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Determination of the Bacteriocin-like Substances Produced By *Enterococcus hirae* Isolated from Traditional Egyptian Food (Koskos)

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The isolated organisms are screened for their antimicrobial activities against different microbial indicators. These activities expressed in inhibition zones against all tested indicator organisms. PCR products used to assess the DNA similarities and multiple sequences showed 92% identity to *Enterococcus hirae* (accession no.HM584102). Further identification to species level, *E. hirae* was based on pigment and carbohydrate formation. The SDS-PAGE analysis was indicated that bacteriocin of *E. hirae* is a two peptides of approximately 42 and 52 kDa. No antimicrobial activity after treatment of the cells with NaCl at pH 2.0.The leakage of K^+ ion increased as the concentration of bacteriocin increased. The bacteriocins had a negative effect on the respiration, where the amount of consumed oxygen decreased by increasing the bacteriocins. No toxic effect against *Artemia salina* larva was recorded. Complete inactivation of antimicrobial activity was observed with Proteinase K and trypsin, respectively, after incubation at 37°C. No antimicrobial activity was recorded at pH values from 5 to 10. On the other hand, high antimicrobial activity was obtained at pH 3. Heat treatment on bacteriocin showed stability whereas non-significant results at 70°C or 100°C after 15 minutes while complete loss after incubation at 70°C, 100°C and by autoclaving.

Key words: *E. hirae,* bacteriocin, antimicrobial substances, characterization, Egyptian food Koskos.

The bacteriocins comprise a subgroup within the far larger body of natural commercial food preservatives. They are produced by bacteria and possess antibiotic properties, but are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics that can potentially illicit allergic reactions in humans (Cleveland *et al*., 2001). The production of bacteriocins is in general, closely associated with growth of the producer organisms. Bacteriocins activities decrease more or less sharply at the end of the growth phases as a result of degradation by proteases (Hur *et al*., 2000). Bacteriocins are ribosomlly synthesized antimicrobial protein in nature produced by eubacteria (Chen and Hoover, 2003). Bacteriocin production is also affected by the medium composition and culture conditions, such as pH, temperature and agitation (DeKwaadsteniet *et al*., 2005). Therefore, the optimization of environmental conditions is very important for the enhancement of bacteriocin production. Some bacteriocins inhibit food spoilage and pathogenic microorganisms. Bacteriocins have inhibitory effects towards sensitive strains and are produced by both Gram positive and Gram negative bacteria (Tambekar and Bhutada, 2010; Laukova *et al*., 2013). Many

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bacteriocins are produced by lactic acid bacteria (LAB) and are active against food borne pathogens such as *Listeria monocytogenes* or *Clostridium botulinum* (Cleveland *et al*., 2001; Deegan *et al*., 2006). Bacteriocinogenic LAB strains may play a role in the food industry as starter cultures, co-cultures, or bio-protective cultures, to improve food quality and safety (Deegan *et al*., 2006; De Vuyst and Leroy, 2007).

Enterococcus, a member genus of the lactic acid bacteria (LAB) is found in various environments, but more particularly in the intestines of humans and other animals, also it is found in vegetables like- plant materials and fermented foods products (Giraffa, 2002; Koehler, 2007; Helena *et al*., 2014). Bacteriocins are extremely heterogeneous group of antibacterial substances. Although chemically diverse, the one unifying property is the presence of an essential protein component. Some bacteriocins appear to be either simple proteins, glycoproteins (Dicks *et al*., 1992) or lipo-carbohydrate protein complexes (DeVuyst and Vandamme, 1994). In this study, we used sample of the cooked source (Koskos) was left several days to be fermented and then used. Koskos is wheat flour with some butter and hot water cooked together which eat as fresh. The isolation, identification and characterization of *Enterococcus hirae* from traditional Egyptian food and bacteriocin producing ability of LAB and its inhibitory effect against food-borne pathogenic microbes (Gram positive and Gram negative bacteria plus fungus *Candida albicans*) were studied.

MATERIALS AND METHODS

Source of bacterial isolates

A sample of the cooked source (Koskos) was left several days to be fermented and the five gram from cooked fermented sample were suspended into 100 ml of sterile physiological distilled water, serially diluted and then 100 µl was spread onto the surface of MRS (De Man *et al*., 1960). Plates were incubated overnight at 30°C ± 2°C for 24h. Colonies were randomly selected and screened for production of antimicrobial compounds by using the Indicator organisms (Gram positive, Gram negative and *Candida albicans*).

Indicator organisms

Different Gram-positive and Gram-negative bacteria in addition to a fungus were used as indicator organisms in this study. Gram-negative bacteria (*Proteus vulgaris* 1753, *Escherichia coli* 1357, *Enterobacter cloacae*, *Serratia marcescens* 921/79 and *Klibsella pneumonia)* and Gram-positive bacteria *(Citrobacter freundi*as, *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus)*, in addition to *Candida albicans* as a fungus. These microorganisms were kindly provided by Bacteriology Unit, Botany Department, Faculty of Science, Tanta University. The indicator organisms were grown on nutrient agar plates at 37°C for 24h (Lapage *et al*., 1970).

Assay for antibacterial activity

Antimicrobial activity was confirmed by using the agar-spot test method. Overnight cultures of the test organisms were prepared in MRS broth at 30°C ± 2°C then, centrifugated at 7000 rpm for 30min at 4°C and the cell-free supernatants were filter sterilized (Millipore/0.22 µm). Overnight culture of the indicator organisms were prepared and 5×10^5cfu ml^{-1} in 100 µl Potassium-Sodium-Phosphate buffer, (pH: 7) were spread onto nutrient agar plates. 100 µl of sterilized cell free supernatant of the test organisms (adjusted to 6.0 with sterile 1 N NaOH) was loaded into a well made in the center of inoculated plates. The plates were incubated at suitable temperature (37°C) overnight. The antimicrobial activity was showed a clear zone of growth inhibition (Ivanova *et al*., 1998). The largest inhibition zone of the indicator bacterium indicates the most antimicrobial-producing test organisms.

Strain identification

Strain with largest inhibition zone against indicator organisms was selected for further studies. Strain was identified according to the physiological and biochemical characteristics described by (Devriese *et al*.; 1992 and Stiles and Holzapfel, 1997). Pigment formation was tested on Tryptic Soy Broth (Merck) as described by Facklam and Collins (1989). Test for motility was done according to Ball and Sellers (1966). Catalase test was performed as recommended by Koneman *et al*., (1992), also Mannitol salt agar (MSA) (Bachoon and Dustman, 2008); and hemolytic activity (Beecher and Wong, 1994) were tested. Sugar fermentation reactions were recorded by using the API 20 Strep and compared with reactions listed

for enterococci (Devriese *et al*., 1992).

Determination the molecular weight mass by (SDS-PAGE)

Enterococcus hirae was grown in MRS broth for 20h at 30°C. The cells were harvested by centrifugation (7000 rpm, 30min) and the bacteriocin was precipitated from the cell-free supernatant with 70% saturated ammonium sulphate (Sambrook *et al*., 1989). The precipitate was resuspended in 25mM ammonium acetate (pH: 6.5), desalted by using a 1000 Da cut-off dialysis membrane and separated by SDS–PAGE, as described by Garfin (1990). A low molecular weight marker with sizes ranging from 2.5 to 45 kDa was used. The gels were fixed and stained, the molecular weight of bands were determined.

Identification of the antimicrobial material by sequencing the 16Sr RNA

Bacterial genomic DNA is extracted from the cells by DNA extraction kit (Qiagen DNeasy DNA extraction protocol) and used as template for PCR reaction. After DNA extraction method, the DNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify 1500 bp fragment of the 16s rRNA region according to The forward primer was 5'AGA GTT TGA TCT GGC TCA G3' and the reverse primer was 5'TAC GGT ACC TTG TTA CGA CTT 3'. Agarose gel of 0.8g was prepared. Samples of DNA were mixed with the gel-loading buffer, and the mixtures were slowly loaded into the slots of the gel. The electrophoresis was run at 100 volt. The electrophoresis gel was immersed in ethidium bromide solution (5ìg/ml) for 15min and washed in water. The washed gel was examined under ultraviolet light and photographed.

DNA Sequencing

Automated DNA sequencing based on enzymatic chain terminator technique, developed by (Sanger *et al*., 1977), by using 3130 X DNA Sequencer (Genetic Analyzer, applied biosystems, Hitachi, Japan). The specific emissions were detected and the data were collected for analysis as described by Ke *et al*., (1999).

Chemical structure of the antimicrobial materials (The infrared spectra IR)

The infrared spectra of the antimicrobial material were carried out using infrared spectrophotometer (Perkin-Elmer 1430). Small discs were made from the mixture of about 1mg of the tested material and 300mg of pure KBr, followed by pressing into a disc and used for determination of the infrared spectra. The measurements were carried out at infrared spectra between 400 and 4000nm (Sherborok-Cox *et al*., 1984).

Mode of action of antimicrobial materialdsorption studies

Adsorption of the bacteriocin by producer strain was studied by using the method of (Todorov and Dicks, 2005). After 20h of growth in MRS broth at 30°C, 300 ml of the culture was adjusted to pH 6.0, centrifuged (7000rpm, 4°C 30min), washed in sodium phosphate buffer (pH: 6.5), and resuspended in 10ml of 100mM NaCl (pH 2.0, 4°C) by slowly stirring. After 1h the cell suspension was centrifuged, the pH of supernatant adjusted to7.0 and then tested for antibacterial activity as described by Ivanova *et al*. (1998).

Leakage of potassium ions of indicator organisms

Cells of indicator organisms of each culture were harvested, washed twice with phosphate buffer (pH 7) and resuspended in sterile deionized water. 0.5ml of the suspension (5×10^5 CFU/ml) were mixed to 4.5ml buffer containing 0.04, 0.1 and 5mg/ml of the antimicrobial materials and incubated at room temperature in rotary shaker at 150rpm for 30min. The incubated samples were analyzed for potassium ion using flame photometer (Clinical flame photometer 410C) (Oladunmoyem *et al*., 2007).

Respiration of indicator bacteria

The indicator bacteria were grown in nutrient broth containing different concentrations of the tested materials (bacteriocin) (0.125, 0.250 mg/ml) in rotary shaker (150rpm), then bacterial pellet were collected by centrifugation (7000rpm for 30min at 4°C), and washed twice in phosphate buffer. 0.5g cell pellet was resuspended in 50ml phosphate buffer pH: 7 then, allowed at room temperature for 15min. The quantities of oxygen consumed by indicator bacteria using HI 2400 Dissolved Oxygen Bench meter were measured and compared to control (without bacteriocin) (Jacyn Baker *et al*., 2001).

Toxicity of the antimicrobial material

The immature brine shrimp (*Artemia salina*) was used for determination of the toxicity of bacteriocin produced by *Enterococcus hirae* at two concentrations (10, 100 mg/ml). Sterilized vials containing 5ml of filter sterilized sea water (Meyer *et al*., 1982) about 10 active shrimps were added to

each vial. The numbers of survivors and dead animals were counted after 24h and compared with control (without bacteriocin).

Cell lyses

Ten ml of bacteriocin-containing free cell supernatant of strain, *E. hirae* adjusted to pH: 6.5 with 1N NaOH, was added to a 100 ml culture of indicator organisms at the onset of growth (time zero) and again after 1, 2, 3h of growth. The OD (600 nm) of the culture was determined at different time intervals for 11 hours (Todorov and Dicks, 2005).

Proteolytic enzymes treatment

The Proteolytic effect of proteinase K and Trypsin in bacteriocin of the *E. hirae* (pH: 6.5) were incubated with proteinase K at final enzyme concentrations of 25µl of proteinase K (1mg/ml, 1mg/100 µl) or to 25µl of Trypsin (1mg/ml, 1mg/100µl) to give concentrations of 0.1 and 1mg/ml respectively, was mixed and incubate at 37°C for 2h (Ivanova *et al*., 1998).

Effect of pH on produced antimicrobial material activity

The effect of pH on the bacteriocins was tested by adjusting each of the cell-free supernatants to pH values ranging from 3-10 (at increments of 1 pH unit) using sterile 1N NaOH or 1N HCl, and incubated for 30min at 37°C (Oh *et al*., 2000). The samples were readjusted to pH: 6.5 and tested for antimicrobial activity as described by Ivanova *et al*., (1998).

Effect of heat on produced antimicrobial material activity

Cell-free supernatants containing bacteriocin was heated to 70°C, 100°C and 121°C (autoclaving) respectively, for 15 and 30min (20min for autoclaving) (Zamfir *et al*., 1999). After the incubation period, residual bacteriocin activity was tested using the agar well diffusion method at each of these temperatures (Ivanova *et al*., 1998).

RESULTS

The isolated bacteria were screened for their antimicrobial activities against different microbial indicators Gram-negative bacteria (*Proteus vulgaris* 1753, *Escherichia coli* 1357, *Enterobacter cloacae, Serratia marcescens*921/79 and *Klibsella pneumonia)* and Gram-positive bacteria *(Citrobacter freundi*as, *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus)*, in addition to *Candida albicans* as a fungus. Antimicrobial activities expressed in inhibition zones against all of the tested indicator organisms. The antimicrobial-producing isolate was identified by biochemical test by using API20 and DNA analysis shown as Gram-positive cocci, absence of catalase, hemolytic activity and mannitol salt agar was showed as negative results. The

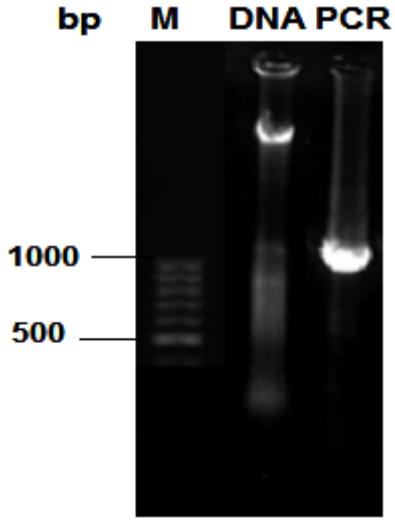

Fig. 1. Agarose gel electrophoresis for PCR products of 16S rRNA analysis of *E. hirae*

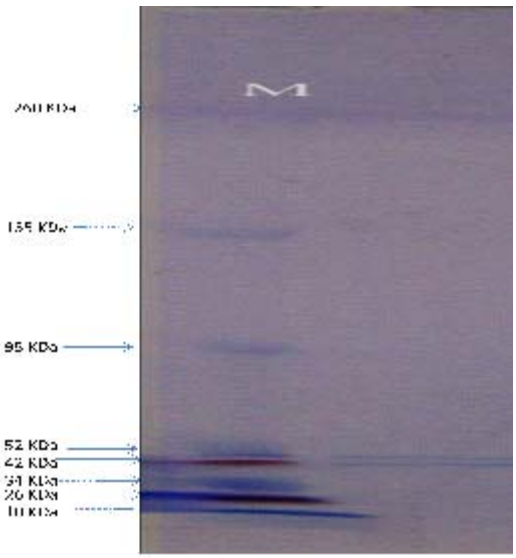

Fig. 2. SDS-PAGE of antimicrobial material produced by *E. hirae*. M, protein marker and lane 1is antimicrobial material of *E. hirae*

sequencing of PCR products (using species-specific primers) and the Blast program used to assess the DNA similarities and multiple sequence a alignment and molecular phylogeny showed 92% identity to *Enterococcus hirae* (accession no.Gu460396) as shown in (Fig. 1). Further identification to species level, *E. hirae* was based on pigment formation on Tryptic Soy Agar, and carbohydrate fermentation reactions recorded (not shown). The cells were non-motile, which distinguished the strain from *Enterococcus* strains.

The SDS-PAGE analysis of the purified antimicrobial material was performed (Fig. 2). Separation by SDS–PAGE indicated that bacteriocin of *E. hirae* is a two peptide of approximately 42 and 52 kDa compared to marker proteins. The maximum activity has been recorded at the beginning of stationary growth have performed on cultures of 24-h-old. The recovery of bacteriocin after ammonium sulfate precipitation was approximately 80%. Active fractions collected from the equilibrated column of Sephadex G-150. The separation of these fractions yielded an active peak which eluted at 40–45min (Fig. 3). No

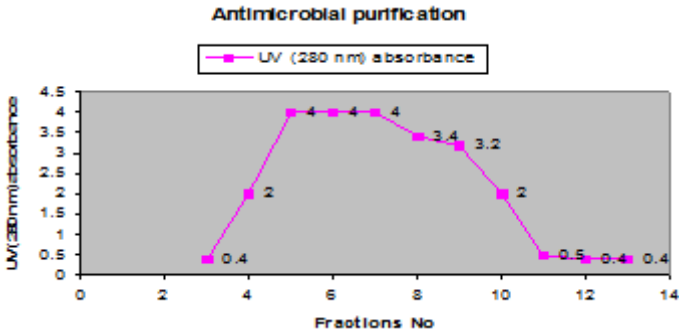

Fig. 3. Elution profile of *E. haire* antimicrobial material obtained by gel filtration on Sephadex G-150 with flow rate of 5 ml/4 min and fraction volume of 5 ml

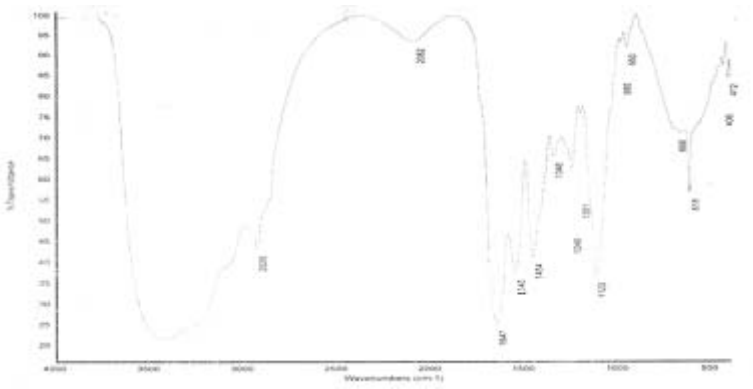

Fig. 4. Infrared spectrum of isolated antimicrobial material produce by *E. hirae*

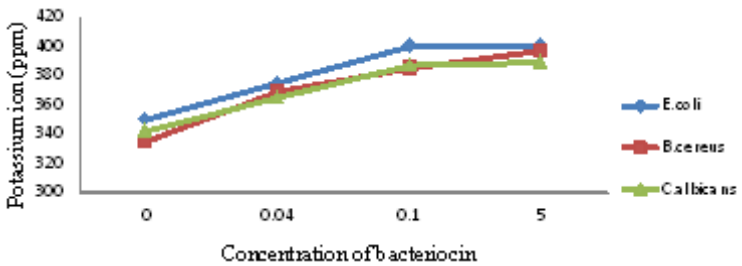

Fig. 5. Effect of bacteriocin produced by *E. hirae* on potassium ion leakage from some indicator organisms

antimicrobial activity was recorded after treatment of the cells with NaCl at pH: 2.0 (data not shown), suggests that antimicrobial material (bacteriocin) does not adhere to the cell surface. The structure of antimicrobial materials compound under investigation, it is necessary to have an assignment for the IR-absorption bands corresponding to the active groups in the compound. IR spectra of the antimicrobial material showed the functional groups (Fig. 4). The spectrum was subdivided into different regions: The bands in the region of 3000-3500 cm^{-1} was amide (amino group), the band in the region of 2926 cm^{-1} was hydroxyl groups (OH) , 2082 cm^{-1} was aliphatic group (C-H), the band in the region 1647 cm^{-1} was (C=O) and (C=N), 1548 cm^{-1} was (C=C), 1346-1201 cm^{-1} was aromatic system and region 1120-618 cm^{-1} was aliphatic group (C-H).

Different concentration of the bacteriocin produced by *E. hirae* affected on flow of K^+ outside the cells of some indicator organisms. Figure (5) showed that the leakage of K^+ ion increased as the concentration of bacteriocin increased. Effect of bacteriocins produced by *E. hirae* on respiration of some indicator organisms with different crude bacteriocin concentration (0.0, 0.125 and 0.250mg/ml). The data presented in (Fig. 6) indicated the bacteriocin had a negative effect on the respiration of the tested indicators, where the amount of consumed oxygen decreased by increasing the

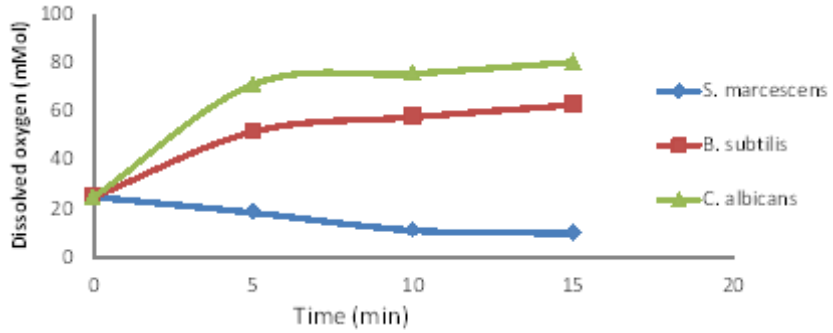

Fig. 6. Effect of antimicrobial material of *E. hirae* on dissolved oxygen (m.Mol) of some indicator organisms

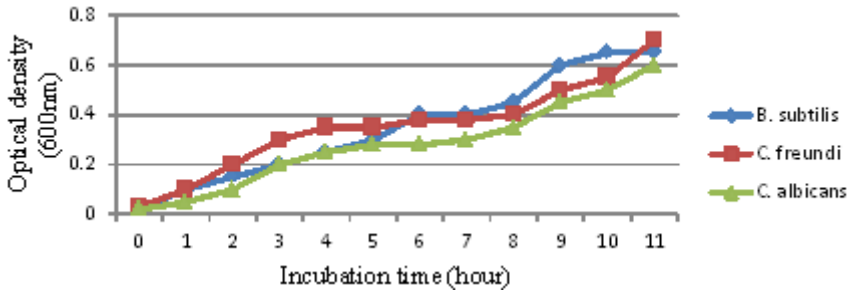

Fig. 7. Effect of bacteriocin produced by *E. hirae* on growth of some indicator organisms

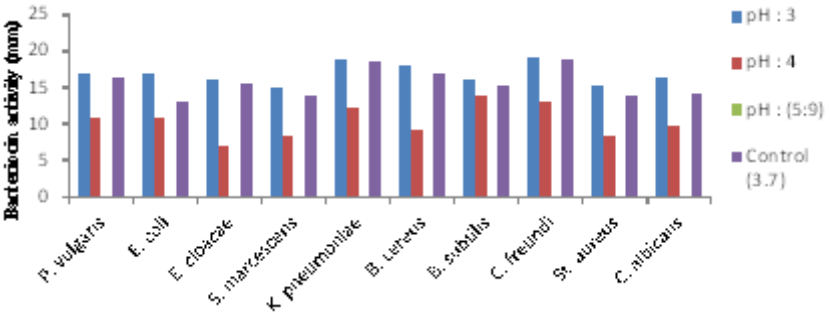

Fig. 8. Effect of pH on antimicrobial material activity of *E. hirae* (mm). (Control = 7)

bacteriocin. The amount of oxygen was determined as dissolved oxygen expressed in mill mole.

No toxic effect of the tested bacteriocin (10 and 100mg/ml) against *Artemia salina* larva was recorded under the experimental conditions. The effect of bacteriocins of *E. hirae* on the growth of indicator organisms was investigated using 3-h-old cultures of the indicators, resulted in growth inhibition for two hours followed by a slow recovery of growth (Fig. 7).

Complete inactivation of antimicrobial activity was observed after treatment of the cell-free supernatant of strain *E. hirae* with Proteinase K and trypsin, respectively, after incubation for 2h at pH settings between 2.0 and 12.0, this indicates that the antimicrobial materials produced is protein in nature (Bacteriocin). The antimicrobial activities at the different pH were determined after cell free extracts of *E. hirae* was adjusted to pH 3, 4, 5, 6, 7, 8, 9 and 10 incubated at 37°C for 20min. The data

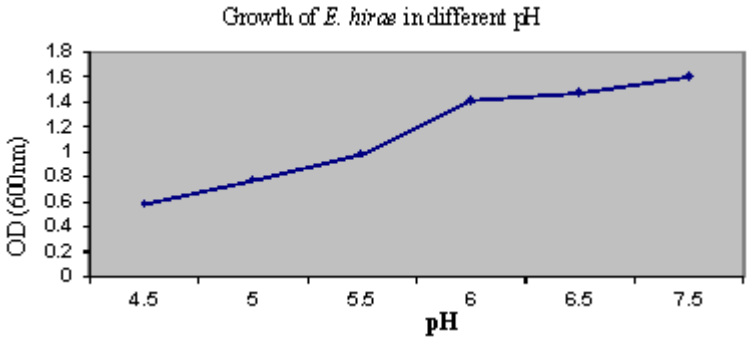

Fig. 9. Growth of *E. hirae* at different pH values

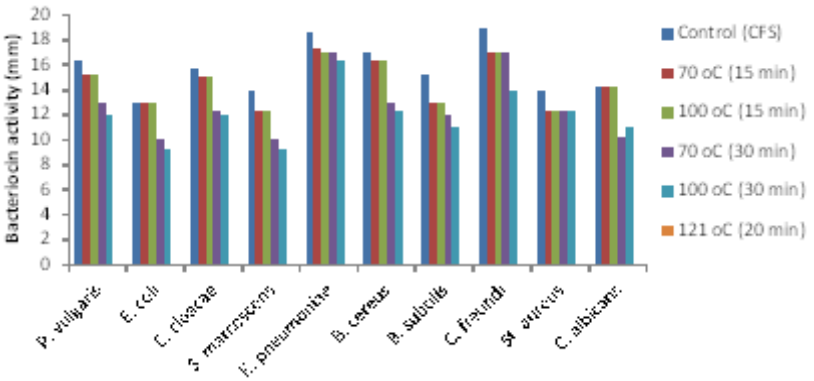

Fig. 10. Effect of heat on antimicrobial activity of bacteriocin produced by *E. hirae* (mm)

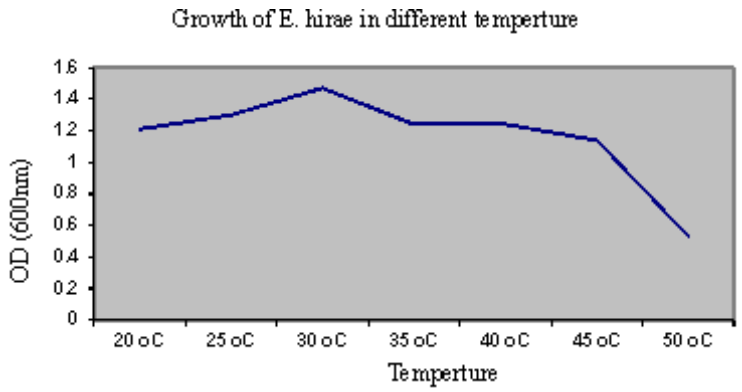

Fig. 11. Growth of *E. hirae* at different incubation temperatures

presented in (Fig. 8 and 9) showed that pH values of the medium had a significant effect on growth and antimicrobial material production of *E. hirae*. No antimicrobial activity was recorded at pH values from 5 to 10. The best levels of antimicrobial material production were obtained at pH (6.5) for. On the other hand, high antimicrobial activity was obtained at pH: 3.

Heat treatment on the activity of the bacteriocins produced by *E. hirae* showed that the incubation Temperature had significant effect on growth and antimicrobial material production of *E. hirae*. A maximum production for antimicrobial material was obtained after 18h at 30°C to 45°C. Non significant decrease in the antimicrobial activity was reported at 70°C or 100°C after incubation for 15min. compared to control (at room temperature) while complete loss of the antimicrobial activity after incubation at 70°C, 100°C for 30min and by autoclaving (Fig. 10 and 11).

DISCUSSION

Bacteriocins are natural antimicrobial agents produced by food fermentative bacteria (Deraze *et al.*, 2005). Bacteriocins and the organisms that produce them have potential in food and feed industry as natural preservatives (Cleveland *et al*., 2001; Laukova *et al*., 2013; Helena *et al*., 2014). In this study, the antimicrobials active isolates were obtained from traditional Egyptian food (Koskos) and were identified as *Enterococcus hirae*. *Enterococcus* a member of the lactic acid bacteria (LAB) that is found in various environments and are also found in vegetables, plant materials and fermented foods products (Giraffa, 2002; Koehler, 2007; Elsilk *et al*., 2015). MRS, pH 6.5, temperature 30°C and incubation period of 20 hours were the optimum condition for the production of bacteriocin indicated that this medium was the most convenient and contains specific nutrients that are required for the production of a desired material (Ogunshe *et al*., 2007). Bacteriocin of *E. hirae* inhibited the growth of indicator bacteria and conforms to the description of a bacteriocin. However, the activity recorded against Gram-negative bacteria is unusual as it inhibits the growth of Gram negative bacteria, and has thus far only been reported for a few bacteriocins of lactic acid bacteria (Todorov and Dicks, 2004; Elsilk *et al*., 2015). As far as we could determine, bacteriocin produced by *E. mundtii* with activity against Gram-positive and Gram-negative bacteria needed that specific nutrients are required for antimicrobial material production (Ogunshe *et al*., 2007). The antimicrobial material obtained from the isolated *E. hirae* was separated by precipitation the cell free supernatant which contains the antimicrobial material by 80% saturation ammonium sulphate. The precipitates were collected, drained and then dissolved in small volume of isopropanol in 25mm ammonium acetate at pH 6.5. The dissolved crude antimicrobial material was purified by passing into a column of Sephadex G-150. The SDS- PAGE analysis of our purified bacteriocins showed the molecular weight of bacteriocin isolated from *E. hirae* was 42 and 52 KDa (De Kwaadsteniet *et al*., 2005). This may indicate that the increase in the total biological activity might result from multimolecular dissociation for *E. hirae* bacteriocin (De Kwaadsteniet *et al*., 2005). The active fractions which contain the active peptide were tested against indicator bacteria and give inhibition zone with a diameter between (8-17mm) for *E. hirae,* these results clearly demonstrated that the antimicrobial materials was produced by traditional Egyptian fermented food bacteria (Tambekar and Bhutada, 2010; Elsilk *et al*., 2015). Infrared (IR) spectroscopy of the antimicrobial materials indicated the presence of many functional groups in the antimicrobial material such as OH, C=O, C-H, C=N, C=C and aromatic group.

The mode of action of the obtained antimicrobial materials not detected after treating with NaCl at pH 2.0, suggesting that the antimicrobial materials did not adhere to the surface of the producer cells. Similar results have been reported for plantaricin ST31 (Todorov and Dicks, 2005; Elsilk *et al*., 2015), bozacin B14 (Ivanova *et al*., 2000) and pediocin ST18 (Todorov and Dicks, 2005). The bacteriocins were destroyed by proteolytic enzymes produced by the indicators (Todorov and Dicks, 2005; Elsilk *et al*., 2015). In the cell lysis showed the growth of indicator bacteria was inhibited for 2h, followed by a slow recovery of growth, Similarity to the bacteriocin produced by *Lactobacillus plantarum* which isolated from molasses. Absence of cell lysis suggests that the mode of action of antimicrobial materials is not impairment of cell wall biosynthesis.

This is in agreement with (Ten Brink *et al*., 1994; Elsilk *et al*., 2015). The common mechanism of action which has been determined for other bacteriocins of lactic acid bacteria (LAB) is disruption of the electrochemical gradient across the cytoplasmic membrane by pore formation (Samelis *et al*., 1994).

The bacteriocin of *E. hirae* had no toxic effect on *Artemia salina*. So, bacteriocin may be safe to use in food industry (Meyer *et al*., 1982). The mode of action also showed that the different concentration of antimicrobial material increased the flow of potassium ion from the susceptible cells by made increase in membrane permeability which allow the passive efflux of ions (K^+) (Ennahar *et al*., 2000). These results agreed with those obtained by (McAuliffe *et al*., 1998; Elsilk *et al*., 2015) found that lactin 3147 interact with cytoplasmic membrane, leading to formation of pores. These pores were shown to be selective for K^+ ions. Also, it was found that lactocin (G) formed small pores, which allowed potassium efflux, resulting in ATP hydrolysis and dissipation of membrane potential. The effect of antimicrobial material on the respiration of indicator bacteria suggests that the respiration of some indicator bacteria decreases in the presence of different concentrations of the antimicrobial materials produced by *E. hirae*.

Treatment of antimicrobial material with proteolytic enzymes proteinase K and Trypsin resulted in complete inactivation (digestion) of antimicrobial materials activity, confirming their proteinaceous nature and also lends support their characterization as bacteriocins (Ghrairi *et al*., 2008). Similar characterization has been reported for the bacteriocin from *Enterococcus* sp. (Ghrairi *et al*., 2008, John *et al*., 2009; Elsilk *et al*., 2015). Antimicrobial material production was strongly dependent on pH and temperature as claimed by (Todorov and Dicks, 2004). Activity of antimicrobial material was observed in our study at acidic and neutral pH levels (5.5:7.5), but maximum antimicrobial materials activity was noticed at pH 6.0 and 6.5. Similar results were observed by (Karthikeyan and Santosh, 2009). The bacteriocin produced by our test bacterium exhibited antimicrobial activity against the indicator organisms at pH 3-4 and no activities were recorded at pH values below 3 and above 4. The finding that the antimicrobial material is pH unstable was perhaps due to more rapidly degraded by subtle changes in pH and it is common among investigators.

The activity of antimicrobial material was observed at different growth temperatures. These observations suggest that the temperature plays an important role in antimicrobial material production, as in case of plantaricin (Li and Song, 2008). The inhibitory activity was seen only in narrow pH range (3:4) and above this range it loses its activity, this result assertion that the antimicrobial material was a protein in nature and as such were a bacteriocin. When the antimicrobial material of *E. hirae* was submitted to heat treatment, most of the activity was maintained at temperatures 70°C and 100°C for 15min. and one third of the activity was lost after 30min, so our bacteriocin considered to be heat stable (Cleveland *et al*., 2001). Heat stability could be considered a very useful characteristic as many of this bacteriocin may find a potential use in food preservation. Similar types of results have been reported for other bacteriocin (John *et al*., 2009; Simova *et al*., 2009), but the activity of antimicrobial material was inhibited after treatment at 121°C for 20min. Also bacteriocin ST15, produced by *Enterococcus mundtii* ST15, bacteriocin N15 produced by *Enterococcus faecium* N15 (Losteinkit *et al*., 2001). Production of antimicrobial material at acidic and neutral pH levels, over a wide temperature range (30°C:45°C), and with inhibition of food-borne pathogens including *E. coli*, *B. cereus* and *Staphylococcus aureus* will be quite promising for development of a wider applications of these antimicrobial material in various foods with control of these pathogens.

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