RESEARCH ARTICLE

Production, purification and characterization of lichenin from Bacillus licheniformis.

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Abstract

Lichenin are bacteriocin produced by Bacillus licheniformis, with probiotic, fungicide and medical importance. Lichenin producing Bacillus licheniformis was isolated from soil sample, collected from research plots of SHUATS, Allahabad. All the gram positive rods was biochemically analyzed and advanced bacterial identification Software showed 89% similarity of isolated bacterial cultures with Bacillus licheniformis. The crude bacteriocin of Bacillus licheniformis, exhibited antagonistic activity against Staphylococcus aureus MCCB 0139 whereas no zone of inhibition ascertained against Escherichia coli MCCB 0017. Ten variables viz. incubation temperature, incubation period, pH and medium components consisting sorbitol, lactose, yeast extract, peptone, NH₄NO₃, K₂HPO₄, and MgSO₄, respectively was optimized from run number 1 to 76. Response surface data showed maximum bacteriocin production by run number 43 at pH 8.0, incubation temperature 50°C, and incubation period 12 hrs. Optimization illustrated that effect of increasing pH from 4.0 to 8.0, incubation temperature from 37.5°C and 50°C have significant effect on bacteriocin activity along with peptone concentration from 0.25% to 0.75% have significant effect on bacteriocin activity. The average value depicted in the top and bottom phase of the cube and left and right face of the cube, respectively showed sorbitol, peptone and MgSO₄ have significant effect on bacteriocin activity. Crude lichenin was partially purified by 80% ammonium sulphate precipitation, dialysis and ion exchange chromatography followed by quantification and estimation by Lowry’s method. SDS-PAGE characterized purified lichenin and revealed < 9 kDa of molecular weight.

Introduction:

Microorganisms primarily yield a protein or compound or substance which exhibit antibacterial properties. These antibacterial properties type peptides are known as bacteriocins (Line et al., 2008). Bacteriocins are bacterial yield ribosomal synthesized protein or antimicrobial peptide. This antimicrobial peptide is classified into broad categories. Colicins and microcins produced by gram negative bacteria whereas bacteriocins formed by gram positive bacteria are classified into different classes (Yang et al., 2007). Bacteriocins are generally produced by bacteria viz Bacillus and Lactobacillus sp. and exist as secondary metabolities (Jack et al., 1995). Bacteriocins are described as compound

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formed by bacteria consists of biologically active protein fraction and bactericidal mechanisms. This action made its use in food industries and medicines. Bacteriocins produced by lactic acid bacteria have been exercising in food industry as natural preservatives. Bacteriocins which are primarily produced by Lactobacillus can also be formed by distinct species and multiple strains like Brevibacillus sp. strain GI-9, produced a novel bacteriocin called laterosporulin (Singh et al., 2012). Bacteriocins produced by lactic acid bacteria are known as nisin and this is only one bacteriocins used as a preservative in foods (Cleveland et al., 2001). Since 1983, nisin is being used as food preservative in 40 countries. Likewise lactic acid bacteria the uttermost studied bacteriocin forming bacteria Bacillus species for example B. subtilis and B. licheniformis are “generally recognized as safe” (GRAS) bacteria (Martiraniet al., 2002). Nisin is FDA authorized bacteriocin, used as preservative in a pasteurized processed cheese (Elayaraajiet al., 2014). An advanced bacteriocin produced by Bacillus licheniformis are named as lichenin which is a bacteriocin like substance (Pattanaik et al., 2001). Bacillus licheniformis is a saprophytic bacterium present in large scale in nature and has been applied in the fermentation industry for the production of antibiotics, proteases and amylases. The first antibiotic produced by Bacillus licheniformis are named as Bacitracin practiced in the field of veterinary and medicine. This demonstrated antibacterial activity in opposition to gram positive species and slight with gram negative bacteria (He et al., 2006). Bacillus licheniformis has been produced hydrophobic peptides such as amoebicins d13-A, d13-B and d13-C evoked antiamoebic action against human pathogenic and non pathogenic species of Naegleri (Galvez et al., 1994). Freshly, three bacteriocins has been produced by Bacillus licheniformiscalled Licidin, Bacillocin 490 and P40 (Pattanaik et al., 2001; Martiraniet al., 2002; Olivera-Cladera et al., 2004).

Bacillus licheniformis are reported to produce antifungal molecule such as fungimycin M4, which inhibits the growth of the various fungus. Bacteriocin production by Bacillus licheniformis are class II bacteriocin includes (0.77-10 kDa), ribosomally synthesized, nonmodified and linear peptides which are large heat and pH stable. The antimicrobial agent produced by Bacillus licheniformis display their antimicrobial activities are used in milk and dairy products (Martiraniet al., 2002). The bacteriocin-like peptides produced by Bacillus licheniformisdemonstrated antagonistic activities against various species of Gram-positive bacteria (He et al., 2006). Bacteriocin production by Bacillus licheniformis are class II bacteriocin includes (0.77-10 kDa), ribosomally synthesized, nonmodified and linear peptides which are large heat and pH stable. The bacteriocin-like peptide produced by Bacillus licheniformis used as medicine, natural bio-preservative, and pesticides for plant diseases. Bacillus licheniformis broad range of metabolic activity made its use in industries for the formation of antibiotics, chemicals and enzymes. Bacitracin produced by B. licheniformiscan also be practiced as animal feed supplement (Murphy et al., 2007). In the present study, we optimized the various parameters in order to study lichenin production by isolated Bacillus licheniformis in which RSM was employed. After medium optimization, the lichenin was purified by Ammonium sulfate precipitation, Dialysis, ion exchange chromatography and estimated lichenin concentration by lowry’s method. Lichenin, molecular weight was determined by SDS-PAGE.

Materials and Methods:-
Sample collection:-
Hundred samples of soil were collected from research plot of SHUATS, Allahabad, in the sterilized sample bottle. Soil sample were taken from 3cm to 5cm depth after removing 5cm from the earth’s surface.

Isolation and identification of bacterium:-
One gram of soil sample was suspended in 9 ml ringer’s solution and vortex vigorously to dissolve the particles. The sample was serially diluted and 1ml from 10⁻³ dilution was added to Bacillus medium plates. The plates were incubated at 37 ± 2°C for 24-42 hrs (Tendulkar et al., 2007). Identification of the bacterium was done by cultural, morphological and biochemical tests as per Bergey’s Manual of Systematic Bacteriology (Claus et al., 1986). The biochemical test such as indole test, voges-proskauer, citrate utilization, catalase, growth at 7% NaCl, 45°C and 65°C temperature, motility, nitrate reduction, starch hydrolysis, casein hydrolysis, gelatin hydrolysis, tyrosine degradation, esculin hydrolysis, egg yolk reaction, urease production, β-galactosidase, oxidase and various sugar fermentation such as glucose, arabinose, raffinose, maltose, sucrose, lactose, ribose, cellobiose, sorbitol, N-acetyl-D-glucosamine, fructose, glycerol, glycogen, meso-inositol, mannitol, D-mannose, melibiose, rhamnose, salicin, sorbitol, sucrose, starch, trehalose and D-xylose was done to identify the isolated organisms. The procedure was followed as given in Anjea (2003).

Evaluation of Antibacterial activity:-
Isolated Bacillus licheniformis were cultivated on Muller Hinton Broth at 200 rpm at 37±1°C for 24-48 hrs. Bacillus licheniformis seeded cultures are centrifuged at 8000 g for 20 min at 4°C and the cell free supernatant was tested for
antibacterial activity by using agar well diffusion. Pre-poured Muller Hinton Agar media plate was equilibrating under anaerobic conditions spread with 10^6 cfu of E. coli and S. aureus and allowed to dry. In Muller Hinton Agar plate, a well of 5 mm diameter was cut by using cork borer. The well was filled with 50 μl of cell free supernatant incubated overnight at 37 ± 1°C.

Optimization of physical and chemical parameters for lichenin production by Bacillus licheniformis:-
Optimization was carried out using different physical and chemical parameters. The variables used were sorbitol, lactose, peptone, yeast extract (YE), NH₄NO₃, K₂HPO₄ and MgSO₄. All the experiments were carried out at different incubation temperature (37.5°C, 25°C and 50°C), pH (4, 6 and 8) and incubation period (12 hrs, 66 hrs and 120 hrs). The response surface methodology (RSM), experimental design applied for optimizing lichenin (bacteriocin) production. Response surface methodology was screened by a design for lichenin production by Bacillus licheniformis and suppression of S. aureus MCCB 0139 and E. coli MCCB 0017 from run number 1 to 76 (Sersyet et al., 2009).

Purification and characterization of lichenin produced by Bacillus licheniformis:-
Ammonium sulphate precipitation
Ammonium sulphate enforced to precipitate lichenin (bacteriocin) was optimized by supplementing alternating concentrations (20%, 50% and 80%) to the crude lichenin (bacteriocin) extract. The best precipitation was proceeded for 1000 ml of crude lichenin extract. 519.1 g of ammonium sulphate was slowly added to crude lichenin by continuous stirring on magnetic stirrer until ammonium sulphate completely dissolved and kept at 4°C for overnight. After overnight incubation, ammonium sulphate supplemented lichenin protein was centrifuged at 6,000 x g for 20 min at 4°C. The supernatant was discarded and pellet was dissolved with phosphate buffer (0.1M, 7.0 pH), stored at -20°C for further purification of lichenin (Henaat et al., 2011).

Dialysis
The ammonium sulphate precipitated pellet was dissolved in 1X phosphate buffer (0.1M) and purified by 1 kDa dialysis membrane tube. 400 μl dissolved pellet was transferred in 1 kDa dialysis membrane and then dialysis tube placed in a beaker containing 1X phosphate buffer (0.1M, 7.0 pH) on a continuous stirring by magnetic stirrer at 4°C for overnight. The purified dialysed lichenin was collected and stored at -20°C for ion exchange chromatography (Henaat et al., 2011).

Ion exchange chromatography
The purified dialysed lichenin was subjected to cation exchange chromatography using Sephadex resin. The lichenin was eluted with 0.1M to 1.0M NaCl gradient in 0.1M phosphate buffer, pH 6.4 using flow rate of 0.5 ml/min. The collected purified lichenin fractions was estimated by U.V spectrophotometer at 280 nm (Dusane et al., 2013).

Estimation of lichenin concentration
The collected ion exchange purified protein fractions was exercised for quantitative protein estimation by Lowry’s method. In 10 tubes, 1ml of purified lichenin was taken. 2 ml of Lowry’s reagent was added, incubated at room temperature for 10 min, 0.2 ml of folins-ciocalteau reagent again added in all tubes, incubated at room temperature for 30 min and then absorbance at 660 nm by visible spectrophotometer were taken for lichenin estimation (Henaet al., 2011).

Molecular weight determination by SDS-PAGE
The purified lichenin was separated by SDS-PAGE as described by Schagger and Jagow (1987). The prestained protein ladder covering a wide range of molecular weight from 10 to 245 kDa (MolBio, Himedia) was used. Proteins bands was detected by silver staining (Dusane et al., 2013). Silver staining was performed by a procedure of Blum (1987) with slight modification in protocol given by proteomic resource centre, the Rockefeller University, New York.

Statistical analysis
The data recorded during the evaluation of antagonistic activity of cell free supernatant of Bacillus licheniformis using variance (ANOVA) to calculate significant effect of crude lichenin (bacteriocin) on gram positive S. aureus MCCB 0139. To optimize the various parameters in order to study lichenin (bacteriocin) production in the media D-optimal design with suitable categoric factors in addition to continuous factors in RSM was employed. The points chosen in this design was algorithmically according to number of factors and desired model (Table 1). The points
chosen were not at any certain positions they are selected to meet the optimality criteria. Linear, quadratic, cubic and other higher order (upto 6th) order models can be fitted to create a good design RSM. This design adds constraints to exclude that particular area where response cannot measure. Those particular area where response cannot be measure can be excluded by adding constraints to the design. The design allows greater control over the level chosen for the design so that discrete factor levels can be specified for numeric factors. A linear equation calculated as proposed for the model to estimate the response of the dependent variable is given in equation 1.0

\[ Y = 9.16 + 0.19x1 - 1.18x2 - 1.85x3 + 3.24x4 - 0.17x5 - 3.92x6 - 0.09x7 + 0.04x8 + 3.73x9 + 215.95x10 + \Sigma \ldots (Eq \ 1.0) \]

Where Y is predicted response, \( x1, x2, x3, x4, x5, x6, x7, x8, x9 \) and \( x10 \) are independent variables.

**Results and Discussion:**

**Isolation and identification of Bacillus licheniformis from the soil sample**

Out of hundred soil sample, only fifty gram positive strain of *Bacillus licheniformis* was isolated on Bacillus medium at pH 7, 37±1°C for 24-48 hrs. Total eight isolated *Bacillus licheniformis* was identified by biochemical analysis and according to Bergey’s Manual of Determinative Bacteriology. Positive growth at 45°C, growth at 7% NaCl and no growth marked at 65°C. Positive citrate utilization, nitrate reduction, utilization of urease, egg yolk, casein hydrolysis, starch hydrolysis, ONPG, esculin hydrolysis, motility and oxidase whereas negative in indole production, voges-proskauer was observed. Acid without gas in sugar fermentation was produced from glucose and from a wide range of carbohydrate viz; mannitol, mannose, starch, cellobiose, fructose, glycerol, maltose, sorbitol, melibiose, ribose, sucrose and trehalose and no acid production was marked from arabinose, salicin, xylose, meso-inositol, lactose and rhamnose respectively. The biochemical observation implemented in Advanced Bacterial Identification (ABIS) software, showed 89% similarity of bacterial isolates and confirmed eight bacterial isolates as *Bacillus licheniformis*.

**Evaluation antagonistic activity of lichenin against test organisms**

The identified eight *Bacillus licheniformis* isolates was assayed for antagonistic activity against the test organisms. Out of eight only seven *Bacillus licheniformis* identified isolates are capable to inhibit the growth of *S. aureus* MCCB 0139 but none against *E. coli* MCCB 001, described in Table 2. The result showed that *S. aureus* was inhibited maximum by isolate no S41 forming a zone of 26 mm at the concentration of fifty μl followed by isolate no S13 (19 mm), S35 (18 mm), T24 (17 mm), S21 (15 mm), C2 (14 mm) and C1 (11 mm) (P<0.05), shown in Fig 1. *E. coli* was resistant against *Bacillus licheniformis* cell free supernatant, shown in Fig 2.

Lichenin (bacteriocin) is known to form pores in the bacterial membranes because of their strong hydrophobic natures. The broad-spectrum inhibitory activity of lichenin against a pathogenic microorganisms *Staphylococcus aureus* could be due to its amphipathic nature (as it contains both hydrophobic and hydrophilic residues) which could cause it to have surfactant–like activity on cell membrane, thereby disturbing cellular function (Chung et al. 2001). Bacteriocins from gram positive are usually ineffective against gram negative bacteria because bacteriocin cannot penetrate the outer membrane (OM). The cytoplasmic membrane of Gram negative bacteria is protected by an outer membrane (OM) composed of a phospholipid bilayer, surrounded by a network of lipids and polysaccharides referred to as lipopolysaccharides. The lipopolysaccharide layer forms a tight shield (Raetz and Whitfield, 2002) and acts as a barrier to many compounds, including antibiotics, hydrophobic compounds, detergents and dyes (Vaara, 1992). Studies have reported that *Bacillus licheniformis* DSM13 culture supernatant against *Staphylococcus aureus* 5G111 and *S. aureus* wood exhibited 1.5 cm and 1.3 cm of inhibition zones respectively (Dischinger et al. 2009). In contrast it have been reported that antibacterial spectrum of Bac-1B 17 produced by *Bacillus subtilis* KIBGE IB against *Eschericia coli* exhibited 18 mm zone of inhibition (Ansari et al., 2012). Bacteriocin produced by *Bacillus subtilis* exhibited antibacterial activity against *S. aureus* IVDC 6538, *S. aureus* IVDC 26003, *S. aureus* IVDC C56005 with antibacterial activities 422.7 AU/ml, 265.9 AU/ml and 452.2 AU/ml and against *E. coli* IVDC C83901, *E. coli* IVDC C83828, *E. coli* IVDC C83709, *E. coli* IVDC C83845 and *E. coli* multi-resistant isolate with antibacterial activity unit as 153.9 AU/ml, 295.4 AU/ml, 211.2 AU/ml, 201.0 AU/ml and 124.7 AU/ml respectively (Xie et al., 2009).

**Optimization of the physical and chemical parameters for lichenin production:**

Optimization was carried out using different production parameters viz; sorbitol, lactose, peptone, yeast extract (YE), MgSO₄, NH₄NO₃ and K₂HPO₄, pH (4, 6 and 8), incubation temperature (37.5°C, 25°C and 50°C) and incubation time (12 hrs, 66 hrs and 120 hrs). Table 3 indicates that maximum zone of inhibition (32 mm) was obtained in run number 43, corresponding to 8 and 19 run numbers at pH 6.0 and 8.0, temperature 37.5°C and 50°C, incubation
period at 66 hrs and 12 hrs with substrate concentration amounting to sorbitol 1.0 % and 1.25 %, lactose 0.05 %, peptone 0.5 % and 0.25 %, yeast extract 3.03 % and 0.06 %, ammonium nitrate 0.85 % and 0.05 %, dipotassium hydrogen phosphate 0.2 % and 0.1 % and magnesium sulphate 0.015 % and 0.005 %, respectively (Fig 3). The normal probability shows that our proposed model was fitting the observation with very high accuracy (Fig 4). Residual vs Predicted graph shows that all the studentized residual were lying within the 3 σ limits which clearly indicate that the whole error part was insignificant (Fig 5). Residual vs Run graph shows that the residual part for each run was also non-significant (Fig 6). All the ten variables for optimization were plotted as Residual vs Factor. Residual vs Factor for pH (Fig 7), temperature (Fig 8), incubation period (Fig 9), concentrations of sorbitol (Fig 10), lactose (Fig 11), peptone (Fig 12), yeast extract (Fig 13), ammonium nitrate (Fig 14), dipotassium hydrogen phosphate (Fig 15) and magnesium sulphate (Fig 16) shows that the model fits well for the present experiment. Absence of outlier was also observed in the plot. It means the present model is good in comparison to prediction.

The Cook’s distance plot depicts the fitting well of the model in this experiment (Fig 17). Cook’s distance is useful for identifying outliers in the x value (observation for predictor variables). It also shows the influence of each observation on the fitted response value. The design for the study was also good since the leverage value falls far away from 1.0 (Fig 18). The Predicted vs actual graph that the model was having high concentration of points along the diagonal indicating the goodness of the model (Fig 19). Selvaraj et al., (2012) reported that the close correlation between the experimental and predicted data indicates the appropriateness of the experimental design. The Box-cox plot for the power transformation shows that the lambda (λ) value of design lies between 0.54 to 0.92 with the least value at 0.73 with the current lambda (λ) values of 1.0, which lies in between the given range. Square root transformation with λ = 0.5 was applied with a constant value of k = 0.032 was used to make response value positive (Fig 20).

Fig 21-30 Illustrate the effect of individual parameters on zone of inhibition. An increasing trend in bacteriocin activity was recorded with increase in temperature from 25⁰ to 50⁰C (Fig 21) and increase in MgSO₄ concentration from 0.01 % to 0.03 % (Fig 22). However the activity of bacteriocin was found to decreasing on increasing the pH from 4.0 to 8.0 (Fig 23) and peptone concentration from 0.25 % to 0.75 % (Fig 24). It have been reported that bacteriocin production by Bacillus sp. Sh10 was studied at different pH values ranging from 4-10, showing inhibitory activity in the acidic and alkaline pH ranges with optimal activity at pH 8. Bacteriocin production in alkaline conditions are now gaining more attention in food industries because several food products vary from natural to alkaline conditions (Shayesteh et al., 2014). It has been cited in literature that nisin is the only commercial bacteriocin used as a food supplement at acidic pH while it is unstable at alkaline pH (Liu and Hansen, 1990). Further, no significant effect on bacteriocin activity by other parameters viz incubation period (Fig 25), concentration of sorbitol (Fig 26), concentration of lactose (Fig 27), concentration of yeast extract (Fig 28), concentration of NH₄NO₃ (Fig 29), and concentration of K₂HPO₄ (Fig 30) was observed. No evident relationship was obtained between growth and bacteriocin production while using different concentrations of carbon and nitrogen source. Shayesteh et al. (2014) reported that bacteriocin production by Bacillus sp. Sh10 at different pH values ranging from 4-10, showing inhibitory activity in the acidic and alkaline pH ranges with optimal activity at pH 8 which was agreeable with the present study. In contrast it have been cited bacteriocin activity was not observed using lactose, starch and sorbitol. Similar studies were reported which investigated the level of lichenin (bacteriocin) production by Bacillus licheniformis induced by lactose (Anthony et al. 2009). Lactose and lactose rich substances like sausage, whey and skimmed milk powder induced bacteriocin production in lactic acid bacteria and other bacteriocin producing bacteria (Cheighet al. 2002; Todorov and Dicks 2006).

The 3D response surface and 2D contour plots were graphical representations of regression equation. They conveniently illustrate the relationship between the responses and experimental levels of each variable and the type of interactions between the two test variables. The shapes of the contour indicate the significance of mutual interactions between the variables. Circular contour plot symbolizes negligible interactions between the corresponding variables whereas elliptical contour plot indicate the significant interactions between the corresponding variables (Zhong and Wang 2010). The contour and surface plot representing regression for activity of bacteriocin is presented in Fig 31-56.

Fig 31-39 depicts the effect of incubation temperature with other optimization parameters individually viz. pH, incubation period (hrs), sorbitol (%), peptone (%), yeast extract (%), lactose (%), NH₄NO₃ (%), K₂HPO₄ (%) and MgSO₄ (%) on bacteriocin activity. Among all parameters evaluated, only incubation temperature was found to have a significant effect on bacteriocin activity. It have been cited that bacteriocin production by B. subtilis BMPO1, was
detected at 37°C, 27°C and 57°C (Bhuvaneswari et al. 2015) whereas bacteriocin production was abolished at 65°C (Ansari et al. 2012). Similar findings also reported that bacteriocin production by Bacillus licheniformisSN2 was observed at 30°C and as well as at 40°C (Sersyet et al. 2009). Bacillus licheniformis were observed to survive at 37°C and also at 50°C; therefore, bacteriocin can be produced at optimum 37°C temperature and at high temperature of 50°C. Bacillus subtilis also shows same features regarding bacteriocin production because B. licheniformis and B. subtilis are closely related bacteria. The chromosome of B. licheniformis has large regions that are similar to Bacillus subtilis and B. subtilis orthologs, it is considered as a part of the subtilis group.

Fig 40-47 showing a negative effect on the activity of the bacteriocin was recorded with increase in pH from 4.0 to 8.0, when the interaction of pH of the medium with other optimization parameters was evaluated individually. Similar research cited in the literature reported that bacteriocin production by B. licheniformisMKU 3 was active over a wide range of pH from 4.0 – 8.0, which is a common characteristic of a number of bacteriocin produced by Lactobacillus such as plantaricins S, T and 35d and acidocin B (Messi et al. 2001). It has been also cited that maximum bacteriocin production from Bacillus megaterium 22 strain at pH 6.0-6.5, resulted in maximum inhibitory effect on the pathogenic strain (Khalil et al. 2009). In agreement, bacillocin 490 from B. licheniformis showed antimicrobial activity between acidic and alkaline pH values (Martirani et al. 2002). From the above study, it can be concluded that pH have a significant effect on bacteriocin production and also related to growth rate. Most of the report of bacteriocin production by Bacillus licheniformis showed its production at slightly acidic and alkaline pH. Lichenin (bacteriocin) activity noted at pH 4, is due to natural adaptation of B. licheniformis at acidic pH. On studying the effect of incubation period with other optimization parameters no significant effect of incubation period on the bacteriocin activity was observed as depicted in Figures 48-56. Shayestehet et al. (2014) detected no bacteriocin activity during exponential growth phase but it was detected at the end of this phase and reached a maximum during the mid stationary phase and decreased at the end of this phase. It can be concluded that incubation period with other optimization parameters a natural phenomenon for Bacillus licheniformis growth kinetics and bacteriocin production.

The cube plot shown in Fig 57 depicts three variables (temperature, pH and incubation period) involved in the activity of isolated bacteriocin. The response means of the factors levels are displayed on the corner of the cube. Low levels of the factor are to the left front or bottom and high levels are at the right back or front of the cube dimensions. The top and bottom phase of the cube had a significant effect on the antimicrobial activity of bacteriocin depicting the effect of temperature and effect of incubation period. The values depicted on the right and left phase of the cubes indicates the effect of pH which was also found to be significant. Further the effect of incubation period as observed by the values indicated on the vertices of the front and the back phase of the cube revealed their effect to be non-significant. The overall response of the three variables indicates incubation temperature and pH to be significant factors in affecting bacteriocin activity. Studying the effect of incubation temperature and incubation period on concentration of substrates like sorbitol (Fig 58), peptone (Fig 59) and MgSO₄ (Fig 60), temperature and substrate concentration were found to have a significant effect on bacteriocin activity as observed by the average values depicted in the top and bottom phase of the cube and the left and right face of the cube, respectively.

In contrast it has been investigated that no significant effect of MgSO₄ on nisin production by Lactococcus lactis (Li et al. 2002). Kayalvizhi et al. (2008) observed MgSO₄ at concentration of 1.0 g l⁻¹ exhibited negative effect on bacteriocin production, whereas sorbitol and peptone exhibited significant positive effect on the production. From the above study it can be concluded that MgSO₄ is an inorganic salt and salts are basic requirement for growth of bacterial cell and for extracting bacteriocin from microorganisms. Its higher concentration may affect cellular action of bacteria, as reported in previously work. 0.015 % and 0.005 % of MgSO₄ concentrations provide adequate amount of inorganic salt therefore resulting in favoring bacterial growth and high cell density beneficial for bacteriocin production as observed in present research work.

The interaction of incubation temperature and incubation period with concentration of other substrates selected for optimization studies revealed the effect of lactose (Fig 61), yeast extract (Fig 62), NH₄NO₃ (Fig 63) and K₂HPO₄ (Fig 64) to be active since significant difference was observed in the average values depicted on the left and corresponding right face of the cube. However the effect due to incubation temperature and incubation period was found to be inactive. Previous study reported that the concentration of K₂HPO₄ exhibited positive effect on the production, specific activity and biomass as K₂HPO₄ provides a buffering action for optimizing media (Kayalvizhi et al., 2008). Higher concentrations of K₂HPO₄ (2.0-10.0 g/l) repressed the bacteriocin activity of plantaricin ST31

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(Todorov and Dicks et al. 2006). Similar research has been reported that a high as well as low concentration of yeast extract (3.03 % and 0.06 %) supplemented medium was marked for bacteriocin production (Anthony et al. 2009).

From the above study it can be concluded that less concentrations of K$_2$HPO$_4$ (0.2 % and 0.1 %) used for bacteriocin production and buffering action for optimizing media. Lactose provide carbon source in bacteriocin production by lactic acid bacteria or other bacteriocin producing bacteria. Yeast extract provide large quantity of free amino acids, short peptides and more growth factors and NH$_4$NO$_3$ are supportive ingredients of bacteriocin production by Bacillus licheniformis.

Fig 65-72 depicts the interaction between pH and incubation period with other optimization parameters. The high variation in the average values of temperature as observed in the left and right face of the cube and pH depicted in the top and bottom surface of the cube suggested the interaction due to these factors to be active. However incubation period had no significant effect on the activity of bacteriocin (Fig 65). Similar effect was observed on studying the interaction of pH and incubation period with sorbitol (Fig 66), peptone (Fig 67) and MgSO$_4$ (Fig 68). On evaluating the effect of pH and incubation period on remaining optimization parameters revealed only the effect due to lactose (Fig 69), yeast extract (Fig 70), NH$_2$NO$_3$ (Fig 71) and K$_2$HPO$_4$ (Fig 72) to be active.

The interaction between pH, incubation temperature and other optimization parameters viz. incubation period (Fig 73) and concentrations of sorbitol (Fig 74), lactose (Fig 75), yeast extract (Fig 76), NH$_2$NO$_3$ (Fig 77) and K$_2$HPO$_4$ (Fig 78) revealed that only the average pH values as depicted in the top and bottom surface of the cube did not differ significantly suggesting the effect to be inactive. However when the interaction between pH, incubation period with peptone (Fig 79) and MgSO$_4$ (Fig 80) was analysed, the overall interaction between all the three variables were found to be active. In contrast it has been investigated that incubation period has a significant role in bacteriocin production. Bacteriocin concentration increased to a maximum at the mid-stationary phase and started declining at the end of the phase indicating it is synthesized as a secondary metabolite. Production of bacteriocin is generally associated with primary kinetics (Shayesteh et al. 2014). In agreement with previous research noted cell growth reached the stationary phase after 12 hrs of cultivation and maximum bacteriocin activity was observed from 15 hrs (Oliver et al. 2004).

According to this reported results the incubation period 12 hrs and 66 hrs had no significant effect on bacteriocin activity and on other optimizing parameters as it comes in a primary kinetics. Getting bacteriocin activity after 120 hrs may be due to the fact that bacteriocin are synthesized as a secondary metabolite that suppressed the growth of gram positive bacteria.

**Purification and Characterization of Lichenin produced by Bacillus licheniformis:-**

Ammonium sulphate precipitation
The culture supernatant of Bacillus licheniformis was purified by ammonium sulphate precipitation. The best lichenin precipitation was noted at 80% ammonium sulphate precipitation.

Similar research reported that formation of frothy floculation and pelicular layer observed with ammonium sulphate (80 % saturation) upon overnight storage at 4°C (a refrigerator temperature) (Pattnaik et al. 2001). The precipitation of an active compound by B. licheniformis isolated from marine sediment, observed at 30-60% ammonium in sulphate precipitation (Smitha and Bhatt 2012). From the above cited results a reason behind of getting frothy floculation at 80%, due to lichenin (bacteriocin) are able to reach saturation level and so that proteins are concentrating in a bulk precipitation. It’s very necessary for a protein to reach a saturation level during ammonium sulphate precipitation process so that proteins can be easily collected as pellet after centrifugation. A protein is hydrophobic in nature as it dissolved with phosphate buffer.

**Dialysis**
One of the most common method in removing salt is that of dialysis. The main feature of dialysis is that, is porous, pore size is such that small salt ions can freely pass through membrane, larger protein molecules cannot that is they are retained. Dialysis proceeds by placing a high salt sample in dialysis tube and putting it into the desired low salt sample as stated in Blaber (1998).
Ion exchange chromatography
Lichenin was purified and distinguished on the basis of their net charge by a procedure called ion exchange chromatography (IEC) using sephadex resin performed as mentioned in Berg (2002). Lichenin (bacteriocin) fractions number 1 to 10 were collected by 0.1 M to 1.0 M NaCl gradient The overall lichenin yield was quantified by UV spectrometer at 280 nm as mentioned in Table 4 and Fig 8, showing elution profile of lichenin from a sephadex column.

It have been reported that bacteriocin from Bacillus subtilis H27 purified by Q-sepharose and sephadex column chromatographies (Kindoliet al. 2012).

Estimation of lichenin concentration
Determination of lichenin concentration in collected fraction was estimated by Lowry’s method given by Lowry et al. (1951). 0.1 mg/ml BSA was used as standard for lichenin concentration estimation. Colour reaction of lichenin concentration by Lowry’s method quantified at absorbance 660 nm, revealed that NaCl gradient from 0.6 M, 0.8 M, 0.9 M and 1.0M contains 0.031 mg/ml, 0.032 mg/ml, 0.097 mg/ml and 0.012 mg/ml lichenin concentration, calculated by obtained linear regression equation, y = 1.710 + 0.038 as shown in Fig 8. Table 5a, showed BSA concentration and Table 5b, showing lichenin concentration.

Similar research reported by estimation of partially purified bacteriocin extracted from Bacillus licheniformisBL8 by Bradford method (Smith and Bhat et al., 2012). Staphylococcin concentration estimated by Bradford method (Hena et al. 2016). Bacillus subtilis cell free protein determined by Lowry’s method. Bovine serum albumim (250µg/ml) was used as standard (Swamy et al. 2012)

The molecular weight of lichenin by SDS-PAGE
The molecular weight of lichenin (bacteriocin) (0.9 M NaCl gradient fraction consisting 0.032 mg/ml lichenin concentration) was determined by running the lichenin (bacteriocin) in SDS-PAGE. Comparison with prestained protein ladder exhibited Bacillus licheniformis bacteriocin lichenin has a molecular weight of about < 9 kDa as shown in Fig 8.

It have been reported that lichenin molecular mass of approximately 1400 Dalton, revealed by tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Pattnaik et al. 2001). A bacteriocin-like protein from Bacillus licheniformisMKU3 with a molecular mass of 1.5 kDa (Chalasani et al. 2015). Less than 3 kDa of peptide produced by Bacillus sp. revealed by silver staining (Kayalvizhi and Gunasekaran 2008).

Summary and Conclusion:-
Bacillus licheniformis was isolated from the soil sample and identified according to Gram’s staining microscopic examination, biochemical identification was based on Bergey’s Manual of Determinative Bacteriology and Advance Bacterial Identification Software (ABIS), confirmed isolates as Bacillus licheniformis. Evaluation of antagonistic activity of crude lichenin by agar well diffusion assay against standard microorganisms S. aureus MCCB 0139 and E. coli MCCB 0017 confirmed lichenin (bacteriocin) producing Bacillus licheniformis. The crude lichenin exhibiting maximum clear zone of inhibition are noted against S. aureus MCCB 0139 was proceeded for optimization of physical and chemical parameters for lichenin production. Different run numbers from 1 to 76, with different media component viz. sorbitol, lactose, yeast extract, peptone, NH4NO3, K2HPO4 and MgSO4 incubation temperature (37.5°C, 25°C and 50°C), pH (4, 6 and 8) and incubation period (12 hrs, 66 hrs and 120 hrs), respectively was optimized. Run no 43 comprising medium components 1.25% sorbitol, 6.65% lactose, 0.25% peptone, 0.06% yeast extract, 0.05% NH4NO3, 0.3% K2HPO4 and 0.025% MgSO4, pH 8 at 50°C for 12 hrs exhibited 32 mm clear zone of inhibition against S. aureus MCCB 0139 and no zone of inhibition against E. coli MCCB 0017 indication of maximum lichenin (bacteriocin) optimized production medium from Bacillus licheniformis. Purification of crude lichenin was proceeded by 80% ammonium sulphate precipitation, dialysis, ion-exchange chromatography. Finally purified lichenin fractions from ion exchange chromatography was characterized by SDS-PAGE.

In this present study, following observations made and conclusion were run number 43 exhibited maximum lichenin antagonistic activity against S. aureus MCCB 0139 alongwith maximum zone of inhibition within pH 8, at 55°C for 12 hrs, medium components amounting sorbitol 1.25 %, lactose 6.65 %, yeast extract 0.25 %, peptone 0.06 %, NH4NO3 0.05 % and MgSO4 respectively are considered to be an optimized production medium composition for
lichenin production. The effect of individual parameters on zone of inhibition revealed a decrease in lichenin (bacteriocin) activity on increasing the pH from 4.0 to 8.0. Further, no significant effect on bacteriocin activity by other parameters viz. incubation period, concentrations of sorbitol, lactose, yeast extract, ammonium nitrate and K$_2$HPO$_4$ was observed. The 3D response surface and 2D contour plot depicts two variables exhibiting effect of incubation temperature ($^\circ$C) and incubation period (hrs) with other optimization parameters viz. pH, incubation period, sorbitol (%), lactose (%), peptone (%), yeast extract (%), NH$_4$NO$_3$ (%) and MgSO$_4$ (%). Among all parameters, only incubation temperature ($^\circ$C) was found to have a significant effect on bacteriocin activity. The cube plot depicts three variables (incubation temperature ($^\circ$C), pH and incubation period (hrs)) involved in the bacteriocin activity of isolated bacteriocin. The effect of incubation temperature ($^\circ$C) and incubation period (hrs) on concentration of substrate shows that temperature and substrate concentration have a significant effect on bacteriocin activity as observed by the average value depicted in the top and bottom phase of the cube and the left and right face of the cube, respectively. However the effect due to incubation period had no significant effect on the activity of bacteriocin. Ion exchange eluted 1 to 10 lichenin fractions number was estimated by Lowry’s method and quantified at absorbance 660 nm, revealed that NaCl gradient from 0.6 M, 0.8 M, 0.9 M and 1.0M contains 0.031mg/ml, 0.032 mg/ml, 0.097 mg/ml and 0.012 mg/ml lichenin concentration, calculated by obtained linear regression equation, $y = 1.710+0.038$.The maximum 0.097 mg/ml lichenin concentration was characterized by SDS-PAGE revealed < 9 kDa. RSM is one of the effective statistical and mathematical techniques used for developing, improving and optimizing the complex process in the experiment. It describes the effect of independent variables, alone or in combination in the process. Lichenin characterization by SDS-PAGE exhibited small < 9 kDa and justifying to be in class II bacteriocins include (0.77-10 kDa), which are ribosomally synthesized, nonmodified and linear peptide which are large heat and pH stable.

Lichenin characterization showed this small peptde to be a model for studying anaerobiosis-specific expression of antibacterial proteins and bacteriocins for studying bacteriocin structure-functions relationships, host-range interaction and the physiology of bacteriocin production and immunity among the obligatory and facultative anaerobic bacteria. However, in depth studies of lichenin are required to explain the mode of action of lichenin.

**Table 1:** Summary of design depicting process variables and levels

| Factors | Name     | Unit(s) | Type    | Subtype     | Minimu m | Maximu m | Coded       | Values     | Mean  | Std.dev |
|---------|----------|---------|---------|-------------|-----------|-----------|-------------|------------|-------|---------|
| A       | pH       | Numer   |ic       | Continuous  | 4         | 8         | -1.00=4.00  | 1.00=8.00  | 5.89  | 1.81    |
| B       | Inc tem  | C       | Numer   | Continuous  | 25        | 50        | -1.00=25.00 | 1.00=50.00 | 37.1  | 11.36   |
| C       | Inc per  | Hrss    | Numer   | Continuous  | 12        | 120       | -1.00=12.00 | 1.00=120.00| 64.5  | 49.07   |
| D       | Sorbito l| %       | Numer   | Continuous  | 0.75      | 1.25      | -1.00=0.75  | 1.00=0.75  | 0.98  | 0.22    |
| E       | Lactose  | %       | Numer   | Continuous  | 0.05      | 6.65      | -1.00=0.05  | 1.00=0.05  | 3.21  | 3.02    |
| F       | Peptone  | %       | Numer   | Continuous  | 0.25      | 0.75      | -1.00=0.25  | 1.00=0.25  | 0.5   | 0.22    |
| G       | Yeast extract| %       | Numer   | Continuous  | 0.06      | 6.00      | -1.00=0.06  | 1.00=6.00  | 2.75  | 2.70    |
| H       | NH$_4$NO$_3$| %       | Numer   | Continuous  | 0.05      | 1.65      | -1.00=0.05  | 1.00=1.65  | 0.81  | 0.73    |
### Table 2: Antibacterial spectrum of isolated *Bacillus licheniformis* against *Staphylococcus aureus* MCCB 0139 and *Escherichia coli* MCCB 0017

| Isolates no | Zone of inhibition on *S. aureus* MCCB 013 | Zones of inhibition on *E. coli* MCCB 0017 |
|-------------|---------------------------------------------|------------------------------------------|
| S41         | 26 mm                                       | -                                        |
| S13         | 19 mm                                       | -                                        |
| S35         | 18 mm                                       | -                                        |
| T24         | 17 mm                                       | -                                        |
| S21         | 15 mm                                       | -                                        |
| C2          | 14 mm                                       | -                                        |
| C1          | 11 mm                                       | -                                        |

Symbol (-) no zone of inhibition of crude lichenin against *E. coli* MCCB 0017

Included well size of 5 mm diameter

F_{cal} = 60.6 < F_{tab} = 1.112

MCCB = Microbial Culture Collection Bank, SHUATS, Allahabad

### Table 3: Variables and their level for optimization of lichenin (bacteriocin) production medium

| Run | Inc. | pH | % Sorbitol | % Lactose | % Peptone | Yeast extract | % NH₄NO₃ | % K₂HPO₄ | % MgSO₄ | Results (mm) |
|-----|------|----|------------|-----------|-----------|---------------|----------|----------|---------|--------------|
| 1   | 50   | 4  | 12         | 0.75      | 6.65      | 0.25          | 6        | 0.05     | 0.1     | 0.025        |
| 2   | 50   | 8  | 12         | 0.75      | 6.65      | 0.25          | 6        | 0.05     | 0.1     | 0.005        |
| 3   | 25   | 4  | 12         | 0.75      | 0.05      | 0.25          | 6        | 0.05     | 0.3     | 0.025        |
| 4   | 25   | 4  | 12         | 0.75      | 0.05      | 0.25          | 6        | 0.05     | 0.3     | 0.005        |
| 5   | 25   | 8  | 12         | 1.25      | 0.05      | 0.25          | 6        | 0.05     | 0.3     | 0.025        |
| 6   | 25   | 8  | 12         | 1.25      | 0.05      | 0.75          | 0.06     | 0.05     | 0.3     | 0.025        |
| 7   | 25   | 4  | 12         | 1.25      | 0.05      | 0.75          | 0.06     | 1.65     | 0.1     | 0.005        |
| 8   | 37   | 6  | 66         | 1         | 0.05      | 0.5           | 3.03     | 0.85     | 0.2     | 0.015        |
| 9   | 50   | 4  | 12         | 1.25      | 6.65      | 0.75          | 0.06     | 1.65     | 0.3     | 0.025        |
| 10  | 25   | 8  | 12         | 0.75      | 0.05      | 0.75          | 0.06     | 0.05     | 0.3     | 0.025        |
| 11  | 50   | 8  | 12         | 0.75      | 6.65      | 0.75          | 0.06     | 0.05     | 0.1     | 0.025        |
| 12  | 50   | 4  | 12         | 0.75      | 0.05      | 0.75          | 6        | 1.65     | 0.1     | 0.005        |
| 13  | 25   | 8  | 12         | 0.75      | 6.65      | 0.25          | 6        | 1.65     | 0.1     | 0.025        |
| 14  | 37   | 6  | 66         | 1         | 3.35      | 0.5           | 3.03     | 0.85     | 0.2     | 0.025        |
| 15  | 25   | 6  | 66         | 1         | 3.35      | 0.5           | 3.03     | 0.85     | 0.2     | 0.015        |
| 16  | 50   | 4  | 12         | 1.25      | 6.65      | 0.25          | 6        | 0.05     | 0.1     | 0.005        |
| 17  | 25   | 8  | 12         | 0.75      | 0.05      | 0.25          | 6        | 1.65     | 0.1     | 0.005        |
| 18  | 37   | 6  | 66         | 1         | 0.05      | 0.5           | 3.03     | 0.85     | 0.2     | 0.015        |
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
19 & 50 & 8 & 2 & 1.25 & 0.05 & 0.25 & 0.06 & 0.05 & 0.1 & 0.005 & 25 \\
20 & 37.5 & 4 & 66 & 1 & 3.35 & 0.5 & 3.03 & 0.85 & 0.2 & 0.015 & 22 \\
21 & 50 & 4 & 12 & 1.25 & 6.65 & 0.75 & 6 & 0.05 & 0.1 & 0.005 & 14 \\
22 & 50 & 4 & 12 & 0.75 & 0.05 & 0.75 & 0.06 & 0.05 & 0.3 & 0.025 & 15 \\
23 & 37.5 & 6 & 66 & 1 & 3.35 & 0.5 & 3.03 & 0.05 & 0.2 & 0.015 & 0 \\
24 & 50 & 8 & 12 & 0.75 & 6.65 & 0.25 & 6 & 0.05 & 0.3 & 0.025 & 21 \\
25 & 25 & 4 & 12 & 0.75 & 6.65 & 0.75 & 0.06 & 0.05 & 0.3 & 0.025 & 22 \\
26 & 37.5 & 6 & 66 & 1 & 3.35 & 0.5 & 3.03 & 0.85 & 0.1 & 0.015 & 14 \\
27 & 37.5 & 6 & 66 & 0.75 & 3.35 & 0.5 & 3.03 & 0.85 & 0.2 & 0.015 & 18 \\
28 & 50 & 4 & 12 & 0.75 & 6.65 & 0.75 & 0.06 & 1.65 & 0.1 & 0.005 & 13 \\
29 & 25 & 4 & 12 & 1.25 & 0.05 & 0.75 & 0.06 & 0.05 & 0.1 & 0.025 & 16 \\
30 & 25 & 4 & 12 & 1.25 & 6.65 & 0.75 & 0.06 & 0.05 & 0.3 & 0.005 & 0 \\
31 & 25 & 4 & 12 & 1.25 & 6.65 & 0.75 & 6 & 0.05 & 0.3 & 0.025 & 11 \\
32 & 25 & 4 & 12 & 1.25 & 0.05 & 0.25 & 6 & 1.65 & 0.1 & 0.025 & 10 \\
33 & 50 & 4 & 12 & 0.75 & 0.05 & 0.25 & 0.06 & 0.05 & 0.3 & 0.005 & 14 \\
34 & 50 & 4 & 12 & 0.75 & 6.65 & 0.75 & 0.06 & 0.05 & 0.3 & 0.005 & 0 \\
35 & 25 & 4 & 12 & 1.25 & 6.65 & 0.25 & 6 & 1.65 & 0.1 & 0.005 & 17 \\
36 & 25 & 4 & 12 & 0.75 & 6.65 & 0.75 & 6 & 0.05 & 0.1 & 0.025 & 17 \\
37 & 50 & 4 & 12 & 0.75 & 6.65 & 0.25 & 0.06 & 0.05 & 0.3 & 0.025 & 31 \\
38 & 50 & 8 & 12 & 1.25 & 0.05 & 0.075 & 6 & 0.05 & 0.3 & 0.025 & 15 \\
39 & 50 & 8 & 12 & 0.75 & 0.05 & 0.25 & 6 & 1.65 & 0.3 & 0.025 & 20 \\
40 & 50 & 8 & 12 & 1.25 & 0.05 & 0.75 & 0.06 & 1.65 & 0.1 & 0.025 & 16 \\
41 & 50 & 4 & 12 & 0.75 & 0.05 & 0.75 & 6 & 1.65 & 0.3 & 0.005 & 14 \\
42 & 50 & 4 & 12 & 1.25 & 0.05 & 0.25 & 6 & 0.05 & 0.1 & 0.025 & 13 \\
43 & 50 & 8 & 12 & 1.25 & 6.65 & 0.25 & 0.06 & 0.05 & 0.3 & 0.025 & 32 \\
44 & 25 & 4 & 12 & 1.25 & 6.65 & 0.25 & 0.06 & 1.65 & 0.1 & 0.025 & 15 \\
45 & 25 & 4 & 12 & 1.25 & 6.65 & 0.25 & 0.06 & 1.65 & 0.3 & 0.005 & 17 \\
46 & 25 & 4 & 12 & 1.25 & 6.65 & 0.25 & 0.06 & 0.05 & 0.1 & 0.025 & 23 \\
47 & 50 & 4 & 12 & 1.25 & 0.05 & 0.25 & 0.06 & 1.65 & 0.3 & 0.005 & 15 \\
48 & 50 & 8 & 12 & 0.75 & 0.05 & 0.25 & 0.06 & 0.05 & 0.1 & 0.005 & 13 \\
49 & 50 & 4 & 12 & 0.75 & 6.65 & 0.75 & 6 & 1.65 & 0.3 & 0.025 & 14 \\
50 & 50 & 4 & 12 & 0.75 & 6.65 & 0.25 & 6 & 1.65 & 0.3 & 0.005 & 17 \\
51 & 50 & 8 & 12 & 0.75 & 0.05 & 0.75 & 0.06 & 1.65 & 0.3 & 0.005 & 12 \\
52 & 37.5 & 6 & 66 & 1 & 3.35 & 0.5 & 3.03 & 0.05 & 0.2 & 0.015 & 15 \\
53 & 50 & 8 & 12 & 0.75 & 0.05 & 0.25 & 6 & 1.65 & 0.1 & 0.005 & 12 \\
\hline
\end{tabular}
| NaCl gradient (0.1 M to 1.0 M) | Absorbance at 280 nm |
|-------------------------------|----------------------|
| 0.1                           | 0.144                |
| 0.2                           | 0.217                |
| 0.3                           | 0.250                |
| 0.4                           | 0.181                |
| 0.5                           | 0.168                |
| 0.6                           | 0.236                |
| 0.7                           | 0.278                |
| 0.8                           | 0.445                |
| 0.9                           | 0.444                |
| 1.0                           | 0.254                |

Table 4: Lichenin quantitative estimation by UV spectrophotometer at 280 nm eluted from sephadex column.
Table 5a: BSA (0.1 mg/ml) concentration estimation by Lowry’s method

| Tubes no | BSA std Stock con. | D/w | Lowry’s Reagent | Follins-ciocalteau Reagent | O.D at 660 nm |
|----------|--------------------|-----|-----------------|----------------------------|--------------|
| 1        | 0                  | 1 ml|                 |                            | 0.000        |
| 2        | 0.1 μl             | 0.9 μl|               |                            | 0.015        |
| 3        | 0.2 μl             | 0.8 μl|               |                            | 0.038        |
| 4        | 0.3 μl             | 0.7 μl|               |                            | 0.174        |
| 5        | 0.4 μl             | 0.6 μl|               |                            | 0.270        |
| 6        | 0.5 μl             | 0.5 μl|               | 2 ml                      | 0.300        |
| 7        | 0.6 μl             | 0.4 μl|               | 0.2 ml                    | 0.311        |
| 8        | 0.7 μl             | 0.3 μl|               |                            | 0.396        |
| 9        | 0.8 μl             | 0.2 μl|               |                            | 0.460        |
| 10       | 0.9 μl             | 0.1 μl|               |                            | 0.478        |
| 11       | 1.0 μl             | 0    |                 |                            | 0.526        |

Table 5b: Lichenin concentration determination by Lowry’s method

| Tubes no | Lichenin Concen (1ml) | Lowry’s Reagent | Follins-ciocalteau Reagent | O.D at 660 nm |
|----------|-----------------------|-----------------|----------------------------|--------------|
| 1        | 1 ml                  |                 |                            | 0.000        |
| 2        | 1 ml                  |                 | 2 ml                      | 0.000        |
| 3        | 1 ml                  |                 | 0.2 ml                    | 0.001        |
| 4        | 1 ml                  |                 |                            | 0.000        |
| 5        | 1 ml                  |                 |                            | 0.031        |
| 6        | 1 ml                  |                 |                            | 0.000        |
| 7        | 1 ml                  |                 |                            | 0.000        |
| 8        | 1 ml                  |                 |                            | 0.032        |
| 9        | 1 ml                  |                 |                            | 0.097        |
| 10       | 1 ml                  |                 |                            | 0.012        |

Fig 1: Antagonistic activity of crude lichenin against S. aureus MCCB 0139. Maximum inhibition zone was demonstrated by isolate no S41.
Fig 2:- Antagonistic activity of crude lichenin against *E. coli* MCCB 0017. Crude lichenin had no effect on *E. coli*

Fig 3:- Run no 43, demonstrated 32 mm, maximum inhibition zone of crude lichenin against *S. aureus* MCCB 0139, optimized at pH 8, 50°C for 12 hrs
Fig 4: Normal plot of residual

Fig 5: Residual vs Predicted graph.
Fig 6 Residual vs Run graph

Fig 7 Residual vs Factor for pH
Fig 8 Residual vs temperature (°C)
Fig 9 Residual vs incubation period (hrs)

Fig 10 Residual vs concentrations of sorbitol
Fig 11 Residual vs Lactose
Fig 14 - Residual vs Ammonium nitrate

Fig 15 - Residual vs Concentration of Dipotassium hydrogen phosphate
Fig 16 Residual vs Magnesium sulphate

Cook's Distance

Fig 17 Cook's distance of the design
Fig 18 Leverage vs Run of the design

Fig 19 Predicted vs Actual graph
Fig 20 Box cox plot for power transformation

Fig 21 Effect of temperature (°C)
Fig 22 Effect of MgSO$_4$

Fig 23 Effect of pH
Fig 24 Effect of Peptone

Fig 25 Effect of Incubation period (hrs)
Fig 26 Effect of Concentration of Sorbitol

Fig 27 Effect of Concentration of Lactose
Fig 28 Effect of Yeast Extract (%)

Fig 29 Effect of Concentration of Ammonium Nitrate (NH₄NO₃)
Fig 30: Effect of Concentration of Dipotassium Hydrogen Phosphate (K₂HPO₄) on Bacteriocin production (%)

Fig 31: Effect of pH and Incubation temperature (°C) on Bacteriocin production (%)

Actual Factors:
- A: Temp
- B: pH
- C: Incub. Period = 48.00
- D: Sorbitol = 1.00
- E: Lactose = 3.00
- F: Peptone = 0.50
- G: Yeast extract = 3.00
- H: NH₄NO₃ = 0.80
- J: K₂HPO₄ = 0.20
- K: MgSO₄ = 0.02
Fig 32 Effect of Temperature (°C) and Incubation period (hrs) on Bacteriocin production (%)

Fig 33 Effect of Incubation temperature (°C) and Sorbitol (%) on Bacteriocin production (%)
Fig 34 Effect of incubation temperature (°C) and Peptone (%) on bacteriocin production (%).

Fig 35 Effect of Incubation temperature (°C) and Yeast extract (%) on Bacteriocin production (%).
Fig 36 Effect of Incubation temperature (°C) and Lactose (%) on Bacteriocin production

Fig 37 Effect of Incubation temperature (°C) and NH₄NO₃ (%) on Bacteriocin production
**Fig 38** Effect of incubation temperature (°C) and $K_2HPO_4$ (%) on Bacteriocin production (%)

**Fig 39** Effect of Incubation temperature (°C) and $MgSO_4$ (%) on Bacteriocin production (%)
**Fig 40** Effect of pH and Incubation period (hrs) on Bacteriocin production (%).

**Fig 41** Effect of pH and Sorbitol (%) on Bacteriocin production (%).
Fig 42 Effect of pH and Lactose (%) on Bacteriocin production (%)

Fig 43 Effect of pH and Peptone (%) on Bacteriocin production (%)
Fig 44 Effect of Yeast extract (%) and pH on Bacteriocin production (%)

Fig 45 Effect of NH$_4$NO$_3$ (%) and pH on Bacteriocin production (%).
Fig 46 Effect of K$_2$HPO$_4$ (%) and pH on Bacteriocin production (%)

Fig 47 Effect of MgSO$_4$ (%) and pH on Bacteriocin production (%)
Fig 48 Effect of Incubation period (hrs) and Incubation temperature (ºC) on Bacteriocin production (%)

Fig 49 Effect of Incubation period (hrs) and pH on Bacteriocin production (%)
Fig 50 Effect of Sorbitol (%) and Incubation period (hrs) on Bacteriocin production (%)

Fig 51 Effect of Lactose (%) and Incubation period (hrs) on Bacteriocin production (%)

Actual Factors:
A. Temp = 37.6°C
B. pH = 6.00
C. Incubation Period
D. Sorbitol = 0.05%
E. Lactose = 3.35
F. Peptone = 0.00
G. Yeast Extract = 0.05
H. NH₄NO₃ = 0.05
J. K₂HPO₄ = 0.20
K. MgSO₄ = 0.42
Fig 52 Effect of Peptone (%) and Incubation period (hrs) on Bacteiocin production (%)

Fig 53 Effect of Yeast extract (%) and Incubation period (hrs) on Bacteriocin production (%)
Fig 54 Effect of NH$_4$NO$_3$ (%) and Incubation period (hrs) on Bacteriocin production (%)

Fig 55 Effect of K$_2$HPO$_4$ (%) and Incubation period (hrs) on Bacteriocin production (%)
**Fig 56** Effect of MgSO₄ and Incubation period (hrs) on Bacteriocin production (%)

**Fig 57** Effect of Incubation temperature (°C), pH and Incubation period (hrs) on Bacteriocin production (%)

**Table:**

| Factor          | Value   |
|-----------------|---------|
| MgSO₄ (%)       | 8.00    |
| pH              | 6.00    |
| Incubation Period (hrs) | 12.00  |

**Actual Factors:**

- A: Temp
- B: pH
- C: Incubation Period

**Factors:**

- A: Temp
- B: pH
- C: Incubation Period
- D: MgSO₄
- E: Incubation period
- F: Yeast extract
- G: Yeast extract
- H: NaNO₃
- J: MgSO₄
- K: MgSO₄

**Response 1:**

- A: Temp
- B: pH
- C: Incubation Period

**Response 2:**

- A: Temp
- B: pH
- C: Incubation Period

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**Fig 58** Effect of Incubation temperature (°C), Sorbitol and Incubation period (hrs) on Bacteriocin production (%)

**Fig 59** Effect of Incubation temperature (°C), Peptone and Incubation period (hrs) on Bacteriocin production (%)

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**Actual Factors**
- A: pH = 6.30
- B: Sorbitol = 1.00
- C: Yeast extract = 0.05
- D: Yeast extract = 0.03
- E: NH₄NO₃ = 0.85
- F: KH₂PO₄ = 0.20
- G: MgSO₄ = 0.02
Fig 60 Effect of Incubation temperature (°C), MgSO₄ and Incubation period (hrs) on Bacteriocin production (%)

Fig 61 Effect of Incubation temperature (°C), Lactose (%) and Incubation period (hrs) on Bacteriocin production (%)

Response 1
X₁ = A: Temp
X₂ = K: MgSO₄
X₃ = C: Incub Period

Actual Factors
E: pH = 6.65
D: Sucrose = 1.00
C: Yeast extract = 0.50
B: NαHCO₃ = 0.65
A: K₂HPO₄ = 0.25

C + : 120.0
A +: 50.00
A -: 25.00
K±: 0.03
E : 0.05
K : 0.01

G: Yeast extract = 0.50
H: NαHCO₃ = 0.65
I: Sucrose = 1.00
J: K₂HPO₄ = 0.25
K: MgSO₄ = 0.25
Fig 62 Effect of Incubation temperature (ºC), Yeast extract (%) and Incubation period (hrs) on Bacteriocin production (%)

Fig 63 Effect of Incubation temperature (ºC), NH$_4$NO$_3$ (%) and Incubation period (hrs) on Bacteriocin production (%)
Fig 64 Effect of Incubation temperature (°C), KH₂PO₄ (%) and Incubation period (hrs) on Bacteriocin production (%)

Fig 65 Effect of Incubation time (hrs), Incubation temperature (°C) and pH on Bacteriocin production (%)
Fig 66 Effect of Incubation time (hrs), Sorbitol (%) and pH on Bacteriocin production (%)

Fig 67 Effect of Incubation time (hrs), Peptone (%) and pH on Bacteriocin production (%)
Fig 68 Effect of Incubation time (hrs), MgSO₄ (%) and pH on Bacteriocin production

Fig 69 Effect of Incubation time (hrs), Lactose (%) and pH on Bacteriocin production (%)
Fig 70 Effect of Incubation time (hrs), Yeast extract (%) and pH on Bacteriocin production (%)

Fig 71 Effect of Incubation time (hrs), NH₄NO₃ (%) and pH on Bacteriocin production (%)
Fig 72 Effect of Incubation time (hrs), \(K_2\text{HPO}_4\) (%) and pH on bacteriocin production (%)

Response 1

\[J = X_1 \times X_2 \times X_3\]

Actual Factors
- \(X_1\): Temp = \(y\)
- \(X_2\): pH
- \(X_3\): Incubation Period

Fig 73 Effect of Incubation time (hrs), Incubation temperature (°C) and pH on Bacteriocin production (%)
Fig 74 Effect of Incubation time (hrs), Sorbitol (%) and pH on Bacteriocin production (%)

Fig 75 Effect of Incubation time (hrs), Lactose (%) and pH on Bacteriocin production (%)
Fig 76 Effect of Incubation time (hrs), Yeast extract (%) and pH on Bacteriocin production (%)

Fig 77 Effect of Incubation time (hrs), NH$_4$NO$_3$ (%) and pH on Bacteriocin production (%)
Fig 78 Effect of Incubation time (hrs), K$_2$HPO$_4$ (%) and pH on Bacteriocin production (%)

Fig 79 Effect of Incubation temperature (°C), Peptone (%) and pH on Bacteriocin production (%)
Fig 80 Effect of Incubation temperature (°C), MgSO$_4$ (%) and pH on Bacteriocin production (%)

Fig 81 Graph illustrating lichenin elution profile from sephadex (cation exchange) at O.D$_{280}$nm
Fig 82 Graph showing Lichenin concentration with respect to BSA standard protein concentration

y = 1.7109x + 0.0384
R² = 0.9694

Fig 83 Lichenin (bacteriocin) molecular weight determination by SDS-PAGE revealed < 9 kDa

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