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Production of Bacteriocin Like Substances as Antipathogenic Metabolites by *Bacillus licheniformis* Isolated from Healthy Human Skin

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Abstract

Different types of bacterial colony were isolated from skin of 30 healthy human and their antipathogenic activity was tested against 9 pathogens. The isolate showed activity against pathogen- *Kl. pneumoniae* subsp. *pneumoniae*, was identified as *Bacillus licheniformis* Variation was found in optimization of cultural conditions (incubation period, incubation temperature and pH) for the most potent antipathogenic metabolites production. *Bacillus licheniformis* showed most potent antipathogenic activity at pH 7, at an incubation period of 48h and at an incubation temperature of 25^0C. Antipathogenic metabolites was then detected as bacteriocin like substances. Samples containing bacteriocin like substances were characterized with respect to their heat and pH stability and susceptibility to denaturation by the enzyme. It showed heat stability up to 80^0C for 30 minutes, stability up to pH 7. Papain treated cell-free supernatant did not show any bacteriocin activity, suggesting that the substances could be antimicrobial peptides. Solvent extraction of bacteriocin was performed by using chloroform and here maximum bacteriocin activity was found in interface layer rather than aqueous and organic layer.

Keywords: Antipathogenic metabolites; Bacteriocin; Antimicrobial peptide; Normal microflora.

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1. Introduction

Microbes that colonize the human body during birth or shortly thereafter, remaining throughout life, are referred to as normal flora [1]. Commensal microflora (normal microflora, indigenous microbiota) consists of those micro-organisms, which are present on body surfaces covered by epithelial cells and are exposed to the external environment (gastrointestinal and respiratory tract, vagina, skin, etc [2]. Human skin is not a particularly rich place for microbes to live. This is an environment that prevents the growth of many microorganisms, but a few have adapted to life on our skin [3]. The effects of the normal flora are inferred by microbiologists from experimental comparisons between "germ-free" animals (which are not colonized by any microbes) and conventional animals (which are colonized with a typical normal flora). The overall beneficial effects of microbes are synthesize and excretion of vitamins, prevention of colonization by pathogens, antagonize other bacteria, stimulation of the development of certain tissues, development of gut associated lymphoid tissue, production of natural antibodies etc [4]. Many mechanisms have been postulated by which Normal flora could produce antipathogenic activity. In addition to their competitive inhibition of the epithelial and mucosal adherence of pathogens and inhibition of epithelial invasion by pathogens, normal flora also show antipathogenic activity by producing antimicrobial sub-stances or stimulating mucosal immunity. Generally normal flora produce three types of antimicrobial substances- H_2O_2, organic acid and Bacteriocin [5]. Bacteriocins are proteins or short polypeptides, which are generally only toxic to bacteria that are closely related to the producing strain [6]. Some researcher found that, [7] *Staph. epidermidis*, a major constituent of the normal microflora on healthy human skin protect skin by producing antimicrobial peptide and Others [8] worked on bacteriocin- like inhibitory substance (BLIS) production by the Streptococcal normal flora of the nasopharynx which found active against pathogens *Strept pneumoniae*, *Strep pyogenes*, *H. influenzae* and *Mor. catarrhalis causing* acute otitis media. In our recent study, we have tried to investigate the ability of normal skin flora to produce antipathogenic metabolites and also tried to reveal the fact that whether the metabolites were bacteriocin like substances or not.

2. Material and Methods

2.1. Samples collection, isolation and purification

Swab samples were collected from the skin of upper arm, under arm, forearm, leg and forehead of 30 healthy human persons free from skin or any other infection during sampling time and with a history of not taking antibiotics or any other antimicrobial drugs in the previous six months. For the collection of normal skin flora, the person who met selection criteria was first taken to laboratory. With the help of sterile moisten cotton bud sample was taken from forearm, upper arm, under arm, forehead and leg of the selected healthy human persons. The swab stick was then soaked in 10ml sterile peptone water from which 1 ml sample was used for pour plate method [9] by using Mannitol salt agar medium and then incubated at 37^0C for 24 to 48 hours. Following incubation, different types of colonies based on differences in size, shape, color and other colony characteristics, were selected and transferred to nutrient agar slant and were further purified by repeated streaking on nutrient agar plates.

2.2. Test pathogens

For our current study, some type strains of skin and intestinal pathogens were collected from Department of Microbiology, University of Chittagong, ICDDR,B and Chittagong Maa-Shishu O General Hospital (CMSOGH) to observe the inhibitory activity of the normal skin flora against these pathogens. The used bacterial test organisms were *Kl. pneumoniae* subsp. *ozaenae* (CMSOGH, urinary tract infection), *Kl. pneumoniae* subsp. *pneumoniae* (CMSOGH, urinary tract infection), *Salm. typhi (*AEI14296), *E. coli* (ATCC 25922), *Ps. aeruginosa (*CRL, ICDDR,B), *Staph. aureus* (ATCC 6538). *Strep. pyogenes* (CMSOGH , pus) *B. subtilis* (BTTC17)

2.3. Primary screening for antimicrobial activity of the isolates

The isolated bacteria (normal flora) were subjected to primary screening procedures to find out the ability of the normal flora to produce antipathogenic metabolites by streak plate method [10]. The isolates (normal flora) were streaked over the surface of petridish containing pre-poured solidified Mueller Hinton media across one side of the plate & incubated at 37^0C for growth and diffusion of antimicrobial metabolites in the growth medium. Then cultures of the test pathogens were streaked right angles to the previously inoculated isolate. The plates were incubated at 37^0C for 24 hours. After the growth of the pathogens, the plates were observed for any zone of inhibition. A clear zone of inhibition appeared against only those organisms which were sensitive to the metabolites produced by the isolates. Those pathogens and respective isolates were selected for secondary screening.

2.4. Secondary screening for antimicrobial metabolite production by the isolates

Primarily selected isolates were subjected to the secondary screening procedures to ensure their antimicrobial activity against the respective pathogen. For this purpose, each normal flora was inoculated in 10ml sterile nutrient broth and incubated at 37^0C for 24 hours. After incubation, culture broth was centrifuged at 12000 rpm for 15 minutes at 4^0C and then filtered through membrane filter. After filtering off the biomass, the antimicrobial activity of the crude filtrate obtained was tested against pathogens using disc diffusion [11] and well diffusion method [12]. All tests were performed in triplicate and the average clear zone greater than10 mm were considered positive results [13].

2.5. Identification

Bacterial isolate (normal flora) which showed positive result on primary screening and secondary screening against maximum pathogens were subjected to biochemical tests and results were compared with standard description given in "Bergey's Manual of Determinative Bacteriology", 8^{th} ed. [14] and 9^{th} ed. [15]. The tests include Gram-staining, Spore staining Acid-fast staining, starch hydrolysis , Voges Proskauer (V-P) Test, Production of hydrogen sulphide , Gelatin liquefaction test, Nitrate reduction test, Indole test Deep glucose agar test, Catalase reaction Methyl-red test, Fermentation test, Urease test Motility test, Oxidase test Cultural and physiological studies were also done. A rapid bacterial identification test kit BBL Crystal™ Identification Systems Gram-Positive Is Kit [16] was also used to identify species of bacteria.

2.6. Optimization of cultural conditions for antipathogenic metabolite production

Different environmental and cultural conditions may influence the antipathogenic metabolite production ability of the selected isolates. Considering this matter, in our present study, an attempt was made to optimize the cultural conditions that favor maximum antipathogenic metabolite production by isolated bacterial strain. To determine the effect of incubation period on antimicrobial metabolite production, 4 pieces 250 ml conical flask was labeled as 24h, 48h, 72h, 96h and 50 ml sterilized nutrient broth was taken on each flask. Selected normal flora strain was inoculated and incubated at 37^0C for 24h, 48h, 72h, 96h. After incubation, culture broth was centrifuged at 12000 rpm for 15 minutes at 4^0C and then filtered through .45µ Cellulose nitrate membrane filter. After filtering off the biomass, using 50 µl and 100 µl of crude filtrate per well the antipathogenic activity was tested against respective pathogens by well diffusion method. The clear zone diameter was measured to determine the antimicrobial activity. To observe the influence of pH on the production of antipathogenic metabolites 8 pieces 250 ml conical flask was labeled as pH 4, pH 5, pH 6, pH 7, pH 8, pH 9, pH 10 and pH 11. 50 ml sterilized nutrient broth was taken on each flask and pH was adjusted acoording to the label in aseptic condition. Selected normal flora strain was inoculated and incubated at 37^0C for optimum incubation period. After incubation, preparation of cell free supernatant and determination of antimicrobial activity was done by following the same method of incubation period optimization. To evaluate the impact of temperature on antipathogenic metabolite production, 3 pieces 250 ml conical flask was taken and labeled as 27^0C, 37^0C and 45^0C. 50 ml sterilized nutrient broth was adjusted to optimum pH in aseptic condition and taken on each flask. Selected normal flora strain was inoculated and incubated at 27^0C, 37^0C and 45^0C for optimum incubation period. After incubation, preparation of cell free supernatant and determination of antimicrobial activity was done by following the same method of incubation period optimization.

2.7. Detection of bacteriocin like substance production

2.7.1. Preparation of bacteriocin samples

Isolated bacterial strain was cultivated in its appropriate culture medium (Brain Heart infusion Broth, Hi Media Ltd) and conditions. Extraction of bacteriocin was carried out using the method of Schillinger and Luke [17]. According to this method, cells from the culture medium were removed by centrifugation at 5000 rpm for 10 minutes at 4^0C followed by filtration through .45µ Cellulose nitrate membrane filter. The pH of the supernatant was adjusted to 6.5 with 5M NaOH and 5M HCl to exclude the antimicrobial effect of organic acid. Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase. The supernatant was serially diluted (2 fold dilution) with filter-sterilized 20mM phosphate buffer and stored at -20^0C.

2.7.2. Bacteriocin bioassay

Antimicrobial activity against indicator organisms was determined by a well diffusion assay. Pre-poured Brain Heart Infusion (BHI) agar plates were overlaid with BHI soft agar containing indicator culture of *Staphylococcus aureus* . Wells of 8 mm in diameter were cut into the agar plate with a cork borer and 100 µl of the culture supernatant fluid was placed into each well. The plates were incubated overnight at 37^0C. After

incubation, arbitrary units (AU) of bacteriocin activities were calculated according to spot-on-lawn method [18]. The antimicrobial activity of the bacteriocin is defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and is expressed in activity units per ml (AU/ml). That means, AU/ml $=2^n \times 1000$ µl /amount of sample per well (µl).

2.8. Determination of bacteriocin activity against test pathogens

Bacteriocin activity of the skin isolates against test pathogens was determined by the same process of bacteriocin bioassay just replacing the indicator organism with the test pathogens. The activity units (AU) of bacteriocins against the pathogens were also calculated.

2.9. Characterization of bacteriocin

The bacteriocin samples were characterized with respect to their stability at or sensitivity to different temperatures, pH, and susceptibility to denaturation by enzyme.

2.9.1. Heat sensitivity of bacteriocin

To observe the heat stability, bacteriocin samples were prepared from culture supernatants of the selected bacterial isolate by following the method of Schillinger and Luke [17]. Then samples were then dispensed at 10 ml per test tube and exposed to various temperatures such as 40, 60, 80 and 100ºC in circulatory water bath and 121ºC in autoclave. Aliquot volumes were then removed after 0, 30, 60 and 90 minutes and assayed for bacteriocin activity.

2.9.2. pH sensitivity of bacteriocin

For the determination of pH stability, samples of bacteriocin were prepared from culture supernatants and pH was adjusted to 3, 4, 5, 6, 7, 8 and 9 with 5N HCl or 5N NaOH. After incubating for 4 hours at room temperature, the bacteriocin samples were adjusted to pH 6.5 and assayed for bacteriocin activity.

2.9.3. Enzyme sensitivity of bacteriocin

The sensitivity of the bacteriocin to enzymes was also checked. Bacteriocin samples, from cell-free culture supernatant fluid of different isolates, at pH 6.5 was treated separately with papain at a final concentration of 1.0 mg/ml. Samples of bacteriocin were incubated with the enzyme for 2 hours at 37ºC. After that, samples of bacteriocin were heated for 3 minutes at 100^0C to inactivate enzyme activity and assayed for bacteriocin activity [19].

2.10. Solvent extraction of bacteriocin and its' bioassay

The extraction of bacteriocin was done by the sample made from cell-free culture supernatant. The extraction was performed following slightly modified method of .Burianek and Yousef [20]. The culture supernatant (100mL) was stirred vigorously for 20 minutes with equal volume of chloroform and transferred to separation

funnel. The aqueous layer, organic layer and the interface layer between the aqueous and organic phases were harvested separately and the residual chloroform was eliminated by rotary vacuum evaporator. Then the bacteriocin activity of the interface layer, aqueous and organic layers was measured separately against the respective pathogen and the indicator organism and the result was expressed in AU/ml.

3. Result and Discussion

3.1. Isolation from skin

In our present study, 10 normal bacterial flora from the skin of upper arm, under arm, forearm, leg and forehead of 30 healthy persons were isolated based on their colony characteristics and abundance on the selective culture medium, Mannitol Salt Agar and subjected to primary screening by cross streaking method.

3.2. Primary Screening

After primary screening, bacterial strain isolated from Upper Arm (UpA) showed inhibitory activity against pathogen *Kl. pneumoniae* subsp. *pneumonia*, and it was selected for secondary screening.

3.3. Secondary screening

Well diffusion and disc diffusion method was used in secondary screening and our selected Isolate showed remarkable result against pathogen *Kl. pneumoniae* subsp. *pneumonia* (Table-1).

Table1: Production of inhibitory metabolites by selected isolates against pathogens in secondary screening.

Pathogen	**Diameter of zone of inhibition (mm)**			
	Aliquots of sample in well diffusion method		**Aliquots of sample in disc diffusion method**	
	100µl	**50µl**	**80µl**	**50µl**
K. pneumoniae subsp. *pneumonia*	20	14	18	13

3.4. Identification of selected isolates

The selected skin isolate was tested for their morphological, cultural and biochemical characters. The characters were then compared with the standard description given in "Bergey's Manual of Determinative Bacteriology", 8th and 9th Edition [14, 15]. The characteristics of each isolates are summarized in Table 2. Data present in Table 2 indicated that the isolate UpA belongs to the genus *Bacillus* and found closely related to the species *Bacillus*

licheniformis, while compared with the standard description given in "Bergey's Manual of Determinative Bacteriology", 9th ed. [15]. For further confirmation, rapid Identification kit BBL Crystal™ Identification Systems Gram-Positive Kit was used. (Figure 1) and found same result with .9999 confidence.

Table 2: Cultural, morphological and biochemical characteristics of selected isolate

Parameter **Biochemical tests**	**Observation**	**Parameter** **Fermentation**	**Observation**	**Parameter** **Agar Colony**	**Observation**
Oxidase	+	Glucose	+	Form	Irregular
Catalase	+	Lactose	+	Elevation	Flat
Deep glucose agar	Aerobic	Sucrose	_	Margin	Rough
Motility	+	Fructose	+	Surface	Rough
Starch hydrolysis	_	Mannitol	_	Color	Off white
Gelatin hydrolysis	+	Arabinose	_	Colony diameter	3mm
Indole	_				
Nitrate reduction	+	Xylose	_	**Staining and**	**Morphology**
Citrate utilization	_	Inulin	+	Gram staining	Gram positive
Voges-Proskauer	_	Raffinose	_	Size	4.48×1.76 µm
Methyl red	+	Galactose	+	Shape	Long Rod
Urease	_	Salicin	_	Form	Single/pair and
H_2S production	+	Maltose	+		chain
		Cellubiose	+		

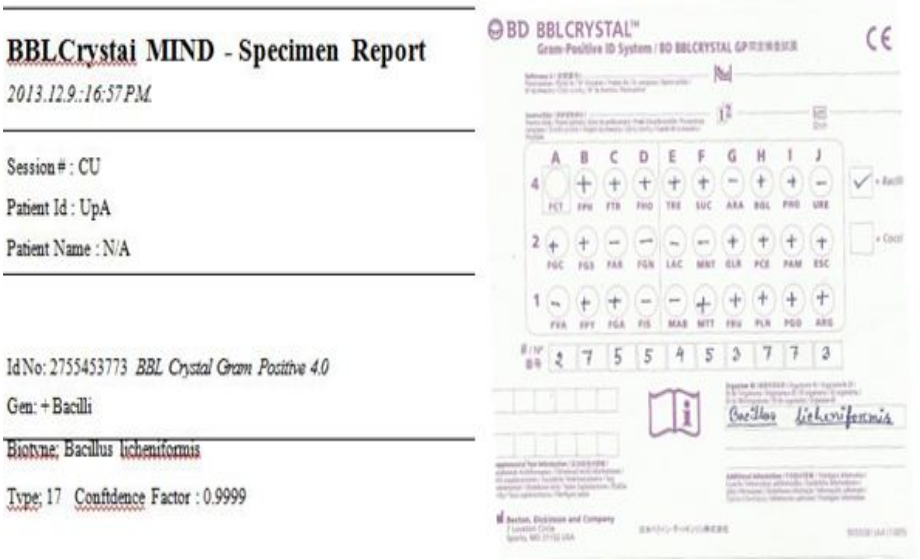

Figure 1: BBL Crystal Identification System Result

3.5. Optimization of cultural conditions for the production of antipathogenic metabolites with maximum activity

Production of metabolites from microorganism varies with the variation in incubation period, incubation temperature, pH, etc. In present investigation, the optimum cultural coditions (incubation period. incubation temperature, pH etc) were studied with the isolate *Bacillus licheniformis* against pathogen *K. pneumoniae* subsp. *pneumoniae* (Table-3)

Table 3: Optimization of different cultural condition (incubation period. pH, temperature) for maximum antipathogenic metabolite production

Optimization parameter	Incubation period				pH						Temperature		
	24h	48h	72h	96h	pH 5	pH 6	pH 7	pH 8	pH 9	pH10	25^0C	37^0C	45^0C
Diameter of zone of inhibition(mm)													
100µl	18	21	15	11	---	10	20	17	16	14	22	20	16
50µl	14	17	11	9	---	8	16	12	10	11	18	16	11

3.6. Detection of Bacteriocin like substance

In our present investigation, we tried to determine whether the metabolites found from normal microflora contain any bacteriocin like substance or not. For this purpose, we used Brain heart infusion broth for bacteriocin production and bacteriocin was extracted by follwing the method of Schillinger and Luke. From this study, we found that the isolates- *Bacillus licheniformis*, produced bacteriocin like substances and showed activities against pathogen or indicator organism (Fig :2) where lowest zone of inhibition was found 8mm for *Staph. aureus* and it was 9 mm for *Kl. pneumoniae* subsp. *Pneumonia*. Chaimanee [21] described isolates- *L. fermentum* and *Strep bovis* showing bacteriocin activity 40 AU/ml against pathogens *Stph. agalactiae* and *Stph. dysgalactia* by following the same method which is closely related to the result we obtained.

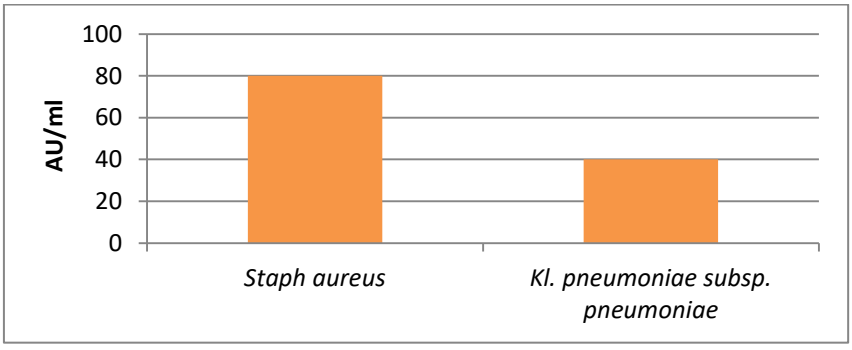

Figure 2: Activity of bacteriocin produced by the *Bacillus licheniformis* Characterization of Bacteriocin

In our present investigation, we tried to characterize the bacteriocin sample with respect to their sensitivity to different pH, temperature and enzyme papain.

3.7. Heat sensitivity of bacteriocin

Samples of bacteriocin were exposed to various heat treatments such as 40, 60, 80, 100 and 121ºC for the periods of 0, 30, 60 and 90 minutes and then assayed for bacteriocin activity. Data presented in Figure 3 & Figure 4 showed that bacteriocin produced by *Bacillus licheniformis* was prominent for it's' heat stability up to 80^0C for 30 mnutes. These results were in agreement with the findings Bizani (2002) [22] who reported that the bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A was stable at 80°C, but the activity was lost when the temperature reached 87°C

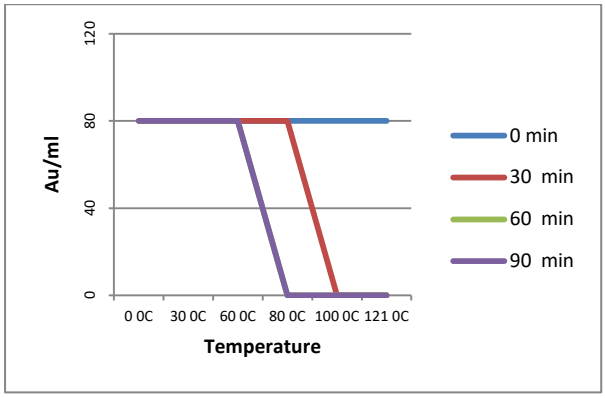

Figure 3: Activity of heat treated bacteriocin produced by the isolate *Bacillus licheniformis* against *Staph. aureus*

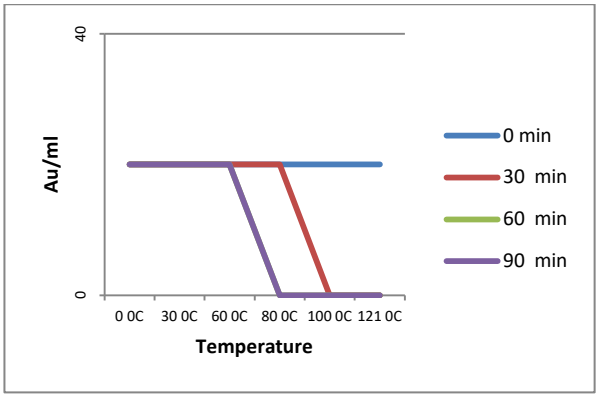

Figure 4: Activity of heat treated bacteriocin produced by the isolate *Bacillus licheniformis* against *Kl. pneumoniae* subsp. *pneumoniae*

3.8. pH sensitivity of bacteriocin

The bacteriocin produced by the isolate *Bacillus licheniformis* did not show any activity at pH-8. It was found stable upto pH-7 (Figure: 5). An investigation about bacteriocin characterization was made by Fatima (2013) [23] where bacteriocin produced from *L. plantarum* and *Ped. pentosaceus* showed pH stability up to pH 2 to pH 6 which was closely related to our findings.

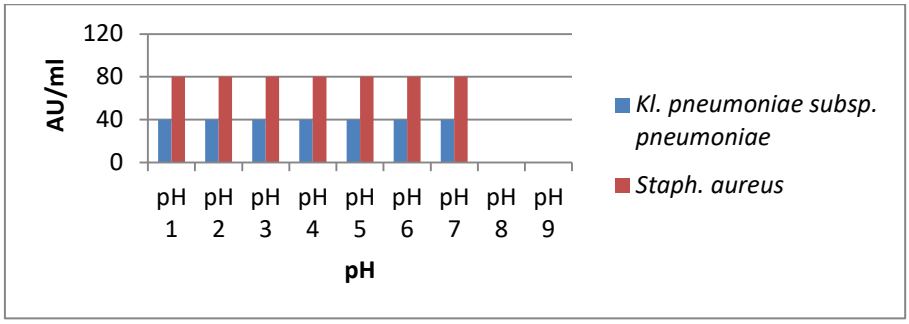

Figure 5: Activity (AU/ml) of *Bacillus licheniformis* produced bacteriocin subjected to different pH.

3.9. Enzyme sensitivity of bacteriocin

The bacteriocin samples from different isolates were exposed to the enzyme papain not only to determine its' sensitivity to enzyme but also to confirm the proteinaceous nature of bacteriocin.. It was found that papain treated cell free supernatant did not show any antimicrobial activity (Table-4), suggesting that the substances could be antimicrobial peptides. Our findings were in agreement with the report of Lauková (1993) [24] describing that bacteriocin-like substance produced by *Ent. faecium* CCM4231 *Ent. faecium* CCM4231 was sensitive to pronase and trypsin.

Table 4: Activity (AU/ml) of bacteriocin produced by *Staph. warneri* after papain treatment

Bacteriocin	**Bacteriocin activity (AU/ml)**	
	Pathogen	
	K. pneumoniae subsp. pneumoniae	***S. aureus***
Bacteriocin without papain	40	80
Papain treated bacteriocin	0	0

3.10. Chloroform Extraction of Bacteriocin like substance

In our present investigation, we tried to extract bacteriocin from crude cell free supernatant. The extraction was performed according to Burianek and Yousef (2000) [20]. Then bacteriocin activity was measured in the interface layer, aqueous and organic phases**.** Highest bacteriocin activity was found in interface layer. Increased bacteriocin activity in the interface layer (Table 5) indicates that this method concentrates the bacteriocin at the interface between chloroform and the aqueous phases of the bacteriocin containing culture supernatant**.**

Table 5: Bacteriocin activity of *Bacillus licheniformis* (AU/ml) against pathogens after in interface layer after chloroform extraction

Skin isolates	**Pathogen**	**Bacteriocin activity (AU/ml)**
B. licheniformis	*K. pneumoniae* subsp. *pneumoniae*	160
	S. aureus	160

4. Conclusion

Microorganisms are getting resistance against conventional antibiotics day by day. That is why, we tried to obtain a potential alternative to conventional antibiotics which will contribute a lot in future research for controlling disease caused by drug resistant bacterial strains though we have some limitation in our research such as Identification of bacteria through 16S RNA Analysis, Chromatographic study of protein etc. Human welfare was at the centre of our research objectives. To reach this final goal, further research including further purification and human trial with the produced bacteriocin is required to prevent the emergence of MDR pathogens and to improve the disease management system

5. Recommendations

Before reaching any final decision to use this metabolites as therapeutics, Chromatographic study of the protein, Human trial and cytotoxic study should be considered.

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References

[1] Salyers Aa, Whitt Dd (2000) Microbiology: Diversity, Disease and the Environment. Bethesda Maryland: Fitzgerald Science Press. Immunol Lett. 2004 May 15;93(2-3):97-108.Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases**.**

[2] Davis Cp. (1996) Baron,s Medical Microbiology. 4th edition chapter 6. Available in http://www.ncbi.nlm.nih.gov/books/NBK7627/

[3] Davidoliver. (2006) MICROBES AND YOU: NORMAL FLORA archive, textbook. Http://www.scq.ubc.ca/microbes-and-you-normal-flora/

[4] Todar, K. (2013) Todar'S Online Textbook of Bacteriology, Part 2, Chapter 2, Page 2-5. Http://textbookofbacteriology.net/normalflora_2.html.

[5] Servin LA (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens FEMS Microbiol Rev. Oct;28(4):405-40.

[6] Chavan Ma, & Riley Ma (2007) Molecular Evolution of Bacteriocins in Gram-Negative Bacteria, In:Bacteriocins: Ecol Evolut. Pp. 5-18,

[7] Gallo Lr, Nakatsuji T. (2011) Microbial Symbiosis with the innate immune defense system of the Skin.

·J Invest Dermatol. 131, pp 1974–1980;

[8] Walls T, Power D, Tagg J (2003) Bacteriocin-like inhibitory substance (BLIS) production by the normal flora of the nasopharynx: J. Med. Microbiol. Vol. 52 no. 9 829-833.

[9] Sanders, E. R. Aseptic Laboratory Techniques: Plating Methods. J. Vis. Exp. (63), e3064, doi:10.3791/3064 (2012).

[10] Florey Hw, Chain Eb, Heatly Ng, Jenninings Ma, Saunders Ag, Abraham Ep, And Florey Me (1949) Antibiot. 1:273-287.

[11] Bauer Aw, Kirby Mm, Sherris Jc, Turuck M (1966) Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol. 45(4):493-496.

12] Magaldi S, Mata-Essayag S, Hartung De Capriles C, Perez C, Colella Mt, Olaizola C, Ontiveros Y(2004) Well diffusion for antifungal susceptibility testing. Int J Infect Dis. Volume 8, Issue 1 , Pages 39-45.

[13] Lima-Filho, J.V.M., A.F.F.U. Carvalho, S.M. Freitas and V.M.M. Melo, 2002. Antibacterial activity of extracts of six macroalgae from the Northeastern Brazilian Coast.Brazil. J. Microbiol., 33: 311-313

[14] Buchanon RE. Gibson NE (1974) Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkins Co. Baltimore. Page:530-534.

[15] Holt JG, Krieg NR, Sneath Pha, Staley JT, Williams ST (2000) Bergey's manual of determinative bacteriology, 9th ed. Lippincott Williams and Wilkins, Baltimore. Pp-532,549-551,559,576,592.

[16] 8809911JAA(01) 2012-03 CLA COMPLEXTY:HGH CDC IDENTIFIER CODES ANALYTE:0412 TEST SYSTEM:07919

[17] Schillinger V. Lucke Fk (1989) "Antimicrobial activity of Lactobacillus sakeisolated from meat". Appl. Environ. Microbiol. 55; 1901-1906.

[18] Yamamoto Y, Togawa Y, Shimosaka M, Okazaki M, (2003) Purification and characterization of a novel bacteriocin produced by Enterococcus faecalisstrain. RJ-11.App Environ Microbiol. 69, 5746–5753.

[19] Narayanapillai Udhayashre, Duraisamy Senbagam, Balakrishnan Senthilkumar, Kanagaraj Nithya, Ramasamy Gurusamy (2012), Asian Pacific Journal of Tropical Biomedicine (2012)S406-S410, journal homepage:www.elsevier.com/locate/apjtb.

[20] Burianek Ll, Yousef AE (2000) Solvent extraction of bacteriocins from liquid cultures. Lett Appl Microbiol.;31(3):193–197.

[21] Chaimanee V, Sakulsingharoj C, Deejing S, Seetakoses P, Niamsup P (2009) Screening and characterisation of bacteriocin-producing bacteria capable of inhibiting the growth of bovine mastitis. Maejo Int J Sci Tech. 3(01), 43-52.

[22] Bizani, D., Brandelli. A. 2002. Characterization of a bacteriocin produced by a newly isolated Bacillus sp. Strain 8 A. J Appl Microbiol. Volume 93, Issue 3, pages 512–519**.**

[23] Fatima, D. and Mebrouk, K. 2012. Characterization and determination of the factors affecting anti-listerial bacteriocins from Lactobacillus plantarum and Pediococcus pentosaceus isolated from dairy milk products. African Journal of Food Science Vol. 7(2) pp. 35-44.

[24] Lauková, A. Mareková, M. Javorský, P. 1993. Detection and antimicrobial spectrum of a bacteriocin-like substance produced by Enterococcus faecium CCM4231 Enterococcus faecium CCM4231. Lett in Applied Microbiol**.** Volume 16, Issue 5, pages 257–260.