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Production of bacteriocin from local isolate Lactobacillus acidophilus and its nanoscale improvement and study of its properties

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Abstract:

The study was conducted on the bacteriocin produced by the isolate of Lactobacillus acidophilus under optimal conditions that were approved according to a previous study after investigating its ability to inhibit a number of test bacteria that divided into two types, including positive bacteria, Staphylococcus aureus Bacillus cereus and negative bacteria Salmonella typhimurium, coli. E The diameter of the damping halo ranged between 9, 8 and 6, 6, respectively .Then the bacteriocin was partially purified by ammonium sulfate with a saturation average of about 70%, and then dialysis using Spectra Dialyzer tubing Cat. No 3787 for 18 hours to get rid of salts and impurities. The obtained bacteriocin was measured so that the kidneys reached about 34500 units, and the total effectiveness was about 115000 units/mg. To improve traits of bacteriocin, it was coated with a chitosan biopolymer, which also has an enhanced role in eliminating microorganisms that cause food spoilage and spoilage through the ionic gelatinization method and to obtain particles with nanoscale dimensions. /ml with a fixed percentage of chitosan has reached about 0.2 g. The reason behind the success of the encapsulation process was due to the ability of the inner cavity of the laboratory-created nano chitosan to accommodate the studied amount of bacteriocin. Nano-characterization of the nanoparticles obtained from bacteriocin coated with chitosan was coundected by scanning electron microscope (SEM). It took the form of smooth, dense and uniform clumps of uniform consistency, compared to the usual bacteriocin, which was in the form of three-dimensional crystals with a monoclinic system, taking the shape of an almost prism. As for the analysis of the chemical composition of the synthetic nanoparticles, they were identified by FTIR spectroscopy at a wavelength of 3435 cm-1, which showed the presence of all the molecules that make up bacteriocin without any change in their arrangement inside the chitosan cavity. The same is true for testing the samples using the X-ray diffraction device, which showed that the match was completely and correctly through the chemical phases of the standard sample with the synthetic material, with the compound not losing its structural integrity during the ion gelatinization process.

Key words: bacteriocin - chitosan - nanotechnology - ionic gelatinization - Lactobacillus acidophilus

Introduction:

Bacteriocin is defined as a compound produced by lactic acid bacteria, a protein with a bacterial effect, but with a narrow inhibitory level that is specific to its constituent bacteria or close to it, for a competitive purpose related to nutritional and environmental conditions that bacteria close to the same species may need for growth and reproduction. Bacteriocin was first discovered by the scientist Gratia in 1925 and

was known as Colicin produced by the isolate (E.coli) Hardy et al. (1986) Lyon et al. (1976). The naming of bacteriocin is based on the genus of the bacteria producing it, and the syllable cin or in is added at the end of the genus name of the bacterium, as in the naming of the bacteriocin Pediocin produced by Pediococcus acidilactici, Yousef et al. (1991). Despite the bacteriocins produced by Gram-positive bacteria on the bacteriocins produced by Gram-negative bacteria, the type produced by Gram-positive bacteria has an inhibitory and lethal effect with a wider range of activity than that produced by Gram-negative bacteria. Savadogo et al. (2006). As described by Delves et al. (1996). There are two types of bacteriocins referred to by Klaenhammer (1988) and Piard (1993) and others, where the first type is known for its ability to act in a limited manner in the sense of narrow spectrum, and the second type them has a broad-spectrum inhibitory ability against types of microorganisms,and based on what was indicated by Mehta et al. (1983), some bacteriocins from Gram-positive bacteria have an antibacterial effect against other Gram-negative bacteria. Bacterocins were also classified according to multiple classifications, as Nes et al. (1996) classified them based on the physical and biochemical characteristics in a more comprehensive manner, as follows:

First: Bacteriocins have short chains of peptides. This type is called lantibiotics, an example of which is Nisin.

Second: - Bacteriocins with short chains of thermostable peptides with a molecular weight of less than ten thousand daltons and they are free of lanthionine and are termed small heat stable non lantiboitic, as three sub-divisions fall under this category:

- Bidiosin, similar to bacteriocin
- Dipeptide bacteriocins:- They are bacteriocins with two peptide chains, as the alpha chain is composed of an inactive bacteriocin and becomes effective in the beta chain, an example of this is Lactococcin.

-Sec-mediated secretion of bacteriocin: It includes the asadine produced by Lactobacillus acidophilus.

Third: Large bacteriocins with a molecular weight of more than thirty thousand daltons, which are termed as Large stable protein, are produced by Lactobacillus casei bacteria, for example Caseicin.

Fourth: - Bacteriocin complex consisting of proteins, fats and carbohydrates, and an example of this is pediosin.

The efficiency of the action of bacteriocin produced by LAB depends on several factors, including the lack of damage by external factors such as high temperatures, as well as oxidation by surrounding oxygen, which is considered to be factors affecting the bioavailability of bacteriocin and weakening its work.

Packaging techniques, whether at the micro- or nano-level, have overcome the above-mentioned problems and worked to increase the efficiency of many biological compounds. In recent years, nanotechnology has specifically entered the field of food industry and systems for preserving biologically active compounds as a result of their small size and large surface area, which made them give physical and chemical properties. Characteristics different from in conventional volume Karthick Raja et al (2015). Nanoencapsulation by biopolymers is considered the most successful and widely used application in this field after being employed in many pharmaceutical industries as a system for drug delivery and its preservation of many effective compounds across biological barriers without causing any harm to human health in

addition to its ability to biodegrade, which is the first factor For her success Márcia R. et al (2008). Given the importance of applied bacteriocin, there was a need to develop it and take advantage of its inhibiting properties of microorganisms. The stages and objectives of the study were:

1. Production of bacteriocins from a local isolate of Lactobacillus acidophilus and its partial purification.
2. Improving the properties of bacteriocin through nanotechnology by encapsulating it with nano chitosan and studying its properties.

Materials and methods:

Bacteriocin production by Lactobacillus acidophilus:

To investigate the efficiency of the isolate Lactobacillus acidophilus isolated from dairy products and to know the extent of its production of bacteriocin, which was sourced from the University of Baghdad - College of Science, Department of Biotechnology, and based on the optimal conditions mentioned by Taqi (2019) in production, some modifications were made in the method used, The isolate was grown in a test tube containing 10 ml of MRS liquid media at 37°C and incubated for 24 hours under anaerobic conditions using an anaerobic jar. After the incubation process, the culture medium was heated in a water bath at a temperature of 70 °C for 30 minutes, then cooled at room temperature, then centrifugation was coundected at a temperature of 4 °C for the bacterial culture at a speed of 4500 x g for 15 minutes, then the isolation filtrate was collected and set to the number pH 6.5 using a sterile 1 mN NaOH solution,Then 0.22 µm diameter microfilters were used and 1 ml of the filtrate was transferred to which 5 mg of caltase was added. The filtrate was cultured in dishes containing MRS Agar and Nutrient Agar media and incubated at a temperature of 37° C for 24-48 hours to ensure that the resulting bacteriocin was free from any bacterial stuck, Then the ability of the obtained bacteriocin to destroy microorganisms was investigated by following the etch-diffusion method described by Tagg et al. 1971)) by spreading 0.1 ml, which is equivalent to 1810 x cells/ml (compared to 0.5 McFarland tube) of the activated test bacteria on the culture medium. assigned to it by means of a sterile glass L-Shape Loop, After that, holes were formed in the center of the dish with a diameter of 4 mm by means of a cork piercing in which 10 microliters of the previously obtained filtered bacteriocin were placed. The culture dishes were incubated at a temperature of 37 °C for 24 hours to inspect the inhibition zone known as the Clear Zone. When producing bacteriocin, 500 ml of liquid MRS nutrient medium was prepared and the pH was set at 6.5, then the medium was inoculated with lactic acid bacteria producing bacteriocin at a ratio of 1810 x cells/ml to be incubated at conditions similar to the previously mentioned temperature and time period using the static incubator (Fixed fermentation method) with nitrogen gas injected during the fermentation time.

Estimation of the protein concentration in the obtained bacteriocin:

To estimate the protein concentration of the bacteriocin extract obtained from Lactobacillus acidophilus, the method described by Bradford et al. (1976) was followed, which is based on the coumassie blue pigment G-250, which has the ability to bind to protein, which contributes to the appearance of color and increased absorbance by means of spectroscopy in the field of rays. visible and ultraviolet, This was done by adding 100 µl of the extracted bacteriocin to 1 ml of the pre-prepared dye solution to be mixed well and kept at a temperature of 30° C for 10 minutes to

read the absorbance at the wavelength of 595 nm. Distilled water was added as a control solution (Blank) instead of the produced bacteriocin, and the standard curve of BSA standard bovine serum albumin was used to calculate the protein concentration through different concentrations of BSA solution that ranged from (0.5-0 mg/ml) through a series of dilutions. In distilled water, 100 µl of each dilution was taken separately and added to tubes containing 1 ml of the previously prepared G-250 reagent dye solution, mixed well, kept at a temperature of 30 °C for 10 minutes, and the absorbance was read at a wavelength of 595 nm.

Partial purification of the product bacteriocin:

Bacteriocin was precipitated from the studied lactic acid bacteria L. acidophilus following the method described by Jayachitra et al. (2012) by saturating the filtrate with 70% ammonium sulfate, then centrifugation at 14000 x g for 60 minutes upon cooling at 4°C. ,Bacteriocin was collected in 15 ml of deionized water, and dialysis was performed for 18 hours using Spectra Dialyzer tubing Cat. No 3787 for removing salts and impurities. The total and qualitative efficacy of the produced bacteriocin was estimated according to the following equations:

Total Effectiveness (Unit) = Effectiveness (Unit/ml) x Volume of Bacterocin Solution (ml(

Specific Effectiveness (unit/mg) = (ml/unit) Effectiveness /((ml/mg) protein concentration(

Lyophilization of bacteriocin:

The bacteriocin was lyophilized to obtain it as a powder using a lyophilizer at a pressure of 0.047 lb/in2 at -80 °C and then preserved by freezing at -18 °C for use in subsequent experiments.

Preparation of chitosan nanoparticles embedded with bacteriocin:

The method described by Namasivayam, K.R.S et al. (2015) was followed with some modifications in preparing chitosan nanoparticles incorporated with pre-prepared bacteriocin, 0.2 g of chitosan was mixed in the form of a suspension solution in 1% acetic acid diluted in 100 ml of deionized distilled water to add 10 ml of bacteriocin suspension prepared from distilled water of different concentrations (6,3,1 mg/ml). The mixture was stirred for three hours by electrophoresis at room temperature with the addition of a few drops of Trisodium Phosphate (TPP).Then the mixture was centrifuged at 10,000 rpm for 10 minutes, the precipitate was collected and washed with deionized water, then re-centrifuged and then frozen at -80 °C for 24 hours to complete the lyophilization process.

The efficiency of the nano-encapsulation process:

The efficiency of chitosan-bacteriocin nano-encapsulation, symbolized by (%EE) for different concentrations (6,3.1 mg/ml) was measured by calculating the difference between the weight of the total chitosan-coated bacteriocin and the weight of the free non-bacteriocin after ultrafiltration based on centrifugation. Centrifugal and using a filter tube (Amicon) Ultra-Centrifugal Filter Unit Ultracel-100 for ten minutes at a speed of 11,000 revolutions per minute at a temperature of 25 ° C.

Characterization of the nanostructure of chitosan incorporated with bacteriocin:

The phenotypic characterization of nanoparticles for the best concentration obtained through the process of synthesis of nanoparticles was conducted by a scanning electron microscope (SEM). 15 kV The dried samples were transferred to a metal wire using a gold-coated double mesh. The images were recorded at different magnifications with an acceleration voltage of 15 kV. The scale bar represents 5 µm.

FTIR: Fourier infrared spectroscopy.

The method described by Kumar et al. (2009) with some modification was used to determine the molecular structure of both free bacteriocin and chitosan-coated bacteriocin by preparing them in the form of tablets by mixing 0.04 g of each sample with 0.12 g of KBr It was mixed well using a ceramic mortar for 5-10 minutes, to take 0.04 g of the mixture and pressurized with a pressure of 10 bar for 60 seconds, which is specific to the FTIR device. Then the obtained discs were dried in a drying oven at a temperature of about 60°C for 6 hours before being analyzed by FTIR device at a frequency of (4000-400) cm^{-1}.

X-ray diffraction measurement:

It aims to characterize the structural properties using an X-ray diffraction device based on X-rays emitted from a copper cathode with a wavelength of 1.5409, a current of 25 kV and a strength of 40 mA.

Results and discussion :

Evaluation of the efficiency of bacteriocin production by Lactobacillus acidophilus:

The isolate Lactobacillus acidophilus was subjected to a detection test for bacteriocin production by growing it in MRS culture medium using the method of diffusion in the pits. The amount of the isolate was tested by measuring the diameter of the Inhibition Zone of a group of Gram-positive test bacteria, which were Staphylococcus aureus, Bacillus cereus and Salmonella cereus. coli.E, which is gram negative. It was noted through Table (1), which is represented by the diameters of inhibition, that all the results indicated a positive investigation into the ability of bacteriocin produced by Lb. acidophilus, and the results obtained showed that the three isolates have the ability to produce bacteriocin, The highest inhibition diameter was 9 mm against Staphylococcus aureus, followed by the Gram-positive isolate B. cereus with a diameter of 8 mm, while for Gram-negative bacteria, the diameter of the inhibition halo for both test bacteria E.coli S.typhimurium was about 6 mm. The obtained results agree with what was indicated by Gupta et al (1998) that the biomass of some types of lactic acid bacteria grown in MRS liquid medium has an inhibitory ability towards a group of Gram-positive and Gram-negative bacteria. The discrepancy in the diameters of the inhibition halo was shown by a study by Tagg et al. (1976) that the weakness of bacteriocin produced by lactic acid bacteria against some test bacteria is due to their lack of receptors that transport it outside the cell, or its production in small quantities may not be sufficient to kill sensitive cells.

Table (1) Diameters of microbial growth inhibition zones by etch-diffusion method due to the antagonistic property of crude bacteriocin extract by Lactobacillus acidophilus

Salmonella typhimurium	*Escherichia coli*	*Bacillus cereus*	*Staphylococcus aureus*	**test bacteria**
6ml	6ml	8ml	9ml	**Diameter aura inhibition**

Partial purification of the product bacteriocin:

Before starting the partial purification process of bacteriocin, both the total and specific activity were measured. For 100 ml of the crude extract, 6400 units and 49230 units/mg at a protein ratio of 1.3 mg/ml were measured either by precipitating the bacteriocin using ammonium sulfate at a saturation rate of 70%. He obtained it by 12.5 ml, and the total effectiveness of the obtained bacteriocin was about 30,375 units, and the specific effectiveness was 50,625 units / mg.When the protein concentration was about 0.6 mg/ml, which increased the levels of effectiveness when performing the purification process through dialysis, it was 34500 units for the total activity and 115000 units/mg for specific activity at a volume obtained of 3 ml and protein with a concentration of 0.3 mg/ml and this is an indication On the importance of purifying bacteriocin to get rid of as many compounds as possible that may affect the quality of bacteriocin or may interfere with it in the antimicrobial action of contaminating microorganisms,This was confirmed by a study by Contreras et al. (1997), which showed that bacteriocin produced by Lb. amylovorus that was purified by ammonium sulfate precipitation method increased the activity of the obtained bacteriocin.

Lyophilization of bacteriocin:

The bacteriocin is dried to obtain it in the form of a powder, which can be obtained in this form in relation to the disposal of carbohydrates that are found in the composition of most types of bacteriocins. Water present in the air and thus a physical change occurs in the composition of bacteriocin that prevents it from drying out. Sebti, I. et al. (2003).

Efficiency of the nano-encapsulation process:

Through the results obtained for the efficiency of the bacteriocin nanoencapsulation process with chitosan biopolymer, after preparing chitosan nanoparticles embedded with bacteriocin, we find that the best concentration of bacteriocin was at 1 mg/ml. by Karthick R.N. et al (2015) worked on encapsulating bacteriocin with chitosan nanoparticles, which ranged between 81-83%, using the same concentration of bacteriocin.While the other concentrations used in the study did not show a good percentage of the efficiency of the encapsulation process, which was 56% and 23% for each of 3 and 6 mg/ml of bacteriocin, respectively, which may be attributed to the ability of the inner cavity of the synthesized nanocapsule to accommodate high concentrations of bacteriocin.

Characterization of the nanostructure of chitosan incorporated with bacteriocin:

Through figure (1) below, which shows the images taken by a scanning electron microscope under different magnification powers, we find that the chitosan in (A) was

in the form of heterogeneous crystal flakes in terms of distribution, which was in the form of layers overlapping each other, while the bacteriocin obtained in the laboratory In (B), it was in the form of three-dimensional crystals with a monoclinic system, taking the shape of an almost prism After the nanoencapsulation of bacteriocin by chitosan, we find that the size of the synthesized particles reached the limits of 50-200 nm, as in Figure (C), in which we also find that the particles were in the form of smooth, dense and uniform clumps of uniform consistency (granular consistency).

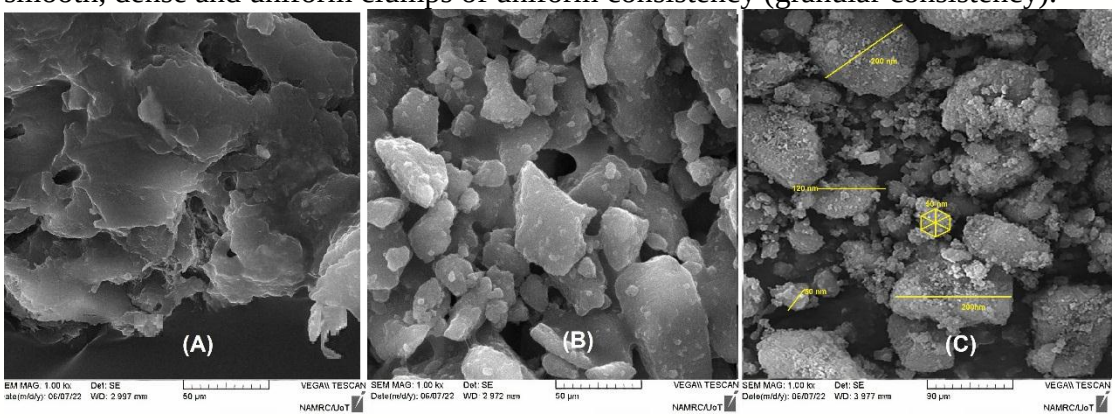

Figure 1: SEM image of (a) chitosan, (b) bacteriocin extracted from L. acidophilus isolate (c) bacteriocin coated with nanoscale chitosan.

FTIR: Fourier infrared spectroscopy.

Through Figure (2), which showed the diagnosis by Fourier infrared spectroscopy FTIR that chitosan has clear absorption bands at wavelength 3290 cm-1 resulting from the amine group and the stretching of the hydroxyl group. FTIR also showed absorption bands at wavelengths 2920-2875 cm-1 Due to symmetric and asymmetric CH-stretching, these bundles are an essential feature of polysaccharides . As indicated by a previous study, the beams that appeared at wavelengths 1325-1645 cm-1 are caused by the stretching of the amide groups (C=O)) and (C-N). The beam was found at wavelength 1580 cm-1 resulting from the bending of ((N-H,The asymmetric stretching of the C-O-C bond also causes the appearance of a beam at wavelength 1250 cm-1 and the beam at wavelength 1025-1056 cm-1 indicating C-O absorption which was recorded by the researchers.As for the spectrometry of bacteriocin nano-coated with chitosan by ionic gelatinization method, the absorption bands reached their maximum at wavelength 3435 cm-1, which occurred due to the stretching vibrations along the hydroxyl (O-H) bond.As a result of the occurrence of a link between the bacteriocin part inside the nanobody through a physicochemical reaction, and the other evidence for the occurrence of this link between the bacteriocin and nano chitosan is the appearance of an absorption band at 1649 cm-1 resulting from the stretchy vibration of amide groups (C=O) with a displacement of 4 cm - 1 of the 1645-1649 cm-1 bundle compared to the absorption bundle of chitosan alone, Where it was observed that the hydroxyl group (O-H)) of the anionic polymer interferes with the amine group in chitosan and the formation of an ionic complex and this change was reflected on the absorption bands of the amine and carboxyl groups and the amide bonds in the spectrometer.

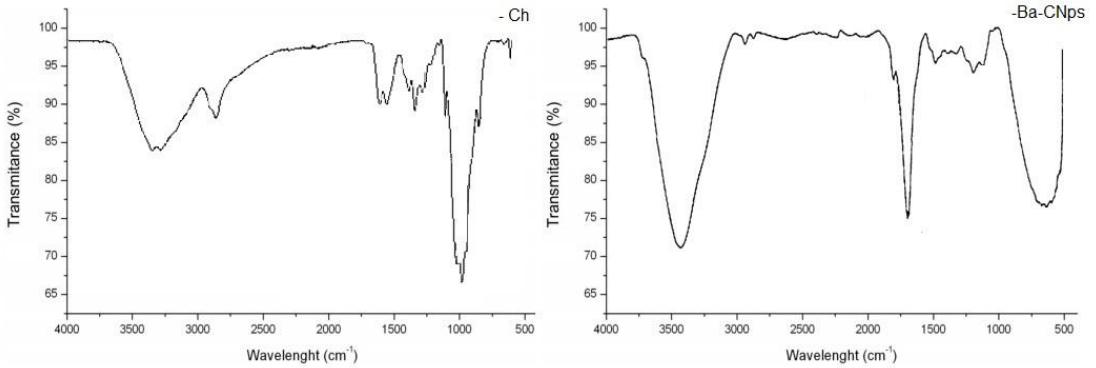

Figure (2) Infrared absorption spectroscopy of bacteriocin coated with chitosan nanoparticles prepared by ionic gelatinization method in comparison with the chitosan model.

X-ray diffraction measurement:

The results of the X-ray diffraction in Figure (3) showed that the bacteriocin particles coated with nano chitosan by ionic gelatinization method. MDI jade program and what was mentioned by .de Abreu, L.C.L et al. (2016), where it is clear that the congruence of the angles for each vertex was at 30.6998 -53.5998 - 35.1998 - 47.4997, which indicates that the match was completely due to the chemical phases of the standard sample compared with the prepared material that was examined for bacteriocin The nucleated chitosan and the peptide are properly encapsulated without losing its structural integrity.

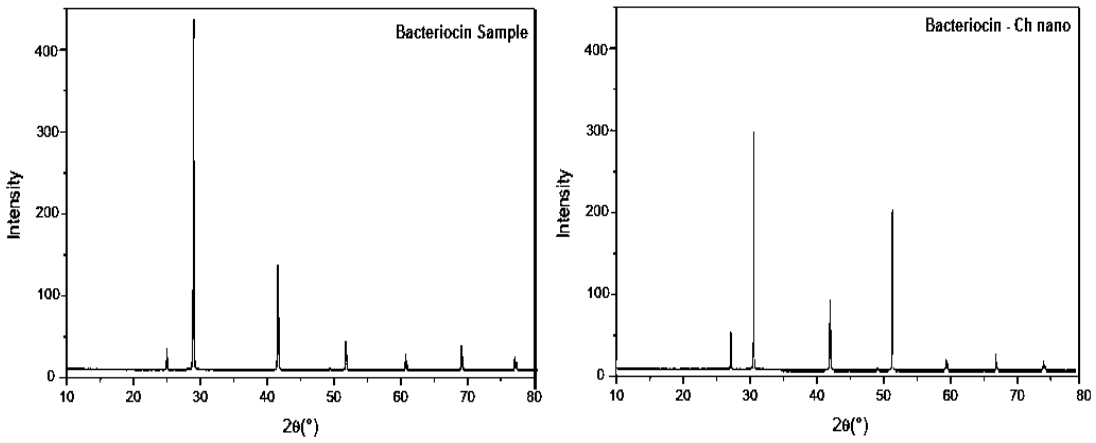

Figure 3. X-ray zig-zag spectrum of bacteriocin coated with nano chitosan prepared by ionic gelatinization method in comparison with the free bacteriocin model.

Conclusions:

It was found that the isolate Lactobacillus acidophilus has the ability to produce bacteriocin, and the partial purification method based on precipitation by aluminum sulfate and then dialysis bags is very efficient and it gave a good percentage of the effectiveness of the obtained bacteriocin. Chitosan-coated bacteriocin showed nanoscale properties when tested by scanning electron microscope and compared with normal bacteriocin by Fourier infrared spectrophotometer.

References:

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein – dye binding. Anal. Biochem. ,72: 248–254.

Contreras, B.G.L.; De Vuyst, L.; Devreese, B.; Busanyova, K.; Raymaeckers, J.; Bosman, F.; Sablon, E. and Vandamme, E.J. (1997). Isolation, Purification, and amino acid sequence of Lactobin A, one of the two bacteriocins produced by *Lactobacillus amylovorus* LMG P .13139. Appl. Environ. Microbiol. ,63(1): 13-20.

Delves, B.; Black burn, P.; Evans, R.J. and Hugenholt, Z. (1996). A **Klaenhammer, T.R.** (1988). Bacteriocins of Lactic acid bacteria, Biochem. ,70(30): 337-349.

Gupta, U.; Radramma, R.E. and Joseph, R. (1998). Nutritional quality of lactic acid fermented bitter gourd and fenugreek leaves. International Journal of Food Sciences and Nutrition. ,49(2): 101-108 .

Hardy, K. (1986). Bacterial plasmid. 2nd.Edition. American Society for Microbiology. U.S.A.

Jayachitra, A.; Sukanya, C.M. and Krithiga, M. (2012). Characterization of Bacteriocin from Probiotic *Lactobacillus plantarum*. International Journal Pharmaceutical sciences and Research. ,3(11): 4374-4386.

Karthick Raja Namasivayam,S.,Justine James. Der Pharmacia Lettre. 2015;7:100-111

Lyon, W.J.; Sethi, J.K. and Glatz, B.A. (1993). Inhibition of psychrotrophic organisms by propionicin PLG-1, a bacteriocin produced by *Propionibacterium thoenii*. J. of Dairy Sci. ,76: 1506-1513.

Márcia R, Moura A, Fauze A, Aouada L, Mattos H. Preparation of chitosan nanoparticles using methacrylic acid. Journal of Colloid and Interface Science. 2008;321:477–48

Nes, **I**.; Deipleiv, D. and Brurberg, M. (1996). Biosynthesis of bacteriocins in Lactic acid bacteria. Antonie van leeuwenhock. ,70: 113-128.

Savadogo, A.; Ouattara, C.; Bassole, I.H. and Traore, A.S. (2004). Antimicrobial Activities of Lactic Acid Bacteria Strains Isolated from Burkina Faso Fermented Milk. Pakistan Journal of Nutrition. 3(3): 174-179.

Tagg, J.R.; Dajani, A.S. and Wannamaker, L.W. (1976). Bacteriocins of gram positive bacteria, Bacteriol. Rev. ,40: 722-756.

Yousef, A.E.; Luchansky, J.B.; Degnan, A.J. and Doyle, M.P. (1991). Behavior of *Listeria monocytogenes* in wiener exudates in the presence of *Pediococcus acidilactici* H or pediocin AcH during storage at 4 or 25'C. Appl. Environ. Microbiol. ,57: 1461- 1467.