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ISOLATION, PURIFICATION AND CHARACTERIZATION OF BACTERIOCIN PRODUCED BY *BACILLUS PUMILUS NJ-M2*; A FUTURE BIOPRESERVATIVE

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ABSTRACT

Our research aims to isolate, identify and characterize the native soil-bacterial strains predominant in the soil of Unaizah city, Al Qassim province of Saudi Arabia, which produce bacteriocin antagonistic to *S.aureus ATCC 25923, S.epidermidis ATCC 12228, E.faecalis ATCC 29212 and S.pneumoniae ATCC 49619*, followed by isolation, purification and characterization of bacteriocin. Out of twenty soil samples, only one soil strain was found to have highest antibacterial activities against the test organisms. The soil isolate was identified

as *Bacillus pumilus NJ-M2*, whereas the optimum growth was obtained at temperature 30ºC and pH 7.0. The highest production of bacteriocin was found to be in soybean casein digest broth, at temperature 35ºC, pH 7.0, shaking at 150-220 rpm and 60-72 hours of incubation. The most appropriate concentration of ammonium sulphate for the isolation of bacteriocin was found to be 60-70 %, whereas the partial purification was successfully done by dialysis membrane with 8 KD MWCO. The maximum and minimum antibacterial activities were observed at pH 3.0 and pH 9.0 respectively, whereas the complete denaturation was observed at pH 10. There was no effect of various temperature ranges 50-120ºC on the stability and activity of bacteriocin. The Arbitrary Unit (AU) of cell free broth was found to be 25 AU/mL, whereas the Minimum Inhibitory Concentrations of partially purified bacteriocin were found to be between 32-64 µg/mL. In conclusion, this study could play a significant role in the treatment of different infections caused by *S.aureus, S.epidermidis, E.faecalis and S.pneumoniae* and in the food and dairy industry, as it might be used as a biopreservative. Thus, these results warrant the further investigations to fully standardized and establish the antibacterial profile of this soil isolate.

Keywords: Arbitrary Unit (AU), Bacteriocin, *Bacillus pumilus NJ-M2*, 16S rRNA, Biopreservative

1. INTRODUCTION

Microorganisms compete for the limited space and nutrients, present in the natural ecological niches. Therefore, they have developed many strategies in order to survive; production of bacteriocins is one of them. Gram-positive bacteria, and mainly lactic acid bacteria and *Bacillus spp.* are now being increasingly studied for their production of bacteriocin-like substances **[1]**. Some bacteria produce ribosomally synthesized antibacterial peptides called bacteriocins also known as proteinaceous toxins, which can kill or inhibit the growth of similar or closely related bacterial strains

but will not harm the bacteria themselves by specific immune proteins. Bacteriocins are widely distributed in natural environments. The biodiversity in peptide is supported by many differences in their structures **[2]**. All constitutively synthesized peptides, regardless of sub-classification, have a net positive charge which causes them to fold into an amphiphilic conformation upon interaction with membranes of bacteria **[3]**. Most characterized bacteriocins are stable at different temperatures, nontoxic, and sensitive to degradation by proteolytic enzymes like trypsin, pepsin present in the gastrointestinal tract **[4].** Now days a lot of food products with chemical preservatives are available in the market and increased consumption of foods containing chemical preservatives and these chemical preservatives have some side effects on the health and thus consumer concerns have created a higher demand for more natural and minimally processed foods therefore, there is a high interest in naturally produced bacteriocins that do not produce adverse effects on the body. The antibiotic resistance among the clinical isolates is one of the major challenges in the healthcare industry, which demands the new antimicrobial agents for the treatment of different infections caused by multidrug resistant organisms. Therefore, bacteriocins might play a significant role in the treatment of such infections. Soil has variety of microbes like fungi, actinobacteria and eubacteria. The major constituents of soil are microbes which produce a variety of primary as well as secondary metabolites, which can play an important role in soil ecology and beneficial for human beings. *Bacillus spp.* is one of the most common soil bacteria which produce a variety of bioactive metabolites such as bacteriocins and antibiotics.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Chemicals and glassware

Soybean casein digest agar, Soybean casein

digest broth, Nutrient broth, Luria broth, Mueller-Hinton agar, Mueller Hinton broth were purchased from Oxoid Ltd., Basingstoke, Hampshire, England.

2.1.2 Test organisms

S.aureus ATCC 25923, S.epidermidis ATCC 12228, E.faecalis ATCC 29212, and S.pneumoniae ATCC 49619 were collected from the Department of Microbiology, King Saud Hospital, Unaizah, Al Qassim province of Saudi Arabia.

2.1.3 Soil samples

Twenty soil samples were collected from the different agricultural lands, date farmhouses and desert areas of Unaizah, Al Qassim province of Saudi Arabia. Iron spade was used for the collection of soil samples and soil samples were collected from 5-10 cm. below the ground surface. All samples were collected in sterile polythene bags and proper labeling was done.

2.1.4 Preparation of test medium plates

The soybean casein digest agar was used as a test medium, whereas *S.aureus ATCC 25923, S.epidermidis ATCC 12228, E.faecalis ATCC 29212, and S.pneumoniae ATCC 49619* were used as test organisms. Each plate of test medium was prepared by adding 1 ml suspension of a test organism (turbidity ~ 0.5 McFarland) into a sterile molten soybean casein digest agar for the isolation of soil bacteria **[5, 6].**

2.2. Preliminary screening

2.2.1 Isolation of soil bacteria

Each soil sample was diluted in series by using sterile normal saline as a diluent, and thereafter, 1 ml sample from each dilution (10^{-4} and 10^{-5}) was inoculated into a test medium plate and spreaded by a glass spreader **[5, 6].** The inoculated plates were incubated at 25-30°C for 48-120 hours. Thereafter, the isolated bacterial colonies were screened for antibacterial activities and data was recorded. The desired isolated colonies of soil bacteria were picked up and purified for further investigations.

2.3 Primary screening

2.3.1 Determination of antibacterial

activity of soil isolate(s) via spot inoculation or cross streak method

The Mueller Hinton agar was used as a test medium, whereas the test medium plates were pre-inoculated with the test organisms. The antibacterial activities of selected soil isolates were redetermined by spot inoculation on the test medium plates. The inoculated plates were incubated at 25-30°C for 48-120 hrs. The results of antibacterial activities of soil isolate(s) were recorded.

2.3.2 Identification of highest potent soil isolate

The selection of highest potent soil isolate was done on the basis of antibacterial activities against the test organisms, and thereafter, the proper labeling was done. A temporary identification code; KA 18-B was assigned to the soil isolate. The selected soil isolate was identified by 16S rRNA sequence analysis method. The soil isolate was stored at 4°C temperature in glycerol for further analysis **[5, 6, 7, 8, 9, 10].**

2.3.3 Characterization of highest potent soil isolate

2.3.3.1 Effect of pH

The effect of pH on the growth of soil isolate was determined by incubating the bacterial suspensions at constant temperature (35°C) with different pH values, ranging from 2.0 to 10 for 24 hours. Thereafter, the optical densities of all suspensions were measured at 620 nm, whereas the results were compared with an un-incubated suspension **[11, 12, 13].**

2.3.3.2 Effect of temperature

The effect of temperature on the growth of soil isolate was determined by incubating the bacterial suspensions at various temperatures, ranging from 20 to 50°C for 24 hours, whereas the pH was kept constant i.e. 7.0 throughout the incubation. Thereafter, optical densities of all suspensions were measured at 620 nm, whereas the results were compared with an un-incubated suspension **[11, 12, 13].**

2.4 Secondary screening

2.4.1 Optimization of fermentation conditions

Soybean casein digest broth medium was used for preparing the inoculum by using the master culture of soil isolate. Three different fermentation media; Nutrient broth, Luria broth and Soybean casein digest broth were used for the fermentation purposes and fermentation was carried out in 250 mL of flasks. All fermentation media were seeded by using 5 % inoculum of soil isolate and incubated at 30°C temperature for up to 120 hours with pH 7.0 and shaking at 150-220 rpm **[10, 11, 12, 13].**

Fermented broth samples were collected at various intervals i.e. every 12 hours of incubation; Starting from 0 hours up to 120 hours. All collected samples were centrifuged at 5500 rpm at 4°C temp. for 30 minutes and then supernatants were filtered through a 0.45 µm pore size filter, and thereafter, the antibacterial activities were determined by well diffusion method **[14].**

Mueller Hinton agar plates; pre-inoculated with test organisms and 80 µL sample quantities were used in well diffusion method. All inoculated plates were pre-incubated at room temperature for about 3 hours, followed by incubation at 37°C temperature for 48 hrs. After that, the results were recorded. The best fermentation medium was selected on the basis of highest yield of bacteriocin. The size of the zone of inhibition was directly proportional to the yield of bacteriocin present in the cell free broth.

2.4.2 Effect of temperature on the production of bacteriocin

The fermentation was carried out at various temperatures such as 25°C, 30°C, 35°C and 40°C with shaking at 150-220 rpm for up to 120 hours and then fermented broth samples were collected at different intervals as described in previous step, and thereafter, antibacterial activities were determined by well diffusion method **[13].**

2.4.3 Growth curve (Incubation vs. pH & Cell density (turbidity), Incubation vs. Antibacterial activity)

The data obtained from the fermentation of

soybean casein digest broth was used to prepare the growth curve of *Bacillus pumilus NJ-M2*.The fermentation conditions were kept same as described in previous steps, and then, the fermented broth samples were collected at various intervals as described in previous steps. Thereafter, the optical density, pH and antibacterial activities were determined, whereas well diffusion method was followed for determination of antibacterial activities and optical densities were measured at 620 nm against the unfermented broth **[15, 16, 17, 18].**

2.5 Isolation, purification and characterization of bacteriocin

2.5.1 Isolation and purification of bacteriocin

Ammonium sulphate method was followed for the isolation of bacteriocin from the cell free broth. The optimization of ammonium sulphate method was carried out by using different concentrations of ammonium sulphate. Partial purification was carried out by the dialysis method in order to remove the ammonium sulphate salts from the bacteriocin solution. The dialysis was carried out by using dialysis membrane of 8000 Daltons Molecular Weight Cut Off (MWCO) and phosphate buffer (pH 7.0). The total protein of dialysate was determined by Biuret method. The antibacterial dialysate was stored at 4°C temperature for further investigations **[1, 19, 20]**.

2.6 Characterization of bacteriocin

2.6.1 Effect of pH on the stability and antibacterial activity of bacteriocin

The antibacterial dialysate was distributed in to nine different tubes and pH values of all dialysates were changed as follows- 2, 3, 4, 5, 6, 7, 8, 9 and 10 by using 1 N HCl and 1 N NaOH solutions and then all tubes were kept at 4-6°C for 24 hours. Thereafter, the antibacterial activities were determined against the test organisms by well diffusion method by using 80 µL samples from each tube **[13, 20, 21].**

2.6.2 Effect of temperature on the stability and antibacterial activity of

bacteriocin

The effect of temperature on the stability and antibacterial activity of bacteriocin was determined by incubating the antibacterial dialysate in water bath at different temperatures, ranging from 50 to 120°C for 30 minutes, whereas the pH of dialysates were kept constant i.e. 3.0. Thereafter, the antibacterial activities were determined against the test organisms by well diffusion method by using 80 µL samples from each tube **[13, 21, 22].**

2.7 Bio-evaluation of bacteriocin

2.7.1 Determination of Arbitrary Unit (AU) of bacteriocin by modified well diffusion method

Cell Free Broth was serially diluted (twofold) in phosphate buffer solution (pH 3.0) as 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/62, whereas 1 was used to denote the undiluted sample. 100 µL suspensions of each test organism (0.5 McFarland) were spreaded out on sterile Mueller Hinton agar plates separately by using sterile spreader. Then agar wells were made by using sterile cork borer (8 mm size) and then 80 µL of each serially diluted cell free broths were inoculated into their respected wells and then all plates were preincubated for 2-3 hours at room temp. till the full diffusion occurred. Thereafter, all plates were incubated at 37°C for 24 hours. The bacteriocin titre was calculated as the reciprocal of highest dilution (2n) that resulted in inhibition of the indicator bacterial lawn. The arbitrary units of bacteriocin (AU/mL) were calculated by using the formula as- AU/mL= 2n×1000 µL/quantity of cell free broth used **[14, 23]**.

2.7.2 Determination of Minimum Inhibitory Concentration (MIC) by broth Dilution Method

2.7.2.1 Bacteriocin weighting and dilution scheme

The antibacterial dialysate was dried at 80°C temperature for 30 minutes in order to obtain the powder form of bacteriocin. Around 100 mg dry powder of bacteriocin

was dissolved in 10 mL of DMSO (Dimethyl Sulphoxide) and vortexed well. The stock concentration of bacteriocin was equal to 10000 µg/mL. Phosphate buffer solution with pH 3.0 was used for further dilution of stock solution to obtain the different concentrations of bacteriocin, whereas results were compared with the standard antimicrobial agent – Augmentin 50 mg (Purity : >98%). The method described by CLSI was followed for the dilution of bacteriocin and determination of MIC **[23, 24, 25]**.

3. RESULTS AND DISCUSSION

3.1 Preliminary screening

3.1.1 Isolation of soil bacteria

Out of twenty soil samples, ten soil isolates were found to having antibacterial activity against the test organisms.

3.2 Primary screening

3.2.1 Determination of antibacterial activity of soil isolate(s) via spot inoculation or cross streak method

Out of ten soil isolates, only one soil isolate was found to having highest antibacterial activity against the test organisms and then a temporary code; KA 18-B was assigned to this highest potent soil isolate for further investigations.

3.2.2 Identification of ka 18-B by 16s rRNA sequence analysis

Approximately, 700 bp sequences of soil isolate were analyzed and results of 16S rRNA sequence analysis were BLASTED in Gene data bank (GenBank). The accession code for NCBI database is NZ_CP012329.1. Thus, the results of 16S rRNA sequence analysis suggest that, the isolated soil bacterium; KA 18-B was 99 % similar with *Bacillus pumilus NJ-M2*. The phylogenetic tree of *Bacillus pumilus NJ-M2* was illustrated in figure 01.

3.2.3 Characterization of soil isolate; Bacillus pumilus NJ-M2

3.2.3.1 Effect of pH

The results of effect of pH on the growth of soil isolate suggest that, the optimum growth of soil isolate was obtained at pH 7.0.

3.2.3.2 Effect of temperature

The results of effect of temperature on the growth of soil isolate suggest that, the optimum growth of soil isolate was obtained at temperature 30^{o}C.

3.3. Secondary screening

3.3.1 Optimization of fermentation conditions

The results suggest that, out of all 3 fermentation media (Nutrient broth, Luria broth, Soybean casein digest broth), the soybean casein digest broth was the best and Nutrient broth was the worst medium, whereas Luria broth was an intermediate type medium. The soybean casein digest broth was selected for further fermentation.

3.3.2 Effect of temperature on the production of bacteriocin

The results suggest that, the average antibacterial activities (zone of inhibition) of fermented broths against the test organisms were as 14 mm, 17 mm, 20 mm and 17 mm at the different temperatures 25°C, 30°C, 35°C, 40°C at the end of 60-72th hours of incubation. The average highest antibacterial activity (zone of inhibition) was 20 mm at 35°C temperature. Thus, the results suggest that, the temperature 35°C was the optimum temperature for the highest production of bacteriocin.

3.3.3 Growth curve (Incubation time vs. pH, Cell density (turbidity) and Antibacterial activity)

The results of growth curve suggest that, the optical density (o. d.) of fermentation medium was increased during the 0 hr. to till 24 hrs. and then o. d. was declined during the 24-48 hrs. of incubation and then o. d. was increased during the 48-72 hrs. of incubation, followed by continuous declined till the end of fermentation. The pH was declined during the 0 hr. to 12 hrs. of incubation and then pH was slightly increased and remained constant till the 60th hrs. of incubation. Then, the pH was increased during the 60-108 hrs. of incubation, followed by continuous declined till the end of fermentation. The maximum production of bacteriocin was

found to be during 60-72th hours of incubation. The results are illustrated in the figures no.02 and 03.

3.3.4 Isolation, purification and characterization of bacteriocin

3.3.4.1 Isolation & purification of bacteriocin

The results suggest that, the bacteriocin was successfully isolated by the salting out/ammonium sulphate method, whereas the partial purification was successfully done by the dialysis method by using the dialysis tubing of 8000 Daltons MWCO and phosphate buffer (pH 7.0). The maximum yield of bacteriocin was at between 60-70 % concentrations of ammonium sulphate, whereas the total protein concentration of the antibacterial dialysate was 5 gm/L.

3.3.4.2 Characterization of bacteriocin

3.3.4.2.1Effect of pH on the stability and activity of bacteriocin

The results suggest that, the pH affects the antibacterial activity of bacteriocin, as the maximum and minimum antibacterial activities were at pH 3.0 and pH 9.0 respectively, whereas no antibacterial activity was seen at pH 10.0. Thus, the pH is very important factor and highly recommended for the antibacterial activities of bacteriocin. The results are illustrated in figures no.04 and 05.

3.3.4.2.2 Effect of temperature on the stability and activity of bacteriocin

The results suggest that, the effect of temperature on the stability and antibacterial activity of bacteriocin was negligible. Thus, the bacteriocin was highly stable at different temperatures ranging from 50-120°C. The results are illustrated in figures no.06 and 07.

3.4 Bio-evaluation of bacteriocin

3.4.1 Determination of Arbitrary Unit (AU) of cell free broth

The results suggest that, the Arbitrary Unit (AU) of cell free broth was found to be 25 AU/mL. The calculation was as follows:

Quantity of cell free broth used = 80 µL
Highest titre (2^n) showed zone of inhibition = 2
Arbitrary Units (AU)/mL = 2^n x 1000 µL/80 µL
= 2 x 1000/80
= 2 x 12.5
Arbitrary Units (AU)/mL = 25

The results of Arbitrary Unit (AU) are illustrated in the figure no. 08.

3.4.2 Determination of MIC (Minimum Inhibitory Concentration)

The results of MIC suggest that, the MIC values of partially purified bacteriocin were found to be between 32-64 µg/mL, whereas the MIC values of standard antimicrobial agent (Augmentin) were found to be 2-4 µg/mL.

In this research study, it was found that the *Bacillus pumilus NJ-M2* is a predominant bacterium in the soil of Unaizah city of Saudi Arabia, which produces bacteriocin against the pathogenic bacteria *S.aureus ATCC 25923, S.epidermidis ATCC 12228, E.faecalis ATCC 29212, and S.pneumoniae ATCC 49619*. The results of characterization of soil isolate suggest that, the soil isolate was mesophilic in nature and grows well at optimum conditions. The results of optimization of fermentation conditions suggest that, the soil isolate needs a well nutritious medium for the production of bacteriocin and thus, it does not need an ordinary medium for the production of bacteriocin. The results of effect of temperature on the production of bacteriocin suggest that, the temperature is very important factor and plays a key role in the production of bacteriocin. The results of growth curve of soil isolate suggest that, the changes in incubation time, temperature and fermentation medium affect the production of bacteriocin.

The results of isolation, purification suggest that, the ammonium sulphate method was the most appropriate method for the isolation of bacteriocin from the cell free broth, whereas the dialysis tubing of 8 KD MWCO was the most suitable cellulose membrane for the partial purification of bacteriocin. The results of characterization of bacteriocin suggest that,

the bacteriocin was very sensitive to the changes in pH, as the highest antibacterial activity was observed in the acidic pH and the lowest antibacterial activity was observed in the alkaline pH solution. The results of effect of temperature on the stability and antibacterial activity of bacteriocin suggest that, the bacteriocin was highly stable at different temperatures because there was no significant effect of temperatures on its stability and activity.

The results of bioevaluation of bacteriocin suggest that, the Arbitrary Units (AU) of cell free broth and the Minimum inhibitory concentration (MICs) of partially purified bacteriocin were very significant. The bacteriocin might play a significant role in the field of food and dairy industry as the antibacterial activities of cell free broth and partially purified bacteriocin were very significant. This study could also play a significant role in the field of healthcare as the bacteriocin was having high degree of antibacterial activities against the pathogenic bacteria- *S.aureus, S.epidermidis, E.faecalis and S.pneumoniae*.

In previous literatures, the authors have not well described about the antibacterial profile of bacteriocin produced by this soil isolate. Some authors had been described that *Bacillus pumilus* produces substances with the antibacterial activity, but very little work had been done to isolate of these substances. Gilliver had described that the strains of *Bacillus pumilus* produce antibacterial activity, but he did not attempt to isolate the active compound **[25].**

Dvonch and Benedict had isolated an antibacterial agent in 1953, from a strain of *Bacillus pumilus* which they described that, it is similar to subtenolin, an antibiotic produced by *Bacillus subtilis* **[26]**. Borowski and some other authors had isolated an antibiotic from a strain of *Bacillus pumilus*, called tetaine **[27]**. D.S. Bhate had showed that *Bacillus pumilus* produces a noble antibiotic called pumilin but he could not described more about this antibiotic **[28].**

In one of the recent literature, the authors have demonstrated that, *Bacillus pumilus (NKCM 8905)* and *Bacillus pumilus (AB211228)* produce the antimicrobial agents but they have not described the quantitative antibacterial efficacy (AU or MIC) **[29]**. In one more literature, the authors have described that, *B. pumilus CL45* produces antibiotic antagonistic to the plant pathogens but no effort have been made to show the full antimicrobial profile **[30, 31].** Thus, by comparing the results of our research study with the previous literatures, the authors found that, the current research study is more significant than the previous studies as there is no significant information available about the antimicrobial profile of *Bacillus pumilus NJ-M2*.

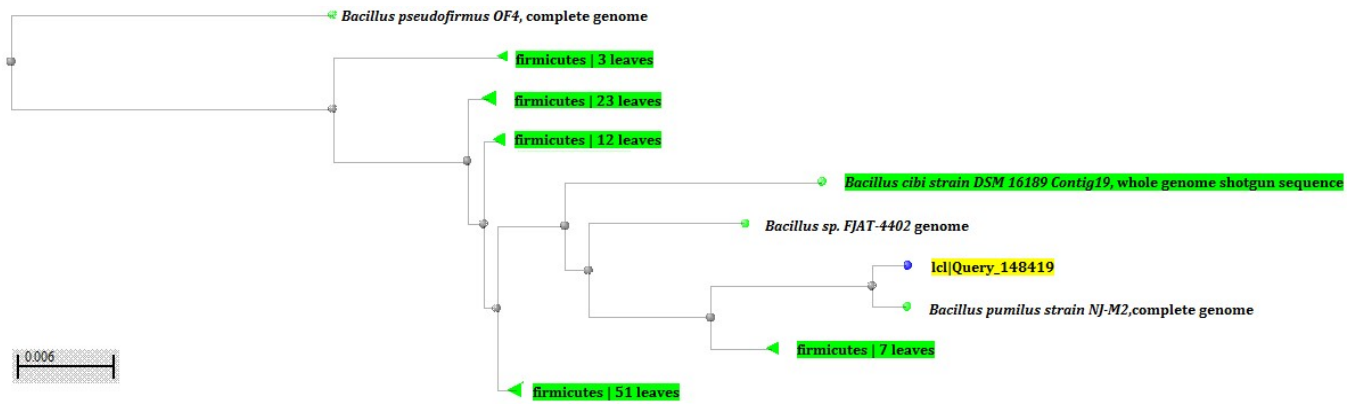

Figure 01: Phylogenetic tree of *Bacillus pumilus NJ-M2*

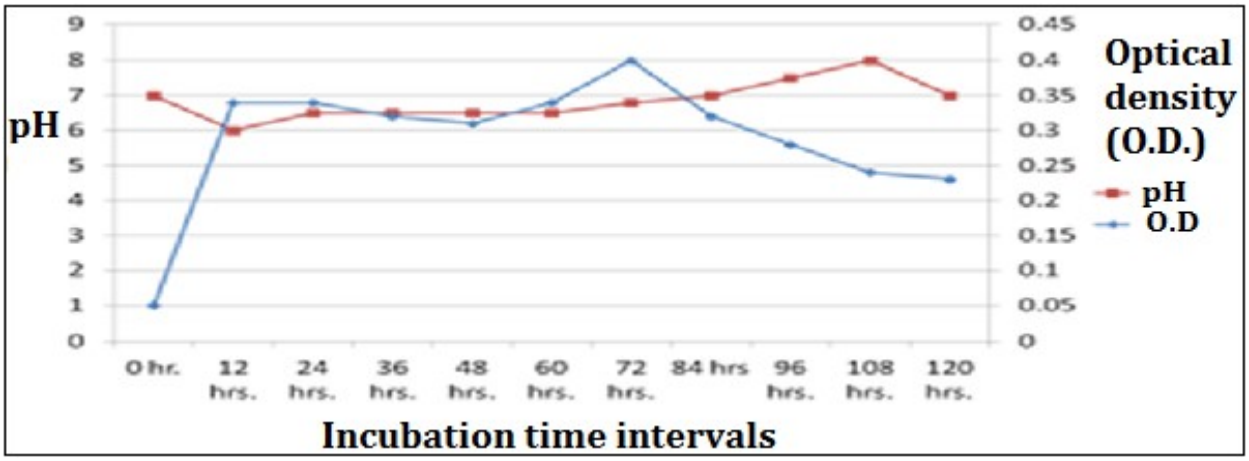

Figure 02: Growth curve of *Bacillus pumilus NJ-M2* (Incubation time vs. O.D. and pH)

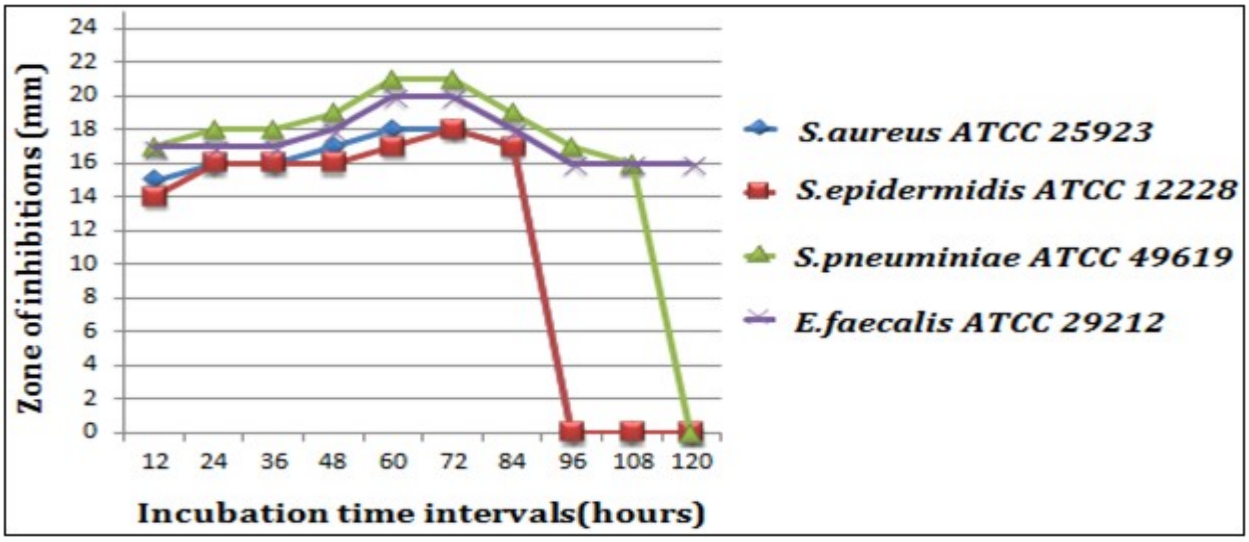

Figure 03: Growth curve of *Bacillus pumilus NJ-M2* (Incubation time vs. Antimicrobia Activity)

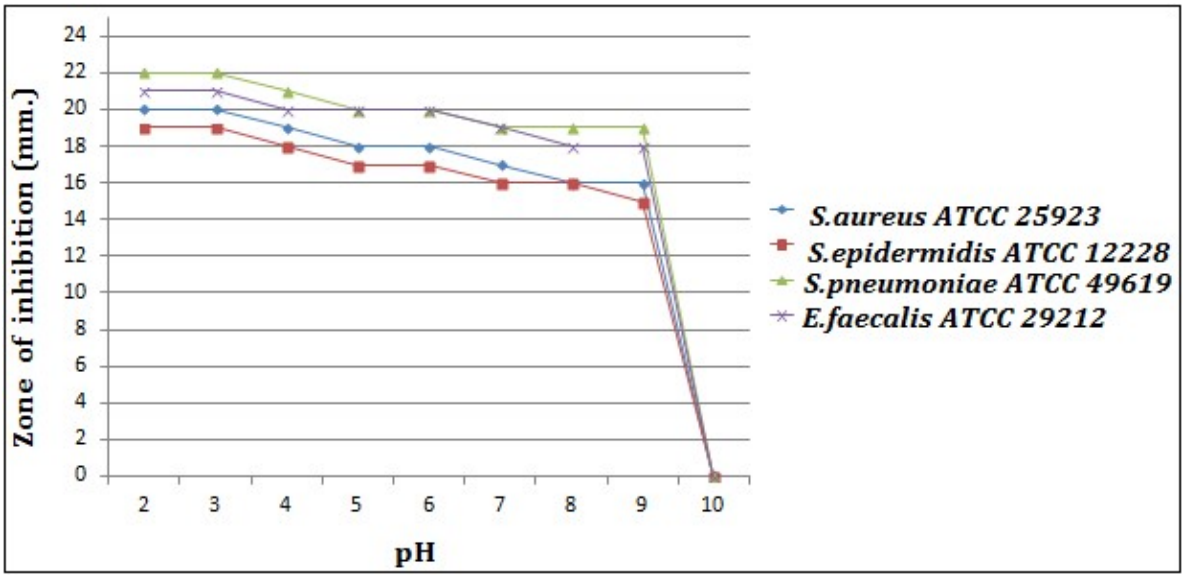

Figure 04: Effect of pH on the stability and activity of bacteriocin

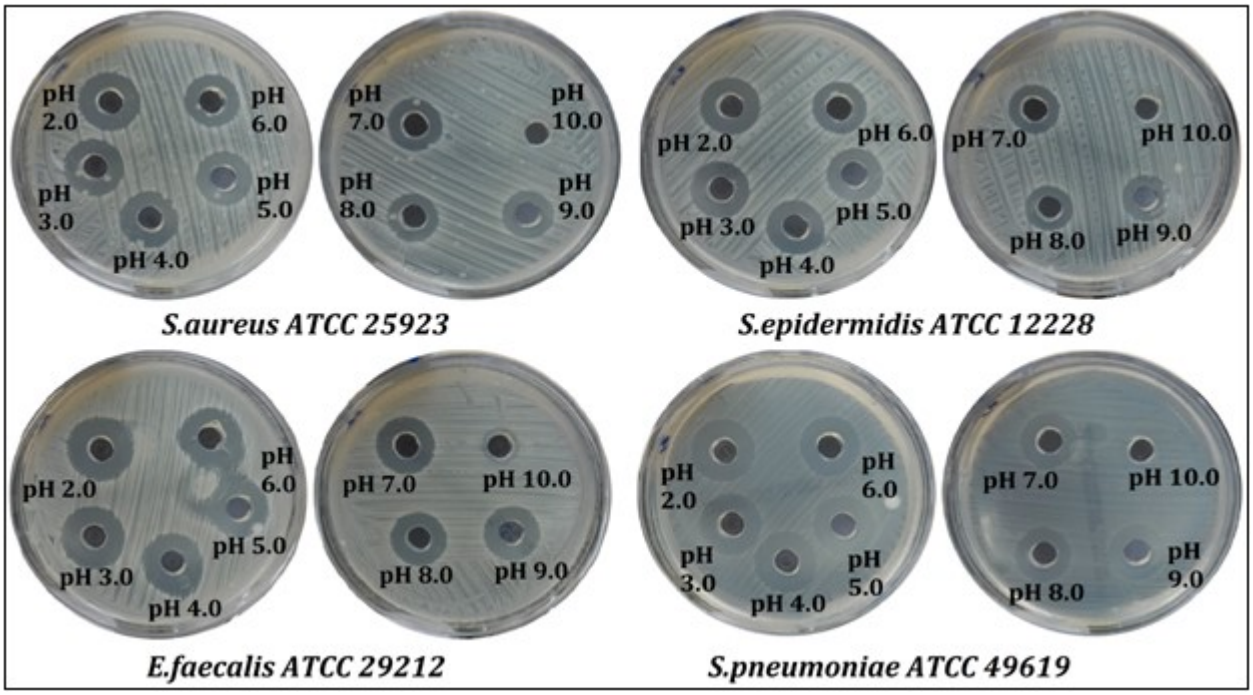

Figure 05: Effect of pH on the stability and activity of bacteriocin

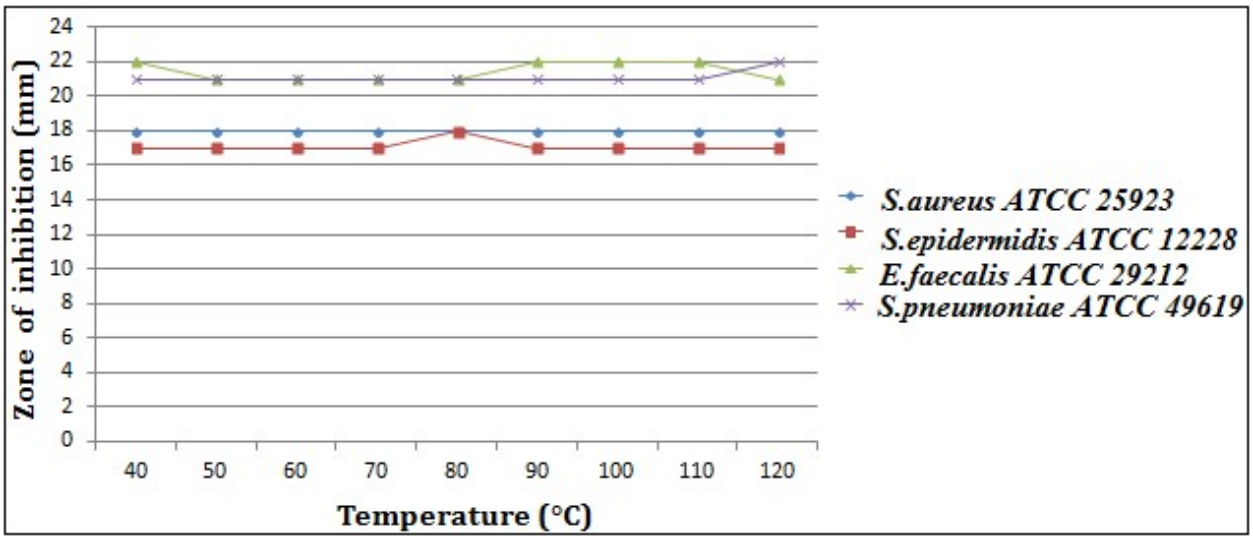

Figure 06: Effect of temperature on the stability and activity of bacteriocin

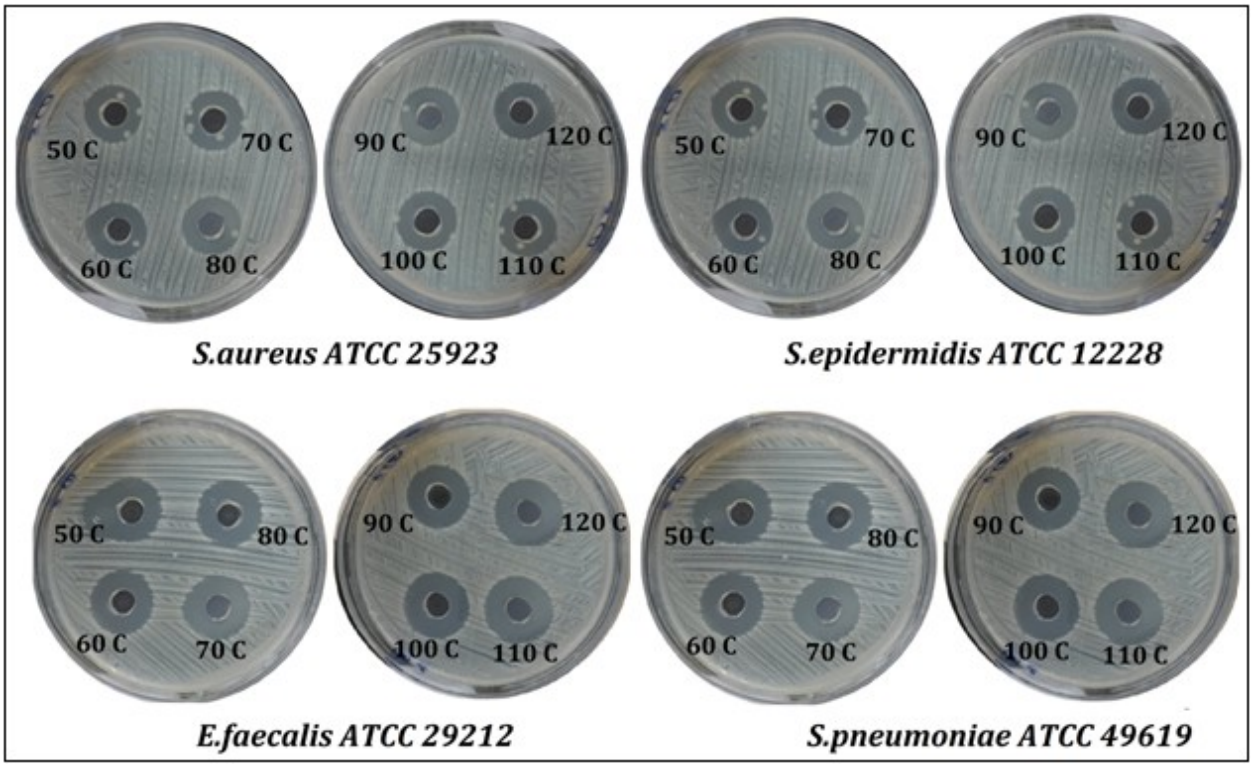

Figure 07: Effect of temperature on the stability and activity of bacteriocin

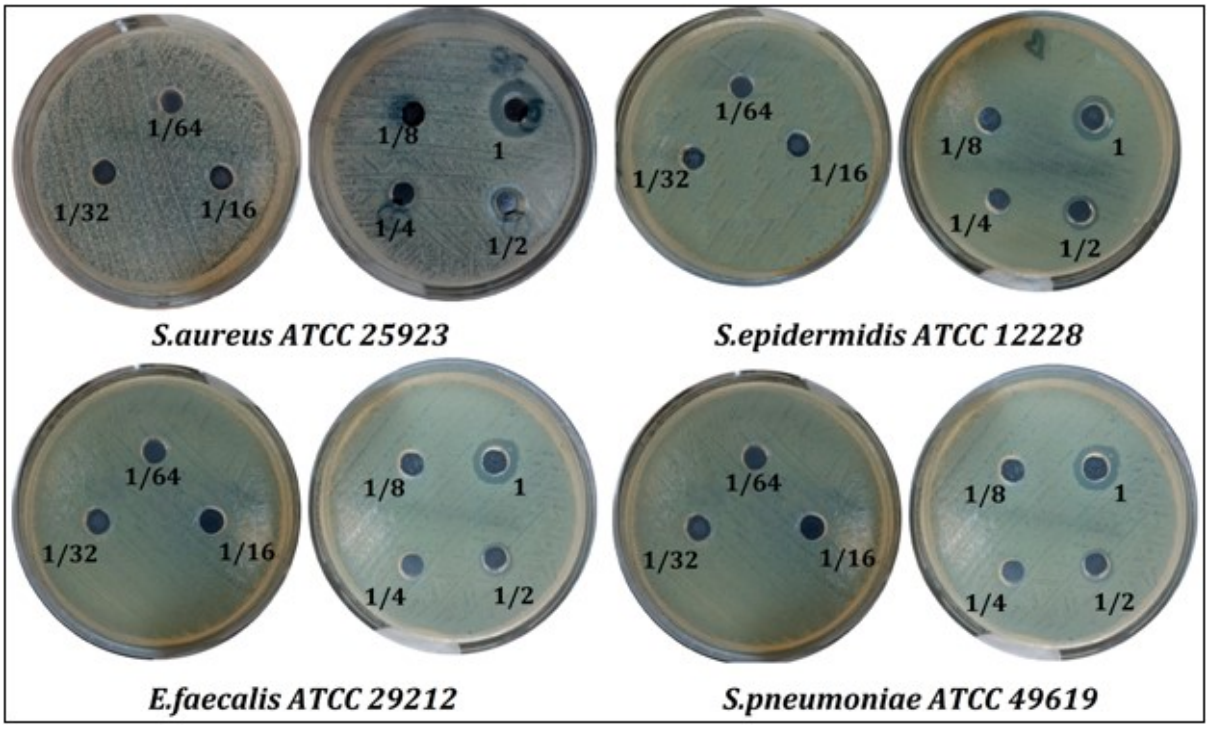

Figure 08: Bio-evaluation: Arbitrary Unit (AU) of bacteriocin

4. CONCLUSION

In conclusion, this present study might be a great endeavour in the fields of food, dairy and healthcare industry. This bacteriocin could be used as biopreservative in the fields of food and dairy industry and as an

antibacterial agent in the field of healthcare industry for the treatment of different infections caused by *S.aureus, S.epidermidis, E.faecalis and S.pneumoniae.* Thus, the results of this current study warrant further investigations to fully standardized and establish the antibacterial profile of this soil isolate; *Bacillus pumilus NZ-M2.*

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