12	18mm	0mm
Enzymes		
Catalase		
1 mg/ ml	17mm	17mm
5 mg/ ml	17mm	17mm
Proteinase K		
0.1mg/ ml	17mm	0mm
1mg/ ml	17mm	0mm
5mg catalase+ 1mg proteinase K	17mm	0mm
Media		
LM17/ 30°C		200 AU/ ml
LM17/37°C		100 AU/ ml
GM17/ 30°C		200 AU/ ml
GM17/37°C		100 AU/ ml
LMRS/ 30°C		400 AU/ ml
LMRS/ 37°C		200 AU/ ml
GMRS/30°C		800 AU/ ml
GMRS/ 37°C		200 AU/ ml
BHI at 30 and 37°C		0 AU/ ml

- **The activity is measured against *Ent. faecalis* and Staph. aureus.**

Table (4): Factors affecting the activity of the purified bacteriocin:

treatment	bacteriocin activity(AU/ml)	% of residual activty
Control	51200	100
Heat treatment		
70°C/30 min	12800	25
100°C/15 min	6400	12.5
100°C/30 min	3200	6.25
121°C/20 min	0	0
pH (after 2h):		
2	+	0
3	12800	25
4	51200	100
5	51200	100
6	51200	100
7	51200	100
8	12800	25
9, 10, 11, 12	0	0
Enzymes (2h/37ºC		
Catalase 5 mg/ml	51200	100
Trypsine 1mg/ml	51200	100
Prteinase K 1mg/ml	51200	0

- **(+) means there was some avtivity of original samle but not after dilution.**

Table (5): Ent. faecalis AK71 bacteriocin minimum inhibitory concentration against indicators

bacteri-ocin prod. strain	Indicators							
	G(+)				G(-)			
	S. aureus	***S. epiderm***	***Ent. faecalis***	***Lis. mono***	***E. aerogenes***	***E. coli***	***Salm. typh.***	***Ser. marcescens***
CFCS	800	**0**	1600	**0**	**0**	**0**	**0**	**800**
Partially purified bacteriocin	3200	**0**	51200	**0**	**+**	**0**	**0**	**8/00**

- **The CFCS was collected after growth of Ent. faecalis into GMRS for 12-14h at 30°C**
- **The determination carried out using serial twofold dilutions of CFCS in d.d.water with exclusion of the effect of acidity and H_2O_2 (by adgusting pH to 6.5 and treatment with catalase(45unit/ml) respectively.**
- **Disc paper diffusion method was used for determination by spotting 10 and 20 µl of neutralized and catalase treated CFCS over the disc stabbed on overlaid indicator(200 µl culture of OD_{620nm}= 0.45 + 5 ml soft agar poured over the prepourd base medium. Antibacterial activity was determined after overnight incubation of plates at 37°C.**
- **(+) means there was some avtivity of original samle but not after dilution.**

DISCUSSION

The isolated bacteriocin AK71 fits the Klaenhammer (1988), definition of bacteriocins. Bacterocin AK71 inhibited the growth of Gram-positive, *Ent. faecalis* 90519, and *St. aureus* 91161 as well as the gram negative *Serr. marscens* 98027. Some inhibition was observed for *Enterobacter aerogenes*

9805. Gram-negative bacteria are usually considered to be resistant to the bacteriocins of LAB and this has been attributed to the impermeability of the outer membrane to these substances (Kordel and Sahl, 1986 and Stevens *et al*., 1991). Similar reports stated that a number of bacteriocins produced by enterococcus ssp. can inhibit a limited number of Gram-negative bacteria.

Bacteriocin AS-48 produced by Ent. faecalis S-48 was the first enterocin described with inhibiting activity against Salmonella and E. coli (Galvez et al, 1986 and Galvez *et al*., 1989). Enterocin ST15 produced by Ent. mondtii (De Kwaadsteniet *et al*., 2005), entericin GM-1, produced by Ent. faecium was active against E. coli and Salm. Typhimurium (Kang and Lee, 2005) and enterocin MR99 was reported to be active towards E. coli, Shigella sonnei and Shigella flexneri (Sparo *et al*., 2006).

Enterocins, bacteriocins produced by enterococci, are known to be generally belong to class II, non lantibiotic, small and heat stable peptide (Franz *et al*., 1999). Bacteriocin AK71 probably belongs to class II since it is about 4 kD (<5 kDa) and resistant to 100°C for 30 min. Further investigation is needed for accurate classification of this bacteriocin.

Best medium for maximum bacteriocin production was GMRS.

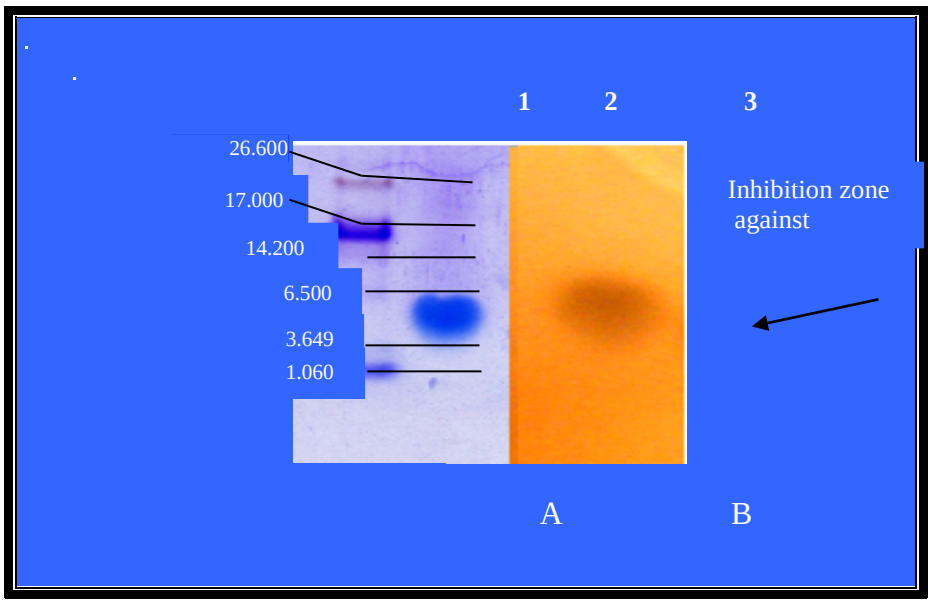

Fig. (4): Tricine-SDS-PAGE of bacteriocin produced by *Ent. faecalis* AK71. Lane A(1): molecular weight marker; LaneA(2): bacteriocin (protein) band ; Lane B(3): inhibition zone corresponding to the position of bacteriocin band.The first half of the gel was stained with Coomassie Blue R250 while the another half was overlaid with *Ent. faecalis* as indicator which imbedded into BHI soft agar.Clear inhibition zone was determined after overnight Incubation of plate at 37°C .

The bacteriocin AK71 was produced after 4 h of growth in GMRS broth, during the logarithmic phase of *Ent. faecalis* growth and the highest level of activity was obtained after 12 h, at the end of log phase and the beginning of the stationary phase of the growth. This was comparable with most other reported bacteriocins produced by LAB (De Vuyst and Vandamme, 1994; Galvez *et al*, 1998; Du Toit *et al*., 2000; Chin *et al*., 2001; Nieto- Lozano *et al*., 2002; Cheigh *et al*., 2002; Aktypis and Kalantzopoulos, 2003 Yamamoto *et al*., 2003; De Kwaadsteniet *et al.,* 2005; Martin-Platero *et al*., 2006 and Kabuki *et al*., 2007). The activity was stable for at least 2 h at the highest level of activity (1600 AU $^{ml-}$) then decreased after prolonged incubation reashing 800Au/ml after 24h. This reduction in activity may be explained either by degradation of the bacteriocin by extracellular proteases, low pH which reached 4.7, or readsortion by the producer cell surface (Parente and Hill, 1992; Parente and Ricciardi, 1994 and Du Toit *et al.,* 2000). During the growth and production of bacteriocin AK71, pH decreased to 5.06 which corresponding to the highest level of activity which accumulated during the first phase of growth before the pH decreases to under 5.0, then the pH reached 4.7 at which the activity decreased and this was reported by many workers (De Kwaadsteniet *et al.,* 2005). It is generally known that growth conditions affect cell growth and bacteriocin production (Juarez-Tomas *et al.,* 2002 and Kabuki et al., 2007). Bacteriocin AK71 is active at pH range between 3.0 and 8.0 (with some activity at pH 2.0). The isoelectric point of all known enterocins are a bove pH 8.3, which implies that the soluability increases with the decreasing pH (Foulquie-Moreno *et al.*, 2003). This cofirms that the bacteriocin AK71 is one of class II and makes it a good candidate for use as preservative in acidic foods.

Bacteriocin AK71 became completely inactive after treatment with proteinase K proving to be a protein and belongs to the bacteriocins family and its safety for application in food preservation. Resistance to treatment with catalase ascribed that the inhibition zone was not due to H_2O_2. Trypsin treatment slightly affected the bacteriocin AK71 activity which is similar to some bacteriocins like enterocin GM-1(Fig. 5) (Kang and Lee, 2005; Kabuki *et al*., 2007).

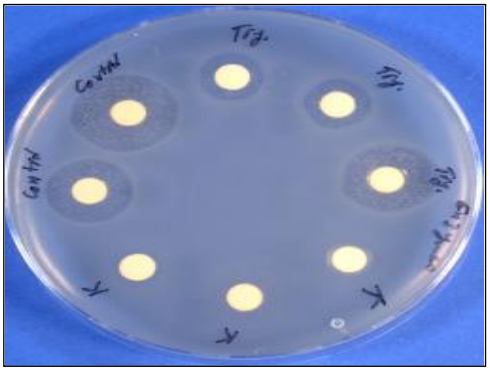

Fig. 5: Effect of enzymes,trypsin and proteinase K, on the antibacterial activity produced by *Ent. Faecalis* AK71.

Partially purified bacteriocin AK71 was heat tolerant even up to 100°C for 30 min at pH 6.5 similar to most bacteriocins (Klaenhammer, 1988; Lee *et al.*, 1999; Okkers *et al*, 1999; Todorov *et al*., 1999; Du Toit *et al*., 2000; Ko and Ahn, 2000; Messi *et al*., 2001; De Kwaadsteniet *et al*., 2005 Martin-Platero et al., 2006 and Kabuki *et al*., 2007). The activity was lost by autoclaving and this trend of behaviour is alike to lactocin MMFII (Ferchichi *et al.*, 2001). Lactocin NK24 (Lee and Paik, 2001) is more tolerant than bacteriocin AK71. Temperature of 100°C for 30min reduced the activity of lactocin by 87% while bacteriocin AK71 was reduced by 94%. Enterocin RJ11 did not lose any activity at 100°C either for 15 or 30 min(Yamamoto *et al*., 2003). On the other hand, enterocin 012 ((Jennes *et al*., 2000), and enterolysin A, produced by Ent. faecalis LMG2333, (Nilsen *et al.,* 2003) are sensitive when treated with heat. These results proved that the isolated bacteriocin can by used for heat preservation of foods.

The bacteriocin AK71 showed a bacteriostatic effect towards, Ent. faecalis 90519, in contrast to most enterocins which displayed a bactericidal effect (Galvez *et al*., 1998). It is well known that the mode of inhibition of bacteriocins depends on the available bioconcentration, and on the nature and the physiological stage of the target strain (Foulquie-Moreno *et al*., 2003).

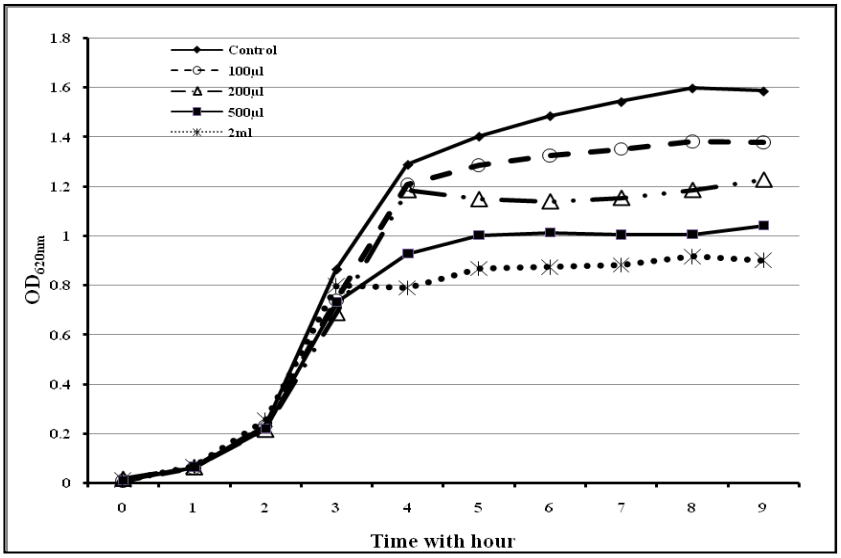

Fig. 6: Effect of different concentrations of bacteriocin AK71 on the growth of Ent. faecalis 90519 after 3h of incubation at 37°C.

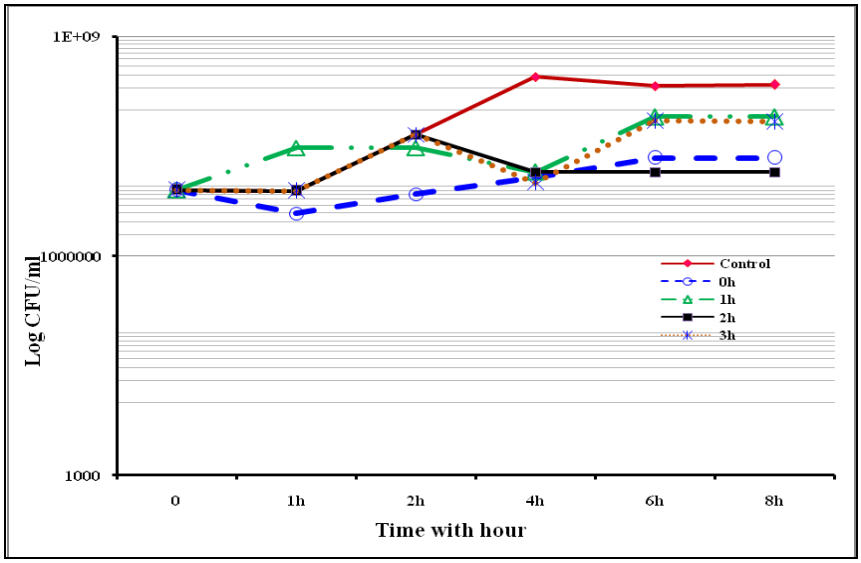

Fig.7: Effect of bacteriocin AK71 (6400 AU ml-1 on the survival of sensitive indicator, Ent. faecalis 90519.

Bacteriocin AK71 is secreted extracellularly to the medium and does not adhere to the cell surface since there was no bacteriocin activity detected after treating of *Ent. faecalis* AK71, producer strain, with NaCL at pH 2.0. Similar results have been recorded for pediocin ST18 (Todorov and Dicks, 2005) and enterocin ST15(De Kwaadsteniet *et al.,* 2005). In the other side, adsorption of bacteriocin plantaricin to the producer strain, Lb. plantarum C19, was reported where tha the adsorption was maximal at pH between 5 and 7, with a complete loss of adsorption and adherence to the cell surface at pH 1.5 and 2.0(Atrich et al., 2001).

Although SDS-PAGE is not accurate technique to determine the molecular mass of small, hydrophobic bacteriocins like enterocins (Cintas et al., 1995), it gives valuable information about the presence of either one or two peptides (De Vyust *et al.,* 1996 and Moreno et al., 2002). For the partially purified bacteriocin AK71, in this study, a single inhibition zone was observed indicating the production of one enterocin with low molecular weight of approximately 3.999 kDa (ca 4kDa) and it can be concluded that the bacteriocin was not inactivated by SDS.

In brief, *Ent. faecalis* AK71 produced an inhibitory substance that is a proteinaceous in nature, resistant to a wide range of pH and heat with low molecular weight and has a broad spectrum of bacteriostatic activity rendering it to be one of the bacteriocin family (named bacteriocin AK71) and a good candidate as a natural food and dairy products preservatives.

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عزل وتنقية ودراسة خواص بكتريوسين (انتيروسين٧١) ذى نشاط فعال ضد بعض البكتريا المرضية الموجبة والسالبة لجرام والمنتج بواسطة Enterococcus faecalis AK71

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استهدف هذا البحث وتمكن من عزل واستغلال أحد السلالات البكتيرية - التي صنفت على على كونها *Enterococcus faecalis* Ak 71 – في إنتاج ماده فعالة مضادة للميكروبات المرضية. حيث تم عزلها من مصدر لبني متخمر مصري وصنفت واستغلت في إنتاج مادة الـ Bacteriocin بتنميتها في بيئة GMRS على درجة تحضين 30 م° لمدة 12 ساعة كأفضل ظروف إنتاجه.

وقد أظهرت هذه المادة قدرة فعالة بلغت قيمها (بـ AU/ml) 51200 ,3200 ,800 ضد البكتيريا المرضية المختبرة الحساسة تجاهها وهى على الترتيب *Enterococcus faecalis* 90519 ، *Staphylococcus aureus* 91161، *Serratia marcescens* 98027. .

كما تمت التنقية الجزئية للـ Bacteriocin (Enterocin AK71) معتمدة على أربع خطوات رئيسة وهى على التوالي: الترسيب باستخدام Ammonium sulphate(50% تشبع) التركيز باستخدام محلول Polyethylene glycol 50%، التنقية باستخدام عمود Chromabond C18 ثم التركيز تحت تفريغ. وقد كان لهذه التنقية أثراً في زيادة فعالية Enterocin AK71 من AU/ml 400 في الـ CFCS إلى 12800 و 51200 و 62277 و 52690 طبقا لتوالي الخطوات السابقة.

هذا وقد تمثلت خصائص الـ Enterocin AK71 في كونها:

1. مادة بروتينية تحللت بواسطة إنزيم Proteinase K والذي أبطل مفعولها بينما أظهرت المادة ثباتاً ولم تبد تأثرا عندما عوملت بأنزيمي Catalase و Trypsin.
2. أظهر Enterocin AK71 ثباتاً على مدى واسع من درجات الحموضة pH (من 3 : 8) بعد ساعتين من التحضين على 37 م° ولم تتغير قدرته الفعالة بينما زالت قدرته عند pH 2، 9 ، 10 ، 11 ، 12.
3. أظهر ثباتاً ضد المعاملة الحرارية حتى درجة حرارة 100 م° زمنية نصف ساعة وإن انخفضت فعاليته بينما انعدمت هذه القدرة الفعالة عندما عومل بحرارة التعقيم (121 م° /20 ق).
4. بلغ الوزن الجزيئي للـ Enterocin AK71 حوالي 4000 دالتون باستخدام SDS-PAGE.
5. القدرة الفعالة للـ Enterocin AK71 قدرة مثبطة Bacteriostatic وليست قاتلة Bactericidal تجاه السلالة المختبرة.
6. هذا النوع من الـ Bacteriocins يتم إفرازه وإنتاجه بشكل كلي وكامل من قبل السلالة البكتيرية المنتجة له إلى خارج خلايا السلالة إلى الوسط الخارجي Extracellular ولا يتم ادمصاصه على جدار الخلايا.