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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 biochemical analyzed and advance 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, NH_4NO_3 , K_2HPO_4 and MgSO_4 , 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 MgSO_4 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.

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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 metabolites (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 bacteriocin used as a preservative in foods (Cleveland *et al.*, 2001). Since 1983, nisin are 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 (Martirani *et al.*, 2002). Nisin is FDA authorized bacteriocin, used as preservative in a pasteurized processed cheese (Elayaraja *et al.*, 2014). An advanced bacteriocin produced by *Bacillus licheniformis* 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* 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 *Naegleria* (Galvez *et al.*, 1994). Freshly, three bacteriocins has produced by *Bacillus licheniformis* called Licidin, Bacillocin 490 and P40 (Pattnaik *et al.*, 2001; Martirani *et 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* 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 (Martirani *et al.*, 2002). The bacteriocin-like peptides produced by *Bacillus licheniformis* demonstrated 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. licheniformis* can 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^{-7} dilution was added to Bacillus medium plates. The plates were incubated at $37 \pm 2^{\circ}\text{C}$ 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 Aneja (2003).

Evaluation of Antibacterial activity:-

Isolated *Bacillus licheniformis* was cultivated on Muller Hinton Broth at 200 rpm at $37 \pm 1^{\circ}\text{C}$ for 24-48 hrs. *Bacillus licheniformis* seeded cultures are centrifuged at $8000 \times g$ 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 μ l of cell free supernatant incubated overnight at $37 \pm 1^\circ\text{C}$.

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), NH_4NO_3 , K_2HPO_4 and MgSO_4 . All the experiments was carried out at different incubation temperature (37.5° , 25° 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 (Sersy 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 $6,000 \times g$ 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 (Henaet 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 μ l 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 (Henaet 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 follins-ciocalteu 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

$$Y = 9.16 + 0.19x_1 - 1.18x_2 - 1.85x_3 + 3.24x_4 - 0.17x_5 - 3.92x_6 - 0.09x_7 + 0.04x_8 + 3.73x_9 + 215.95x_{10} + \Sigma \dots \text{(Eq 1.0)}$$

Where Y is predicted response, $x_1, x_2, x_3, x_4, x_5, x_6, x_7, x_8, x_9$ and x_{10} 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, $37 \pm 1^\circ\text{C}$ 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 (**Pattnaik 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* SG511 and *S. aureus* wood exhibited 1.5 cm and 1.3 cm of inhibition zones respectively (**Dischinger et al. 2009**). In contrast it has been reported that antibacterial spectrum of Bac-1B 17 produced by *Bacillus subtilis* KIBGE IB against *Escherichia 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* multiresistant 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), MgSO_4 , NH_4NO_3 and K_2HPO_4 , pH (4, 6 and 8), incubation temperature (37.5° , 25° 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 3σ 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 $\lambda = 0.5$ 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 25°C to 50°C (Fig 21) and increase in MgSO_4 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 has 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 NH_4NO_3 (Fig 29), and concentration of K_2HPO_4 (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 has 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 *et 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 (%), NH_4NO_3 (%), K_2HPO_4 (%) and MgSO_4 (%) on bacteriocin activity. Among all parameters evaluated, only incubation temperature was found to have a significant effect on bacteriocin activity. It has been cited that bacteriocin production by *B. subtilis* BMPO1, was

detected at 37°C, 27°C and 57°C (Bhuvaneshwari *et al.* 2015) whereas bacteriocin production was abolished at 65°C (Ansari *et al.* 2012). Similar findings also reported that bacteriocin production by *Bacillus licheniformis* SN2 was observed at 30°C and as well as at 40°C (Sersy *et al.* 2009). *Bacillus licheniformis* are 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. licheniformis* MKU 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**. Shayesteh *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 provides 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

(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_2HPO_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_4NO_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_4NO_3 (Fig 71) and K_2HPO_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_4NO_3 (Fig 77) and K_2HPO_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 (Olivera *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 flocculation and pellicular 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 flocculation 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 81**, showing elution profile of lichenin from a sephadex column.

It has 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, $y = 1.710 + 0.038x$ as shown in **Fig 82**. **Table 5a**, showed BSA concentration and **Table 5b**, showing lichenin concentration.

Similar research reported by estimation of partially purified bacteriocin extracted from *Bacillus licheniformis* BL8 by Bradford method (**Smith and Bhat et al., 2012**). Staphylococin concentration estimated by Bradford method (**Henaet al. 2016**). *Bacillus subtilis* cell free protein determined by Lowry's method. Bovine serum albumin (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 83**.

It has 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 licheniformis* MKU3 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, NH_4NO_3 , K_2HPO_4 and MgSO_4 , 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 % NH_4NO_3 , 0.3 % K_2HPO_4 and 0.025 % MgSO_4 , 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 %, NH_4NO_3 0.05 % and MgSO_4 , 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_2HPO_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_4NO_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, $y = 1.710 + 0.038x$. 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 peptide 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	Units	Type	Subtype	Minimum	Maximum	Coded	Values	Mean	Std.dev
A	pH		Numeric	Continuous	4	8	- 1.000=4.0 0	1.000=8.00	5.89	1.81
B	Inc tem	C	Numeric	Continuous	25	50	- 1.000=25. 00	1.000=50.0 0	37.1 7	11.36
C	Inc per	Hrss	Numeric	Continuous	12	120	- 1.000=12. 00	1.000=120. 00	64.5 7	49.07
D	Sorbitol	%	Numeric	Continuous	0.75	1.25	- 1.000=0.7 5	1.000=0.75	0.98	0.22
E	Lactose	%	Numeric	Continuous	0.05	6.65	- 1.000=0.0 5	1.000=0.05	3.21	3.02
F	Peptone	%	Numeric	Continuous	0.25	0.75	- 1.000=0.2 5	1.000=0.25	0.5	0.22
G	Yeast extract	%	Numeric	Continuous	0.06	6.00	- 1.000=0.0 6	1.000=6.00	2.75	2.70
H	NH_4NO_3	%	Numeric	Continuous	0.05	1.65	- 1.000=0.0 5	1.000=1.65	0.81	0.73

I	K ₂ HP O ₄	%	Numer ic	Continou s	0.10	0.30	- 1.000=0.1	1.000=0.30	0.2	0.09
J	MgSO ₄	%	Numer ic	Continou s	0.005	0.025	- 1.000=0.0 05	1.000=0.00 5	0.01	0.009

Table 2:-Antibacterial spectrum of isolated *Bacillus licheniformis* against *Staphylococcus aureus* MCCB 0139 and *Escherichia coli* MCCB 0017

Isolates no	Zone of inhibition	
	Zone of inhibition on <i>S. aureus</i> MCCB 013	Zones of inhibition on <i>E. coli</i> 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

Ru n	Inc te m	p H	In c pe r	Sorbitol %	Lactose %	Peptone %	Yeas t ext %	NH ₄ NO ₃ %	K ₂ HPO ₄ %	MgSO ₄ %	Result s (mm)
1	50	4	12 0	0.75	6.65	0.25	6	0.05	0.1	0.025	13
2	50	8	12	0.75	6.65	0.25	6	0.05	0.1	0.005	19
3	25	4	12 0	0.75	0.05	0.25	6	0.05	0.3	0.025	15
4	25	4	12	0.75	0.05	0.25	0.06	0.05	0.1	0.005	10
5	25	8	12 0	1.25	0.05	0.25	6	0.05	0.3	0.005	0
6	25	8	12 0	1.25	0.05	0.75	0.06	0.05	0.3	0.025	13
7	25	4	12	1.25	0.05	0.75	0.06	1.65	0.1	0.005	13
8	37. 5	6	66	1	0.05	0.5	3.03	0.85	0.2	0.015	25
9	50	4	12 0	1.25	6.65	0.75	0.06	1.65	0.3	0.025	13
10	25	8	12	0.75	0.05	0.75	0.06	0.05	0.3	0.025	0
11	50	8	12	0.75	6.65	0.75	0.06	0.05	0.1	0.025	18
12	50	4	12	0.75	0.05	0.75	6	1.65	0.1	0.005	16
13	25	8	12	0.75	6.65	0.25	6	1.65	0.1	0.025	0
14	37. 5	6	66	1	3.35	0.5	3.03	0.85	0.2	0.025	19
15	25	6	66	1	3.35	0.5	3.03	0.85	0.2	0.015	12
16	50	4	12 0	1.25	6.65	0.25	0.06	0.05	0.1	0.005	13
17	25	8	12	0.75	0.05	0.25	0.06	1.65	0.1	0.005	0
18	37. 5	6	66	1	0.05	0.5	3.03	0.85	0.2	0.015	16

19	50	8	12	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	120	0.75	6.65	0.25	6	0.05	0.3	0.025	21
25	25	4	120	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	120	0.75	6.65	0.75	0.06	1.65	0.1	0.005	13
29	25	4	120	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	8	120	1.25	6.65	0.75	6	0.05	0.3	0.025	11
32	25	8	120	1.25	0.05	0.25	6	1.65	0.1	0.025	10
33	50	4	120	0.75	0.05	0.025	0.06	0.05	0.3	0.005	14
34	50	8	120	1.25	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	120	1.25	0.05	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	120	1.25	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	120	1.25	6.65	0.25	0.06	1.65	0.3	0.005	17
46	25	8	120	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	120	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	120	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

			0									
54	50	8	12	1.25	6.65	0.25	6	1.65	0.1	0.025	11	
			0									
55	50	8	12	0.75	6.65	0.25	0.06	1.65	0.3	0.025	0	
			0									
56	25	8	12	0.75	0.05	0.75	0.06	0.05	0.1	0.005	11	
			0									
57	25	8	12	1.25	0.05	0.75	6	1.65	0.3	0.025	22	
58	37.5	6	66	1	3.35	0.5	0.06	0.85	0.2	0.015	16	
59	25	8	12	1.25	0.05	0.75	6	0.05	0.1	0.005	0	
60	25	4	12	0.75	0.05	0.25	0.06	1.65	0.3	0.025	21	
61	37.5	6	66	1	3.35	0.5	3.03	0.85	0.2	0.025	23	
62	50	8	12	0.75	0.05	0.75	6	0.05	0.1	0.025	10	
			0									
63	37.5	6	66	0.75	3.35	0.5	3.03	0.85	0.2	0.015	17	
64	37.5	6	66	1	3.35	0.25	3.03	0.85	0.2	0.015	17	
65	25	8	12	0.75	0.05	0.75	6	1.65	0.3	0.005	11	
			0									
66	25	8	12	1.25	6.65	0.75	0.06	1.65	0.1	0.005	0	
			0									
67	50	8	12	1.25	6.65	0.75	6	1.65	0.3	0.005	15	
68	25	4	12	0.75	0.05	0.75	6	0.05	0.3	0.005	11	
69	25	8	12	0.75	6.65	0.25	0.06	0.05	0.3	0.005	0	
70	37.5	6	66	1	3.35	0.5	0.06	0.85	0.2	0.015	24	
71	25	4	66	0.75	6.65	0.75	0.06	1.65	0.3	0.005	18	
72	25	4	12	0.75	0.05	0.75	0.06	1.65	0.1	0.025	12	
			0									
73	50	4	12	0.75	6.65	0.25	0.06	1.65	0.1	0.025	12	
74	25	4	12	0.75	6.65	0.25	6	0.05	0.3	0.025	15	
75	25	4	12	1.25	6.65	0.75	6	1.65	0.1	0.005	16	
			0									
76	37.5	6	12	1	3.35	0.5	3.03	0.85	0.2	0.015	18	
			0									

Table 4:-Lichenin quantitative estimation by UV spectrophotometer at 280 nm eluted from sephadex column

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 5a BSA (0.1 mg/ml) concentration estimation by Lowry’s method

Tubes no	BSA std Stock con.	D/w	Lowry’s Reagent		Follins-cioalteau Reagent		O.D at 660 nm
1	0	1 ml	↑ 2 ml ↓	Incubate at R.T for 10 min	↑ 0.2 ml ↓	Incubate at R.T for 30 min	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					0.300
7	0.6 µl	0.4 µl					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-cioalteau Reagent		O.D at 660 nm
1	1 ml	↑ 2 ml ↓	Incubate at R.T for 10 min	↑ 2 ml ↓	Incubate at R.T for 30 min	0.000
2	1 ml					0.000
3	1 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

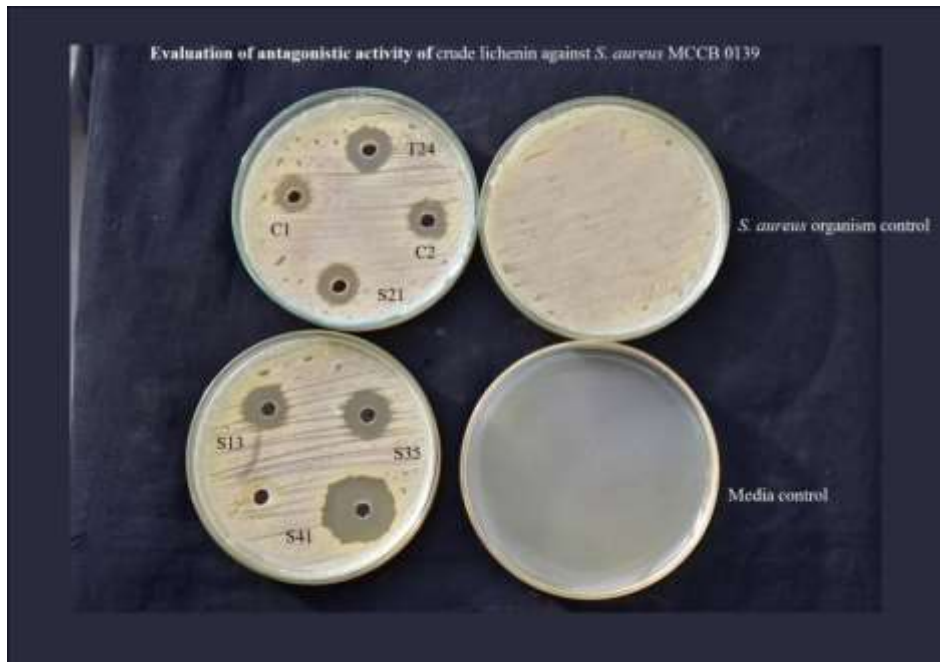


Fig 1:-Antagonistic activity of crude lichenin against *S. aureus*MCCB 0139. Maximum inhibition zone was demonstrated by isolate no S41

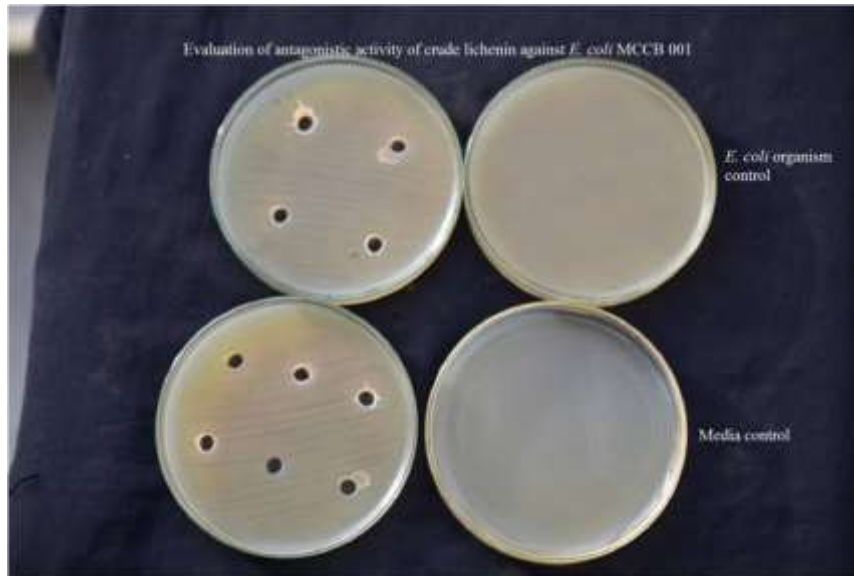
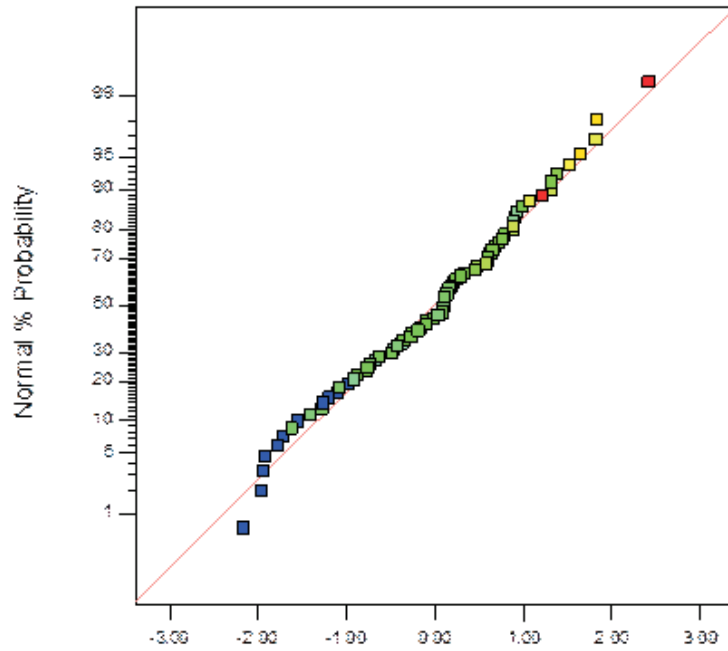


Fig 2:-Antagonistic activity of crude lichenin against *E. coli* MCCB 0017. Crude lichenin had no effect on *E. coli*



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

Color points by value of Response 1:
32
0



Externally Studentized Residuals

Fig 4:-Normal plot of residual
Residuals vs. Predicted

Response 1
Color points by value of Response 1:
32
0

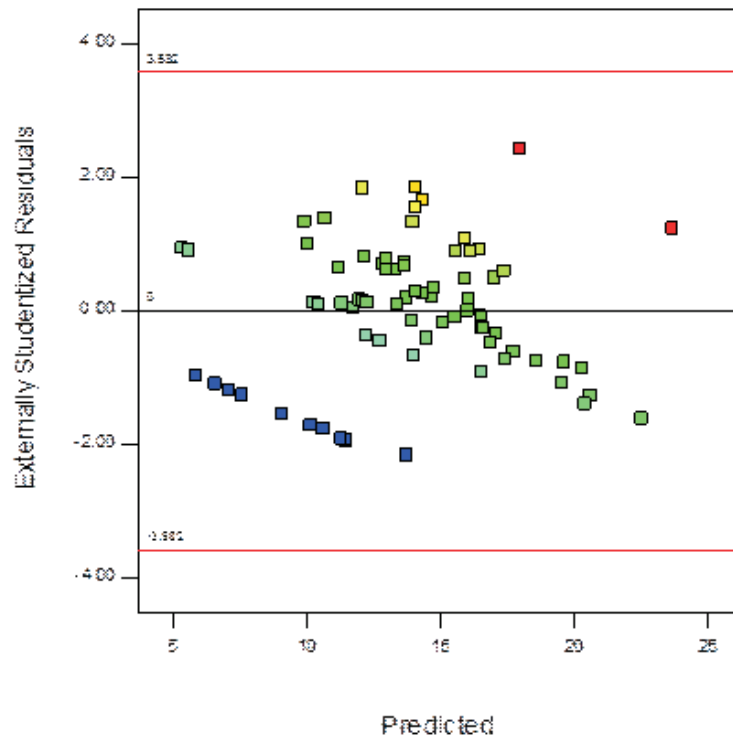


Fig 5:-Residual vs Predicted graph.

Response 1
Color points by value of Response 1:
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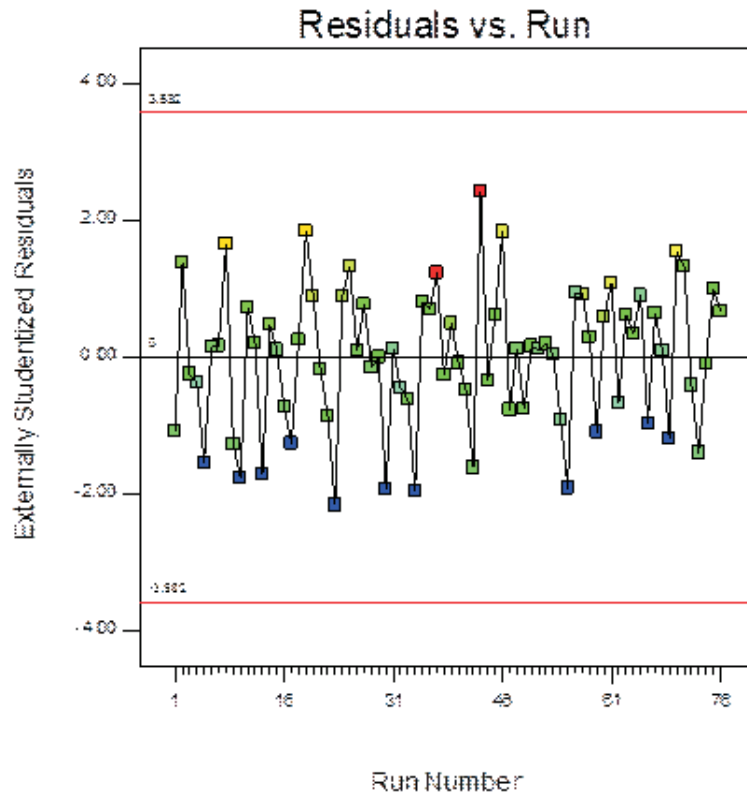


Fig 6 Residual vs Run graph

Response 1
Color points by value of Response 1:
32
0

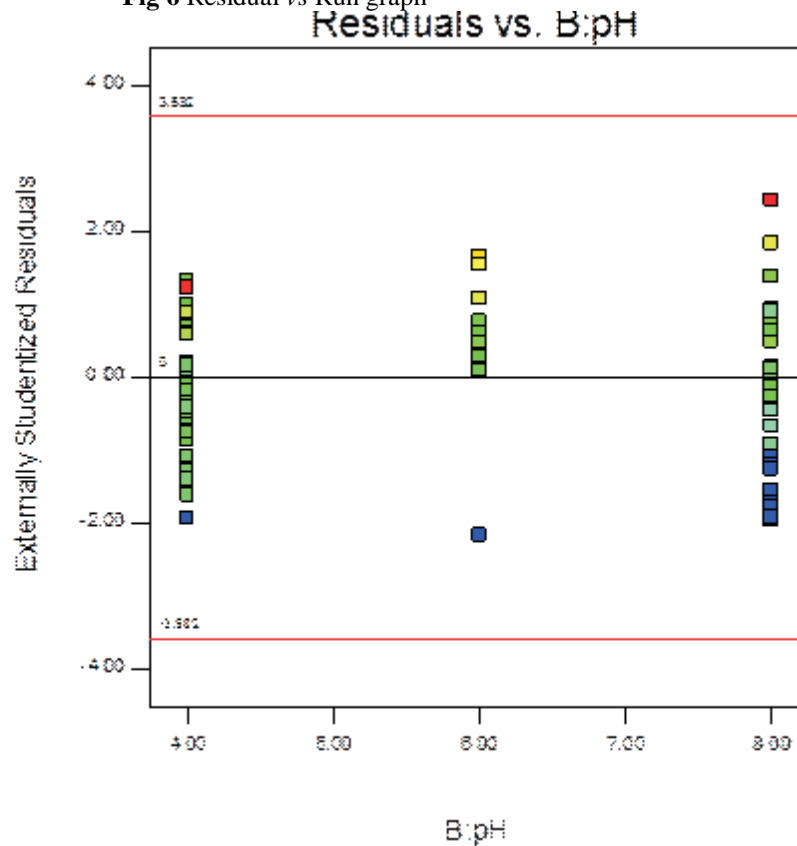


Fig 7 Residual vs Factor for pH

Response 1
Color points by value of
Response 1:
32
0

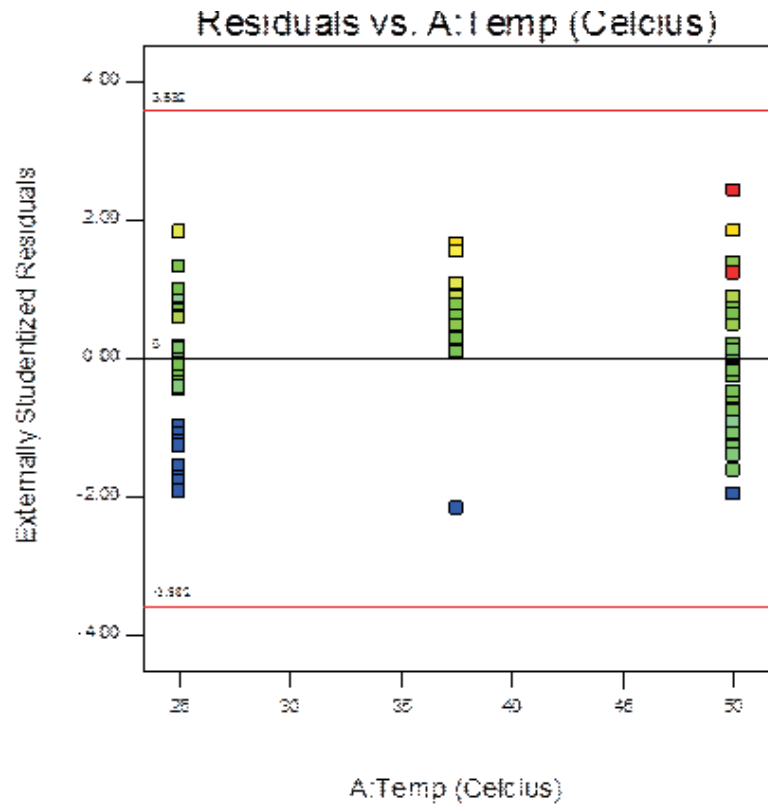
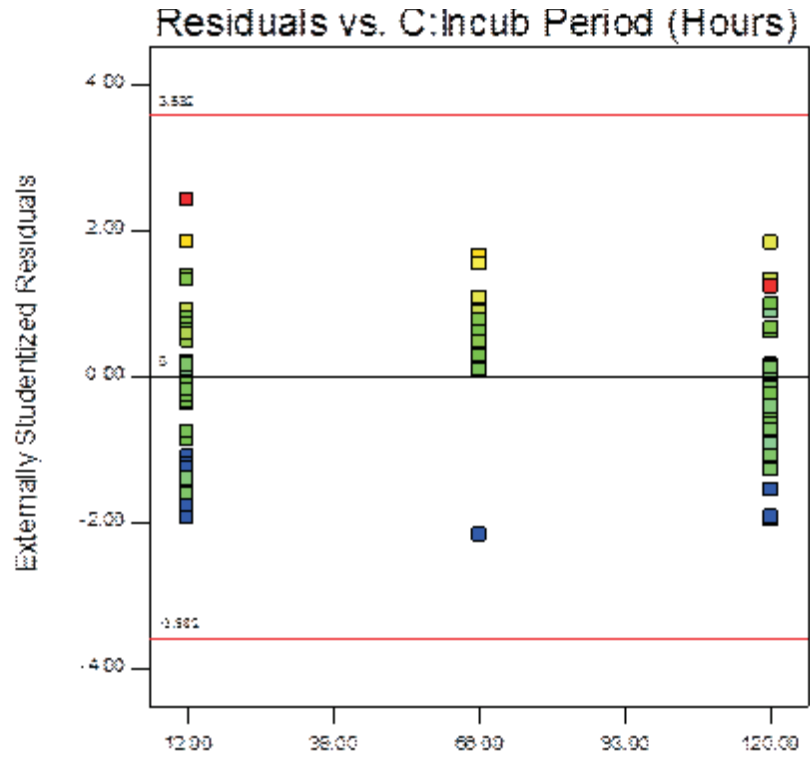
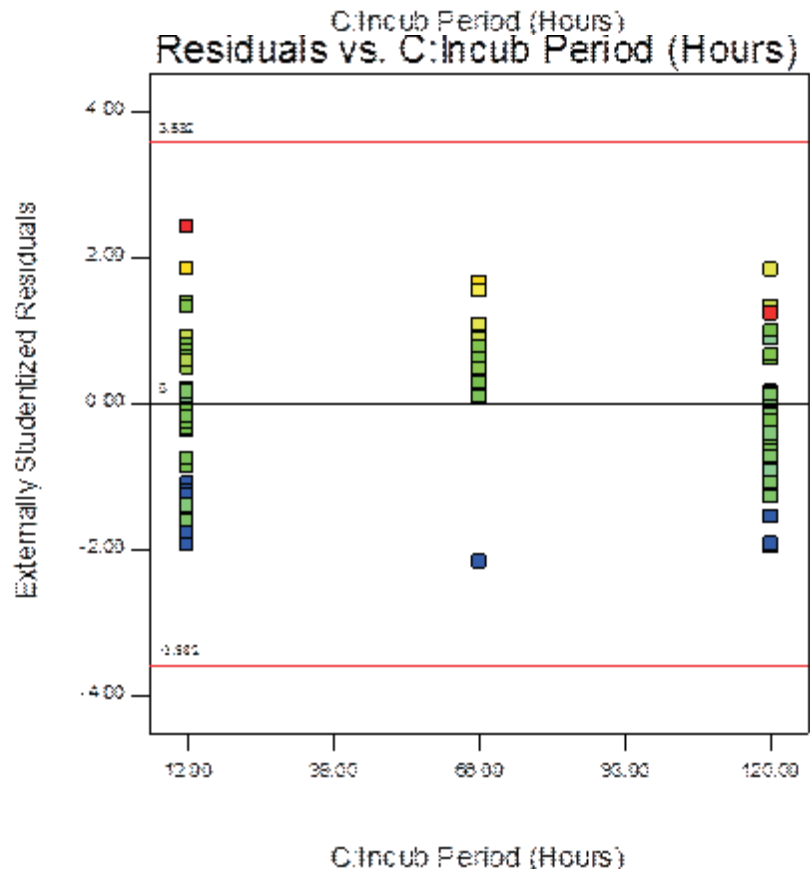


Fig 8 Residual vs temperature (°C)

Response 1
Color points by value of
Response 1:
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0



Response 1
Color points by value of
Response 1:
32
0



Response 1
Color points by value of Response 1:
32
0

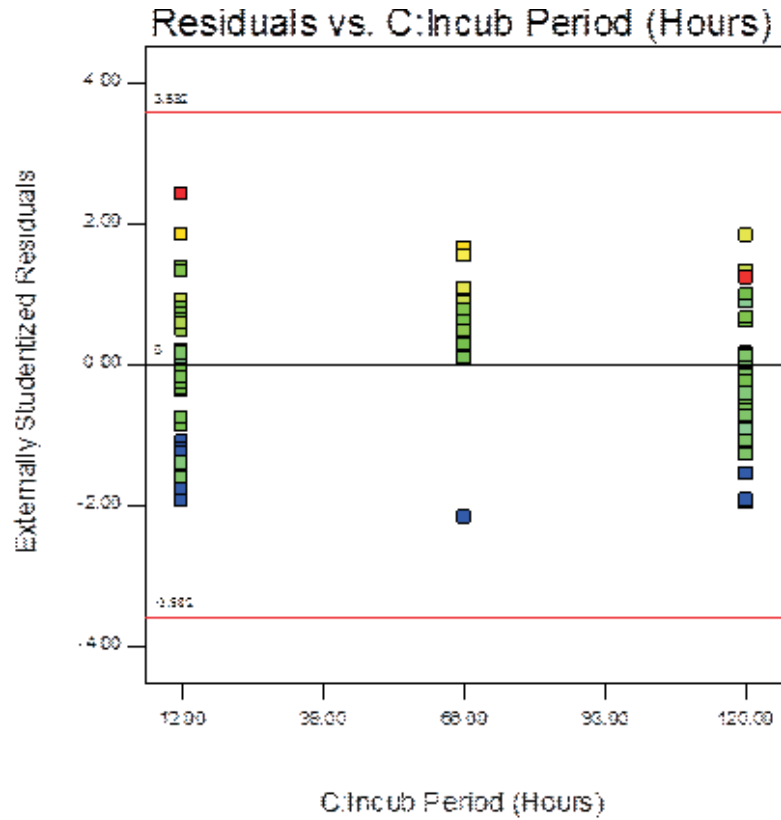


Fig 9 Residual vs incubation period (hrs)

Fig

Response 1
Color points by value of Response 1:
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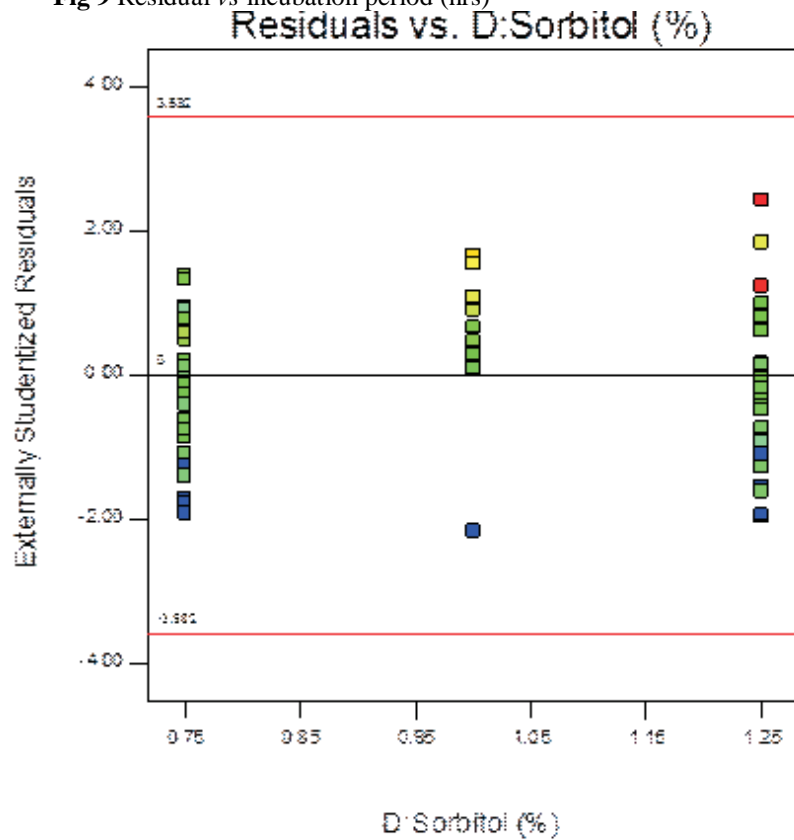
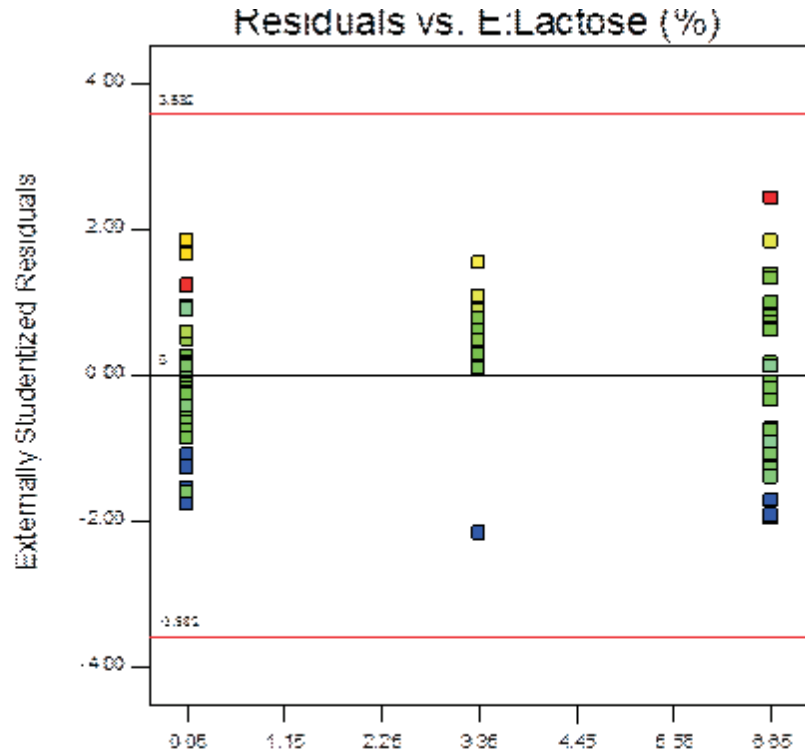


Fig 10 Residual vs concentrations of sorbitol

Response 1
Color points by value of
Response 1:
32
0



Response 1
Color points by value of
Response 1:
32
0

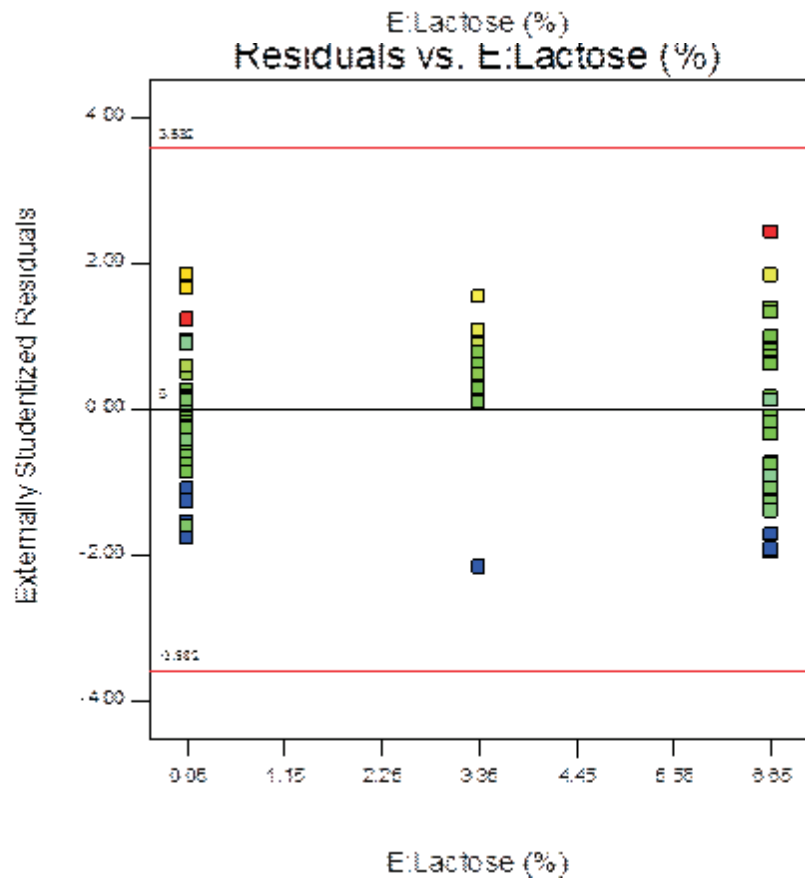


Fig 11 Residual vs Lactose

Response 1
 Color points by value of Response 1:
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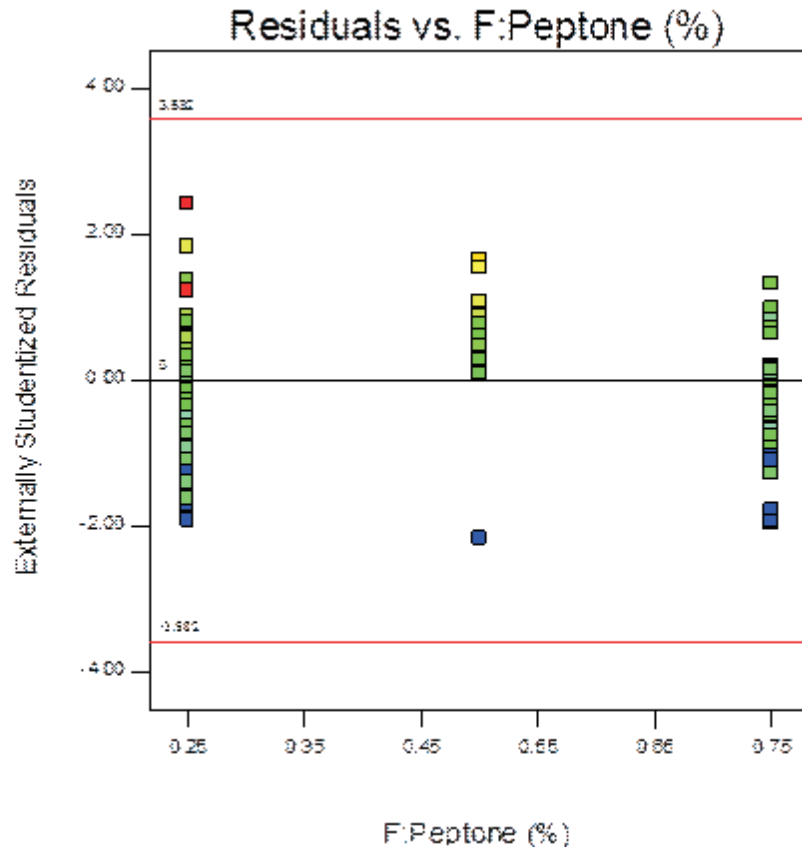


Fig 12 Residual vs Peptone

Response 1
 Color points by value of Response 1:
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 0

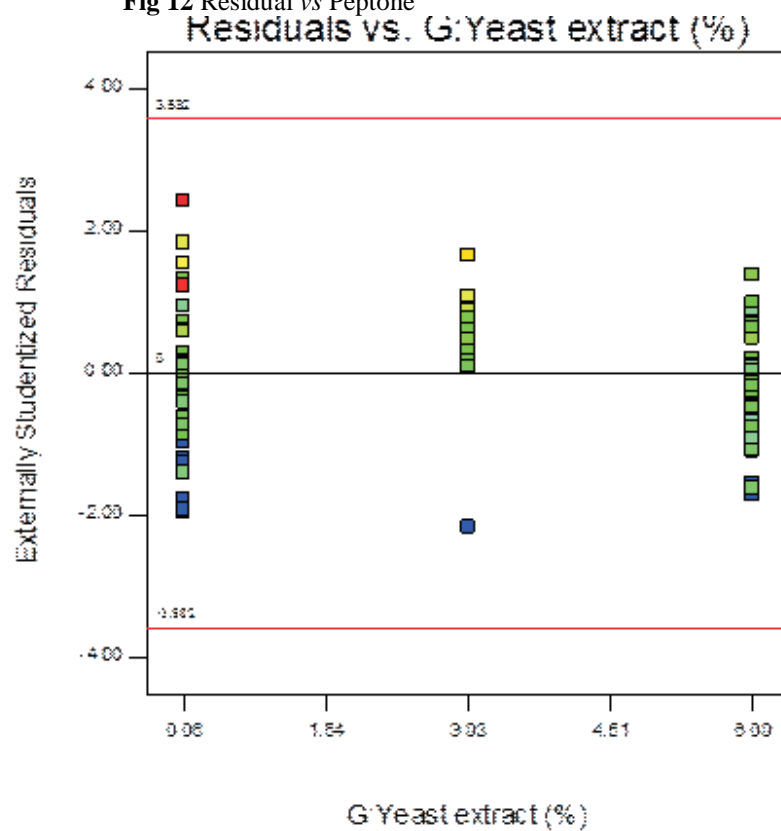


Fig 13 Residual vs Yeast extract

Response 1
Color points by value of Response 1:
32
0

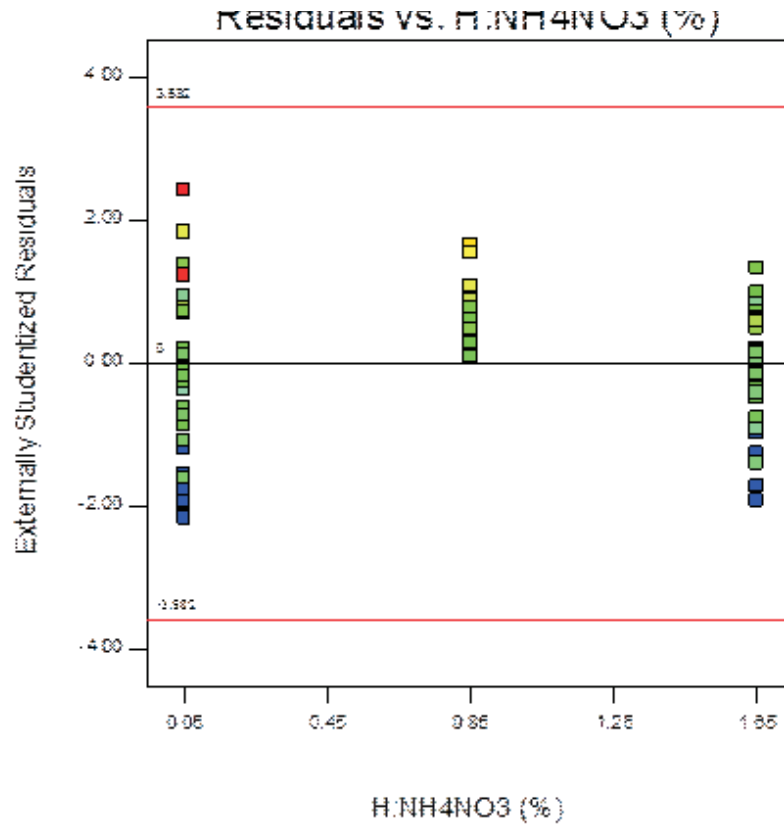


Fig 14 Residual vs Ammonium nitrate

Response 1
Color points by value of Response 1:
32
0

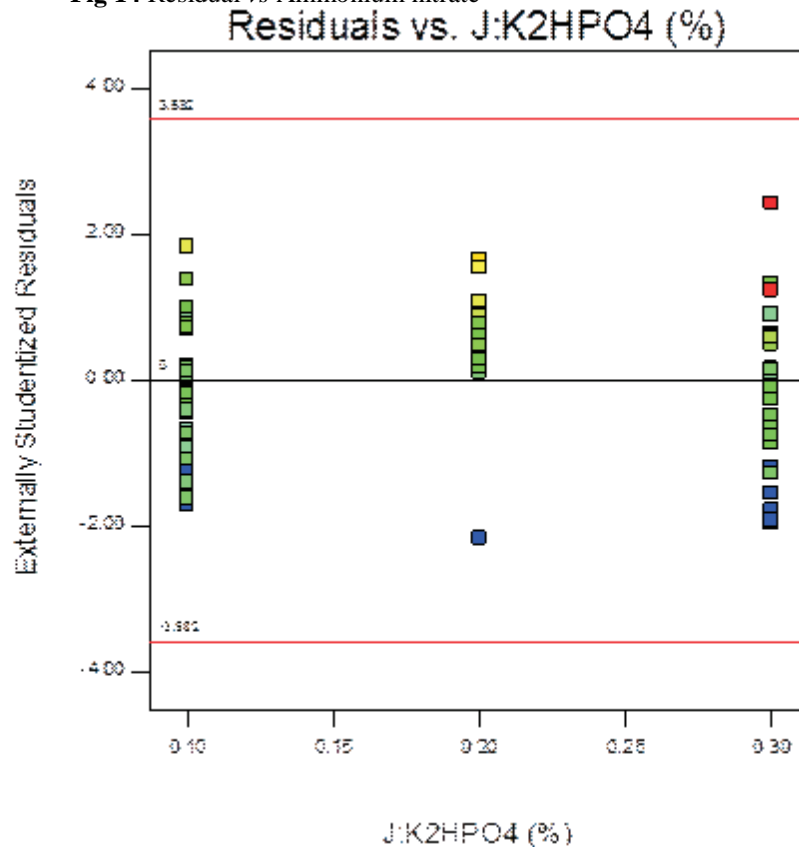


Fig 15 Residual vs Concentration of Dipotassium hydrogen phosphate

Response 1
Color points by value of Response 1:
32
0

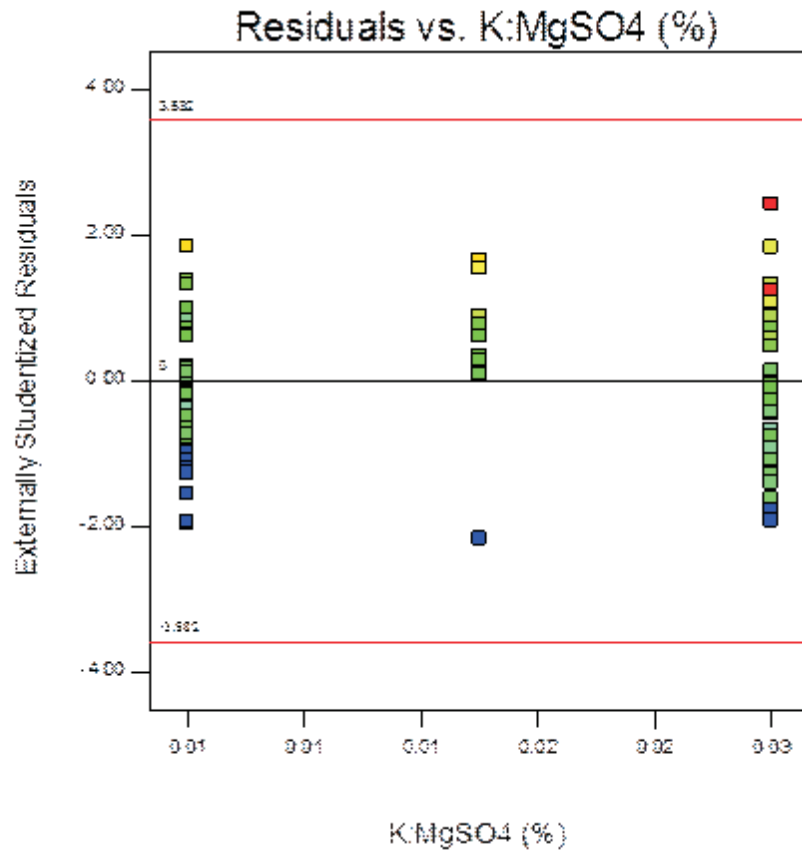


Fig 16 Residual vs Magnesium sulphate

Response 1
Color points by value of Response 1:
32
0

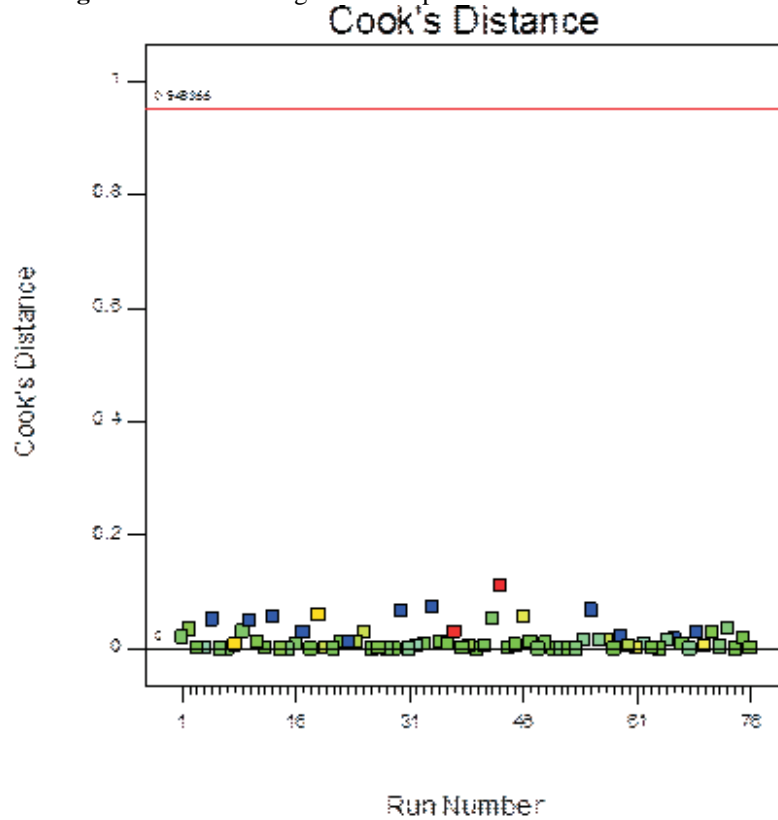


Fig 17 Cook's distance of the design

Design Expert Version 12.0.1
Response 1

Color points by value of Response 1:

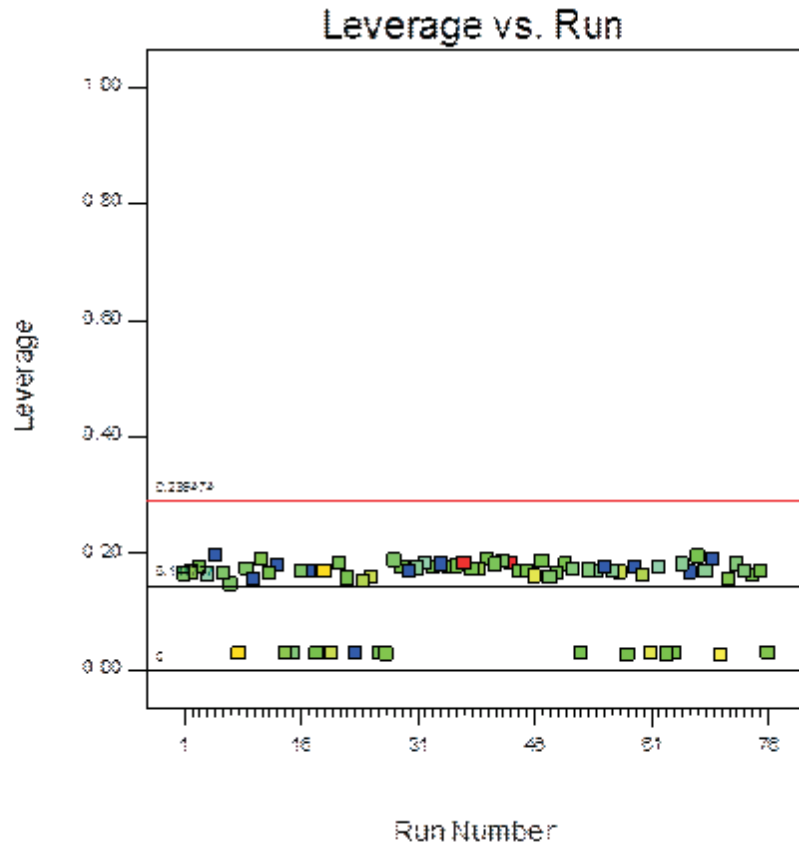


Fig 18 Leverage vs Run of the design
Predicted vs. Actual

Response 1

Color points by value of Response 1:

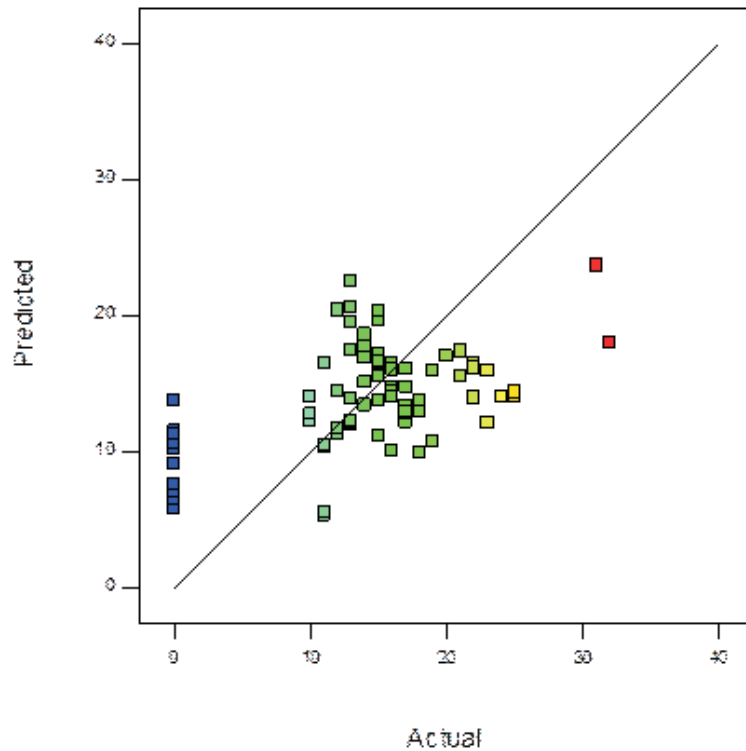


Fig 19 Predicted vs Actual graph

Response 1
Lambda
Current = 1
Best = 0.73
Low C.I. = 0.54
High C.I. = 0.92
Recommended transform:
Square Root
(Lambda = 0.5)
k = 0.032
(used to make
response values
positive)

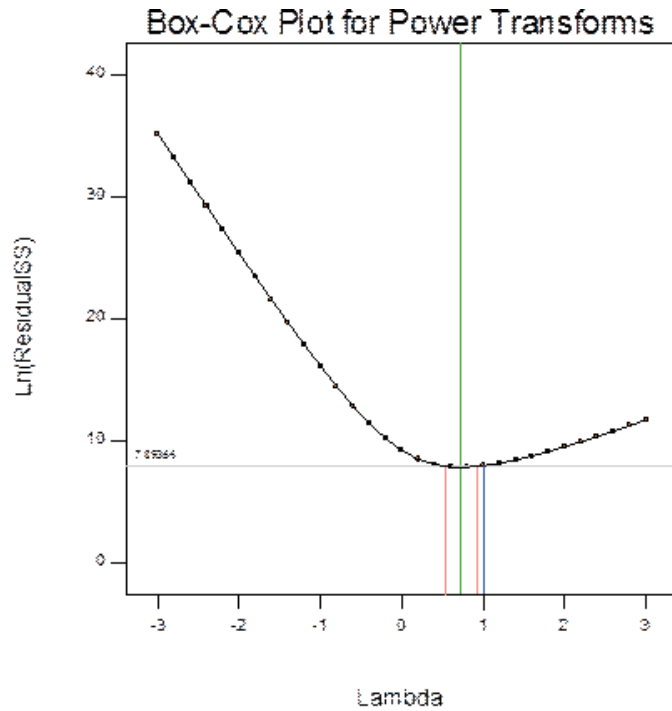


Fig 20 Box cox plot for power transformation
One Factor

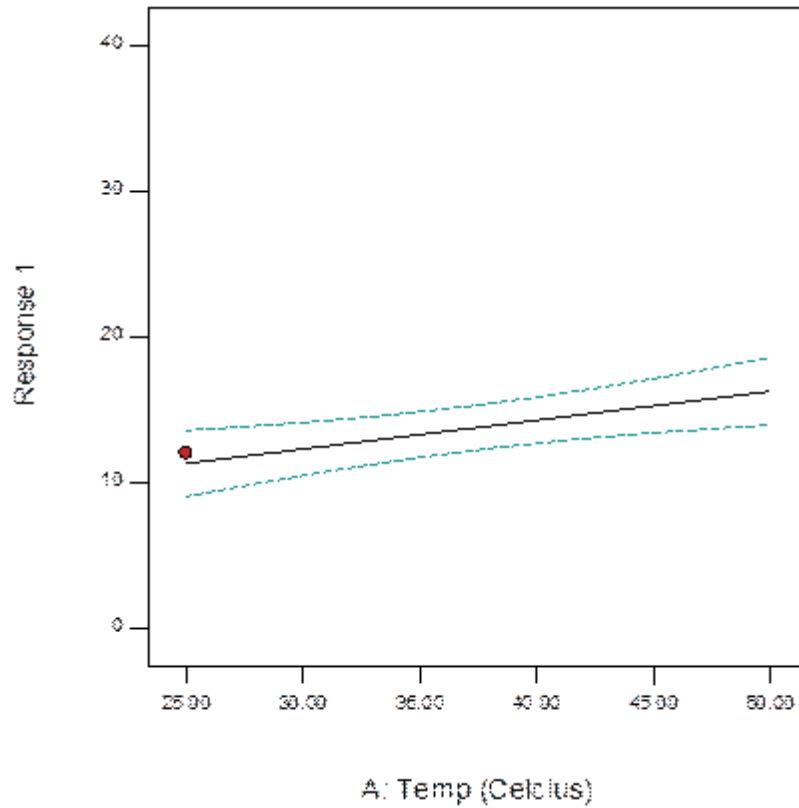


Fig 21 Effect of temperature (°C)

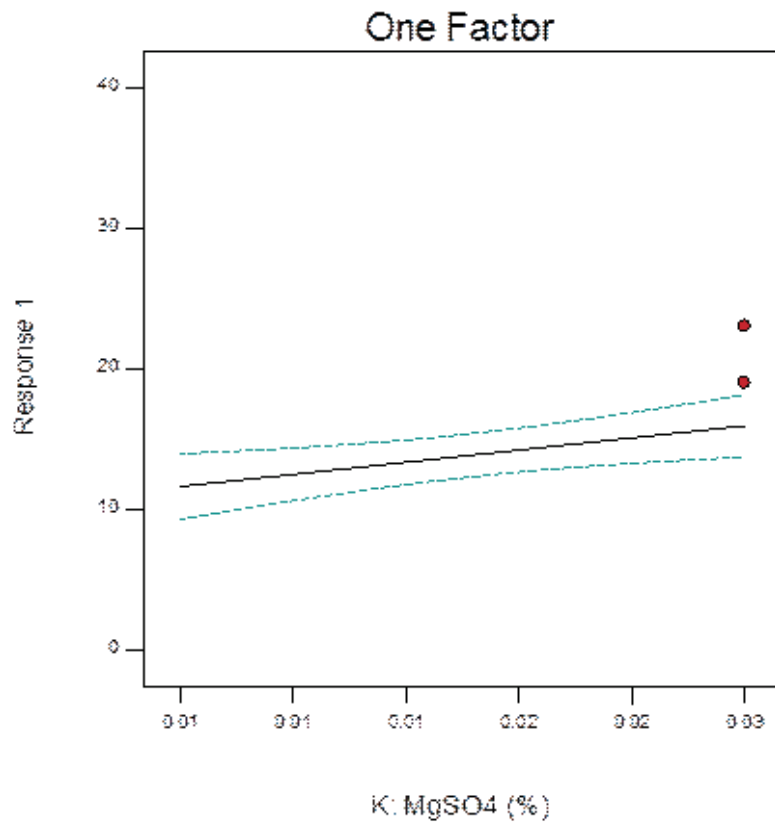


Fig 22 Effect of MgSO₄

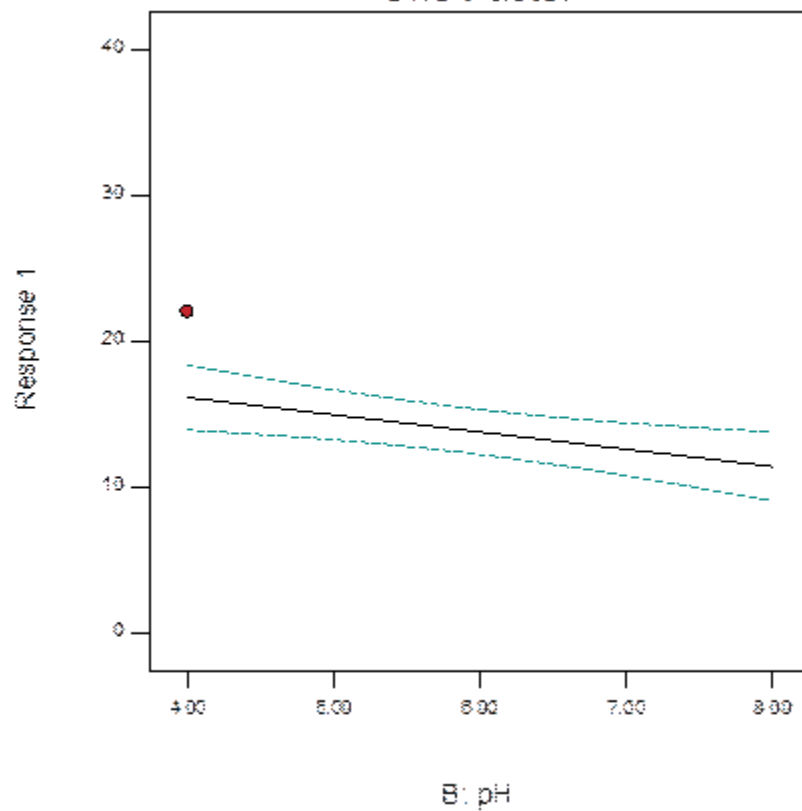


Fig 23 Effect of pH

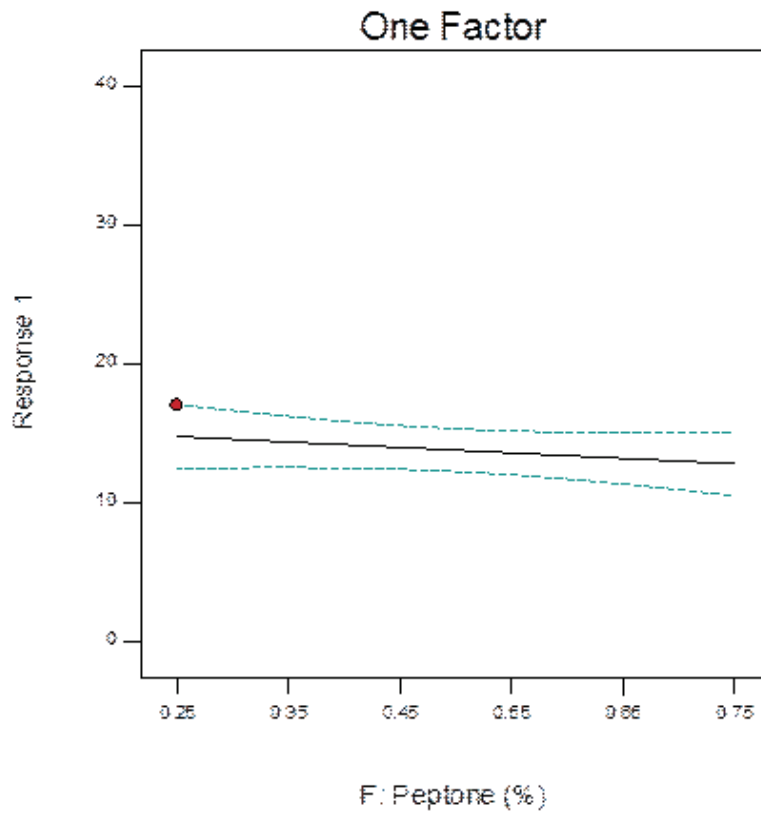


Fig 24 Effect of Peptone

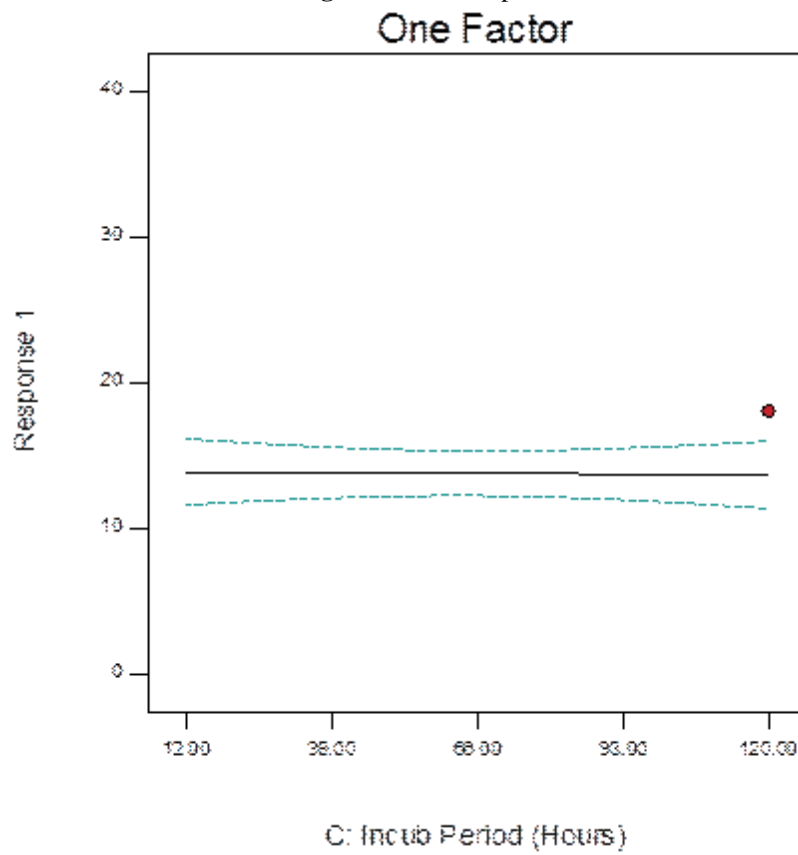


Fig 25 Effect of Incubation period (hrs)

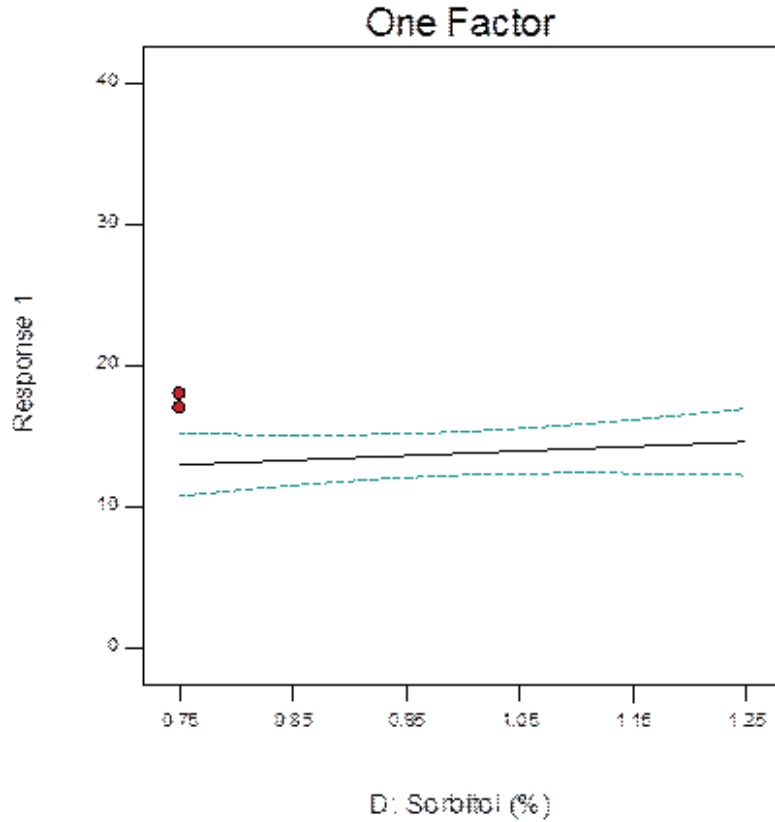


Fig 26 Effect of Concentration of Sorbitol

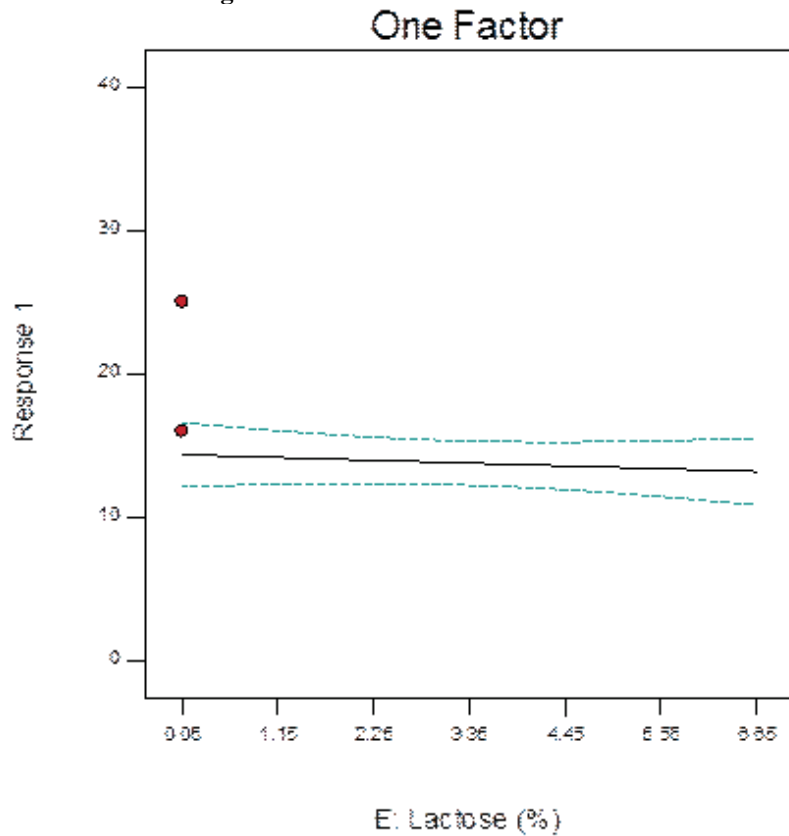
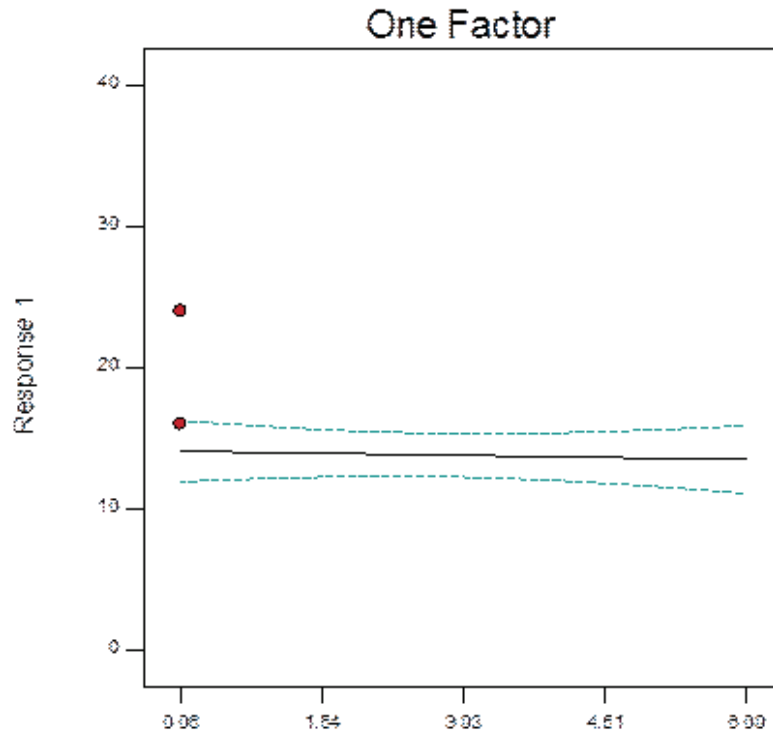
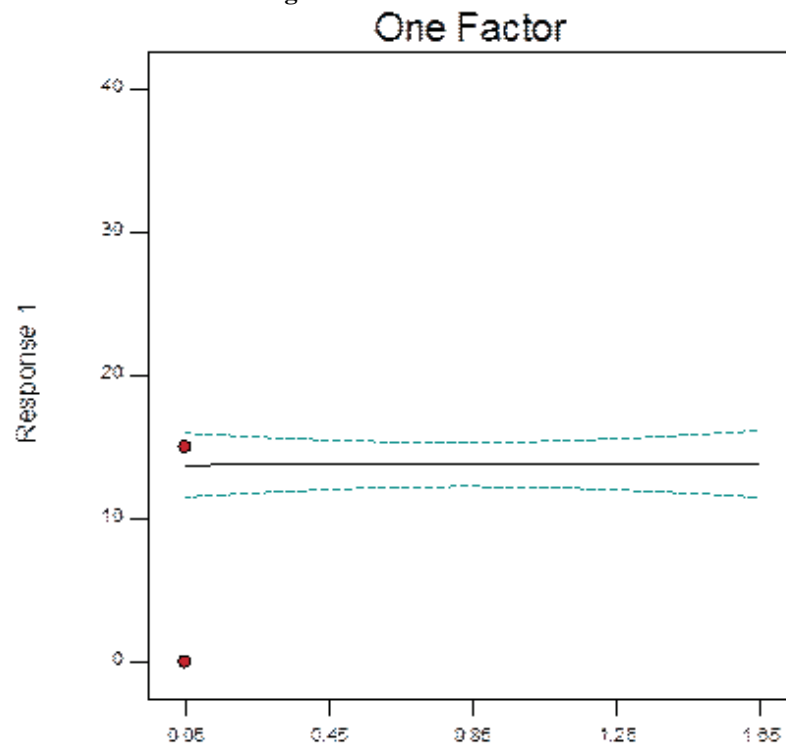


Fig 27 Effect of Concentration of Lactose



G: Yeast extract (%)

Fig 28 Effect of Yeast Extract



H: NH4NO3 (%)

Fig 29 Effect of Concentration of Ammonium Nitrate (NH₄NO₃)

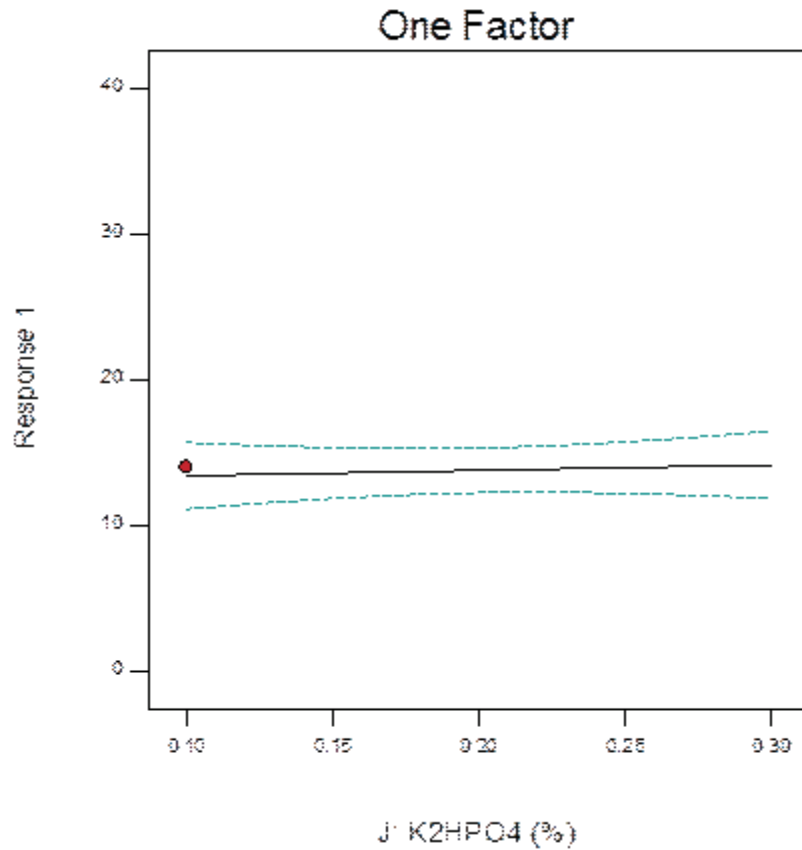


Fig 30 Effect of Concentration of Dipotassium Hydrogen Phosphate (K₂HPO₄)

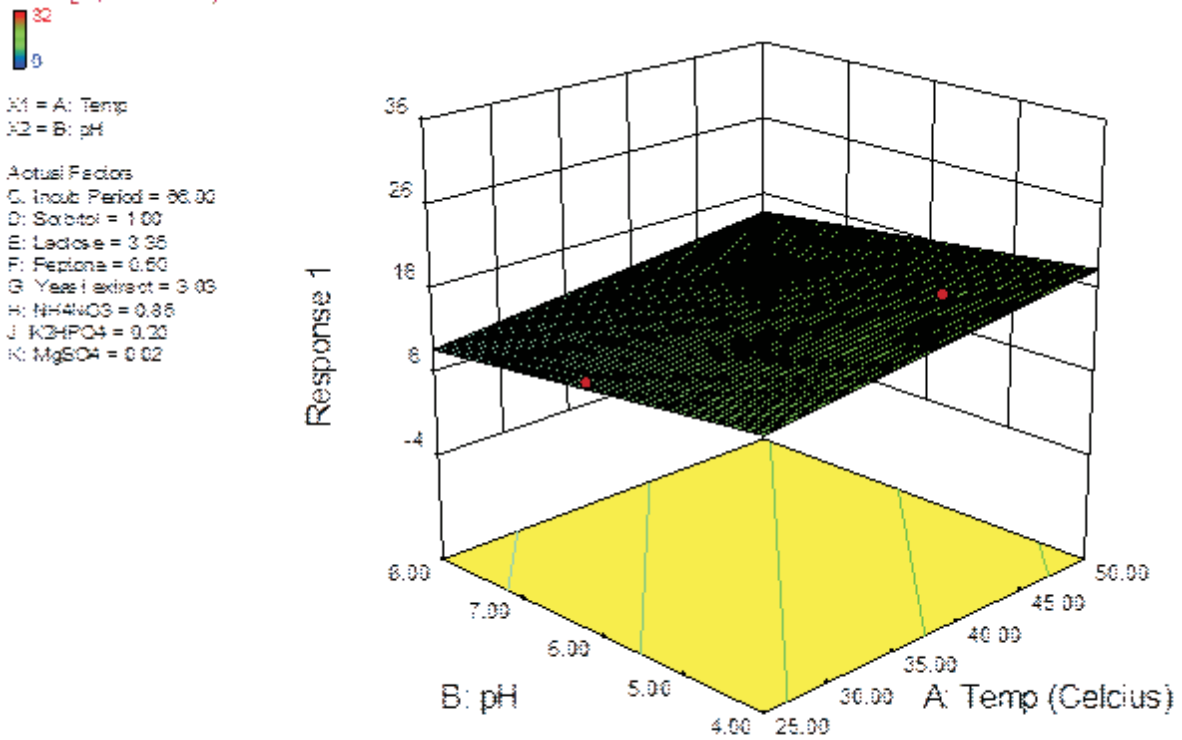


Fig 31 Effect of pH and Incubation temperature (°C) on Bacteriocin production (%)

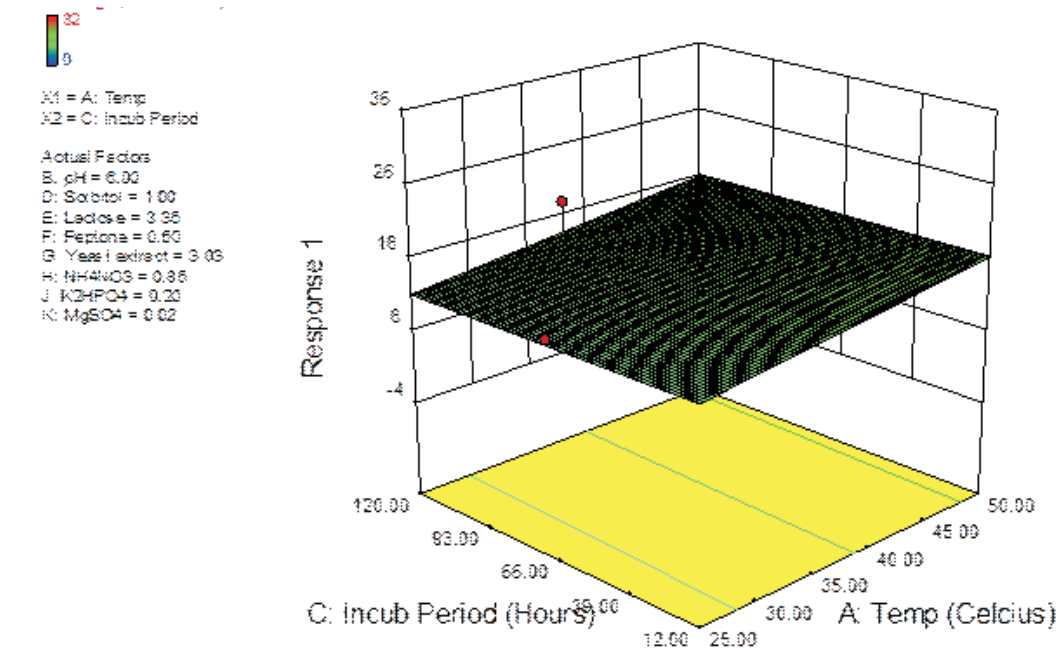


Fig 32 Effect of Temperature (°C) and Incubation period (hrs) on Bacteriocin production (%)

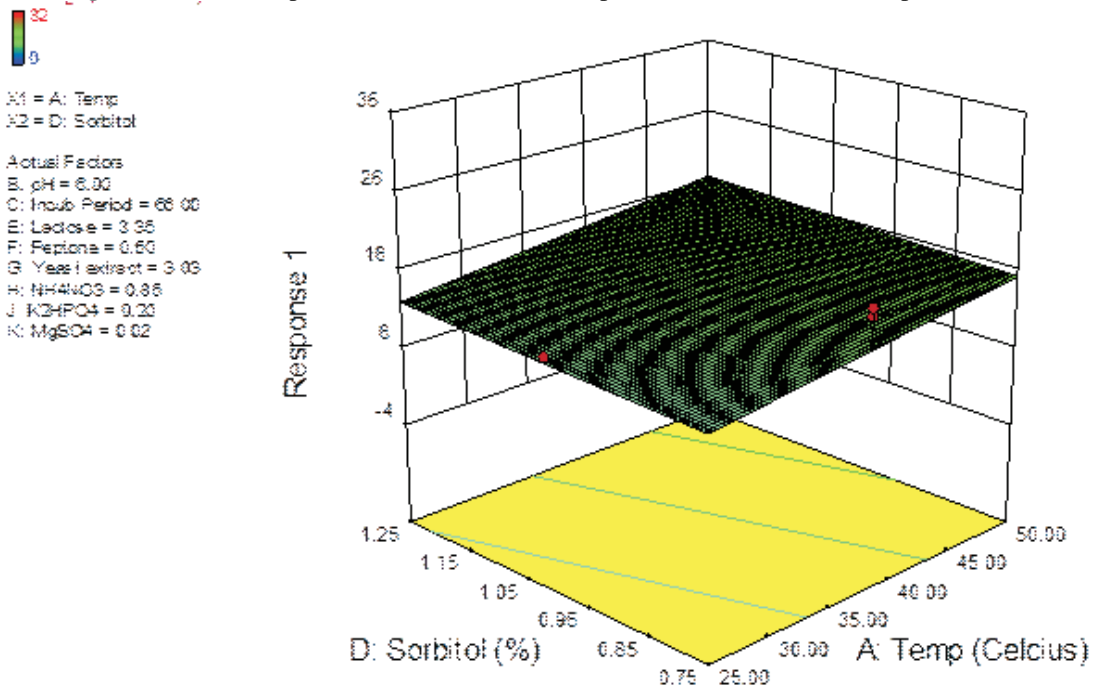


Fig 33 Effect of Incubation temperature (°C) and Sorbitol (%) on Bacteriocin production (%)

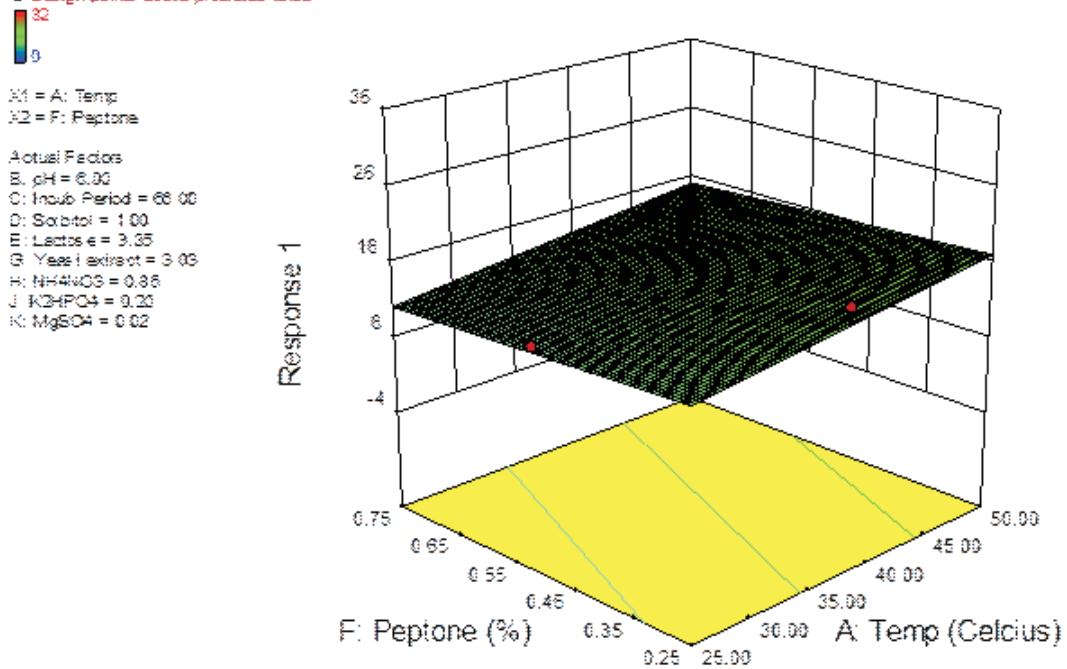


Fig 34 Effect of incubation temperature (°C) and Peptone (%) on bacteriocin production (%).

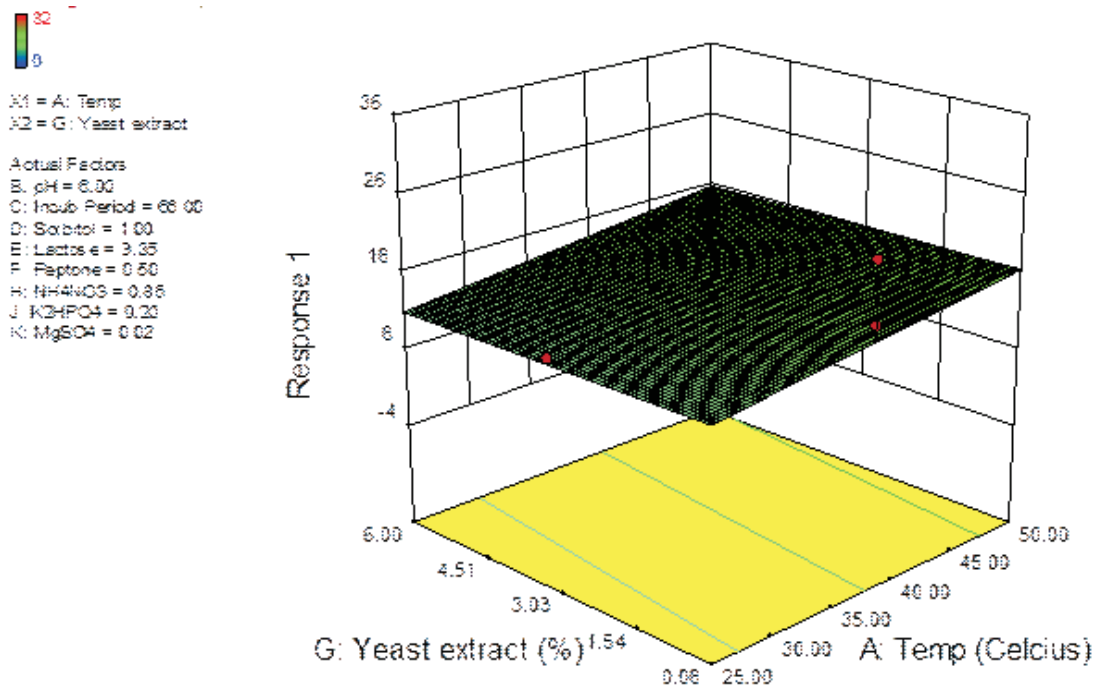



Fig 35 Effect of Incubation temperature (°C) and Yeast extract (%) on Bacteriocin production (%)


 X1 = A: Temp
 X2 = E: Lactose
 Actual Factors
 B: pH = 6.00
 C: Incub Period = 66.00
 D: Sorbitol = 1.00
 F: Peptone = 0.50
 G: Yeast extract = 3.00
 H: NH4NO3 = 0.88
 J: K2HPO4 = 0.20
 K: MgSO4 = 0.02

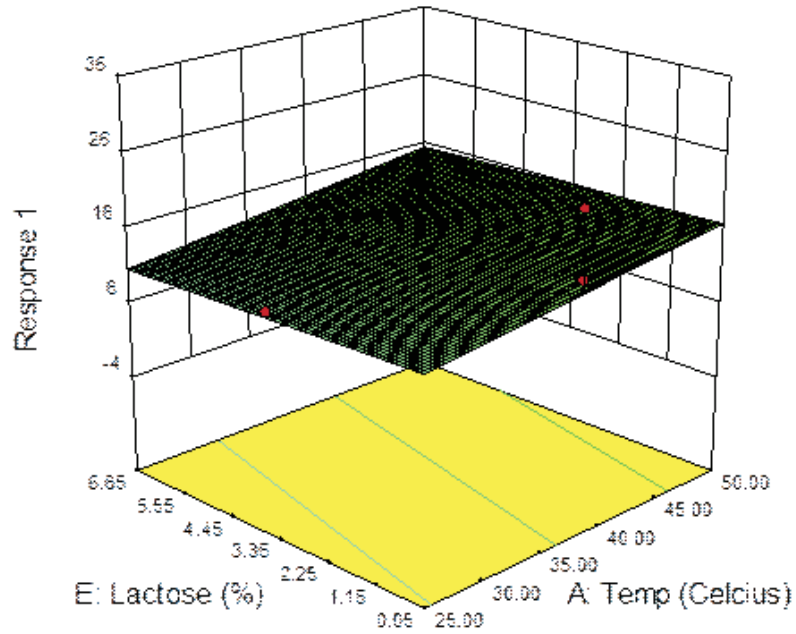



Fig 36 Effect of Incubation temperature (°C) and Lactose (%) on Bacteriocin production


 X1 = A: Temp
 X2 = H: NH4NO3
 Actual Factors
 B: pH = 6.00
 C: Incub Period = 66.00
 D: Sorbitol = 1.00
 E: Lactose = 3.35
 F: Peptone = 0.50
 G: Yeast extract = 3.00
 J: K2HPO4 = 0.20
 K: MgSO4 = 0.02

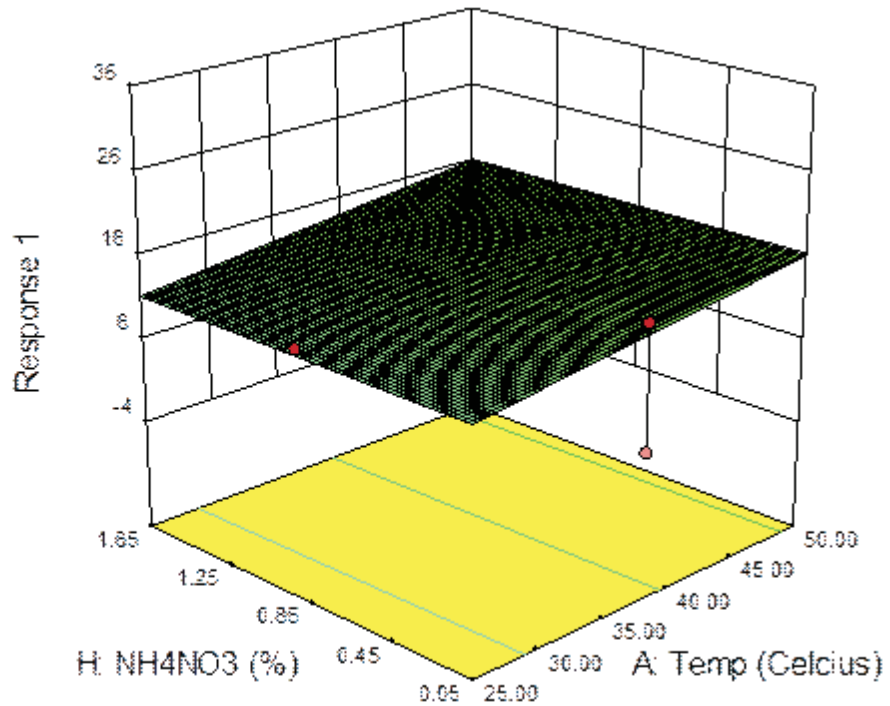


Fig 37 Effect of Incubation temperature (°C) and NH₄NO₃ (%) on Bacteriocin production

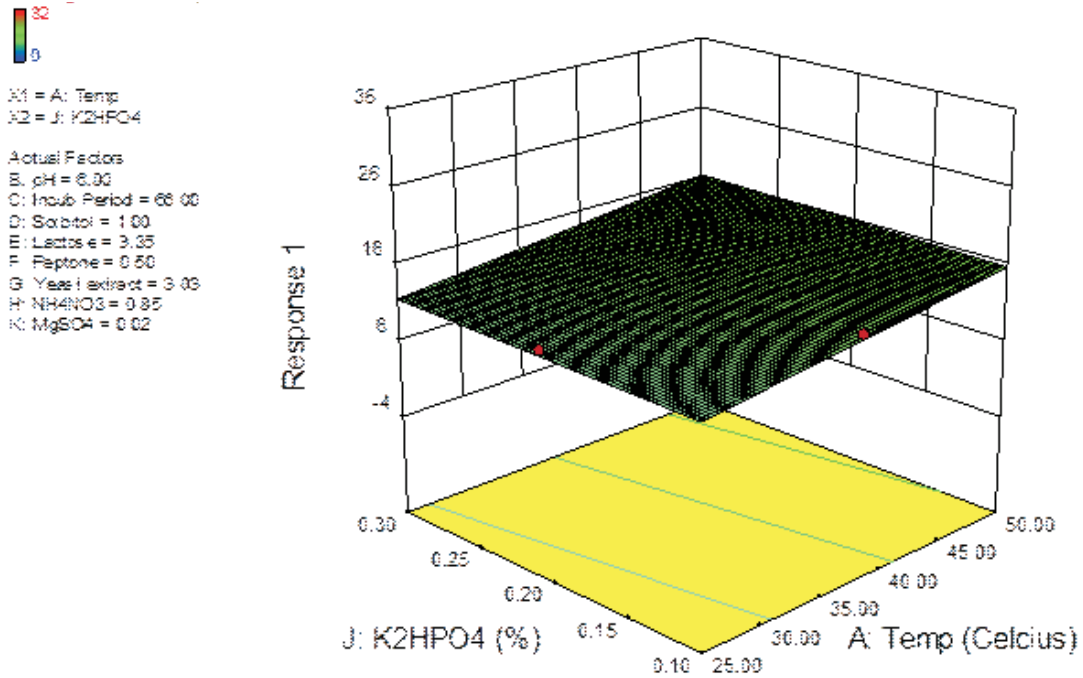


Fig 38 Effect of incubation temperature (°C) and K₂HPO₄ (%) on Bacteriocin production (%)

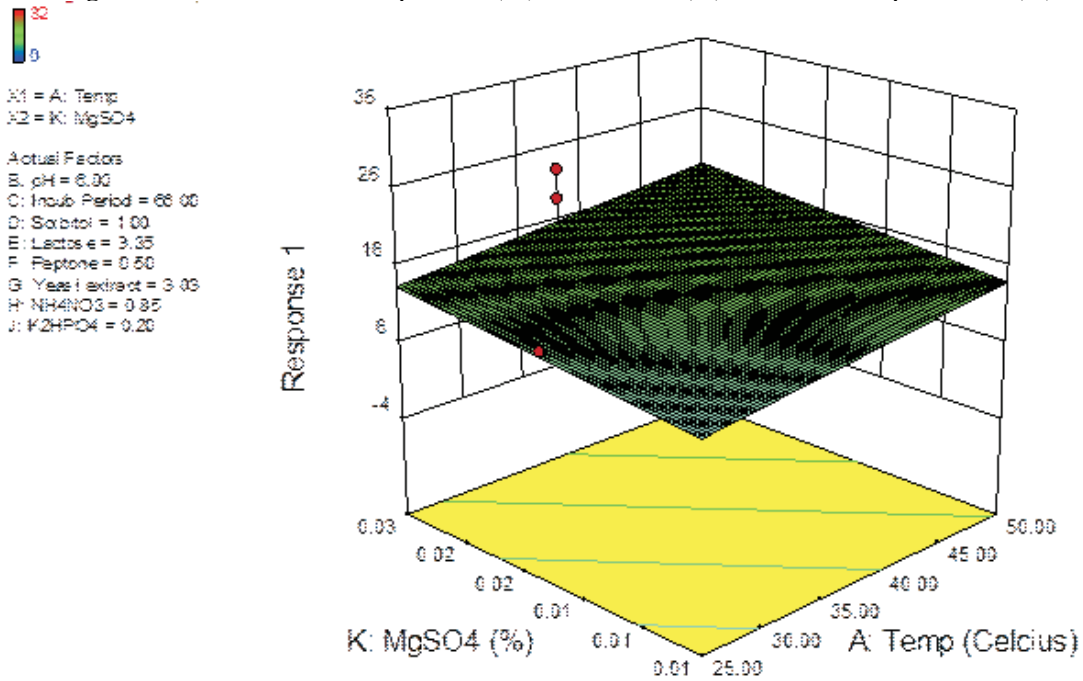


Fig 39 Effect of Incubation temperature (°C) and MgSO₄ (%) on Bacteriocin production (%)

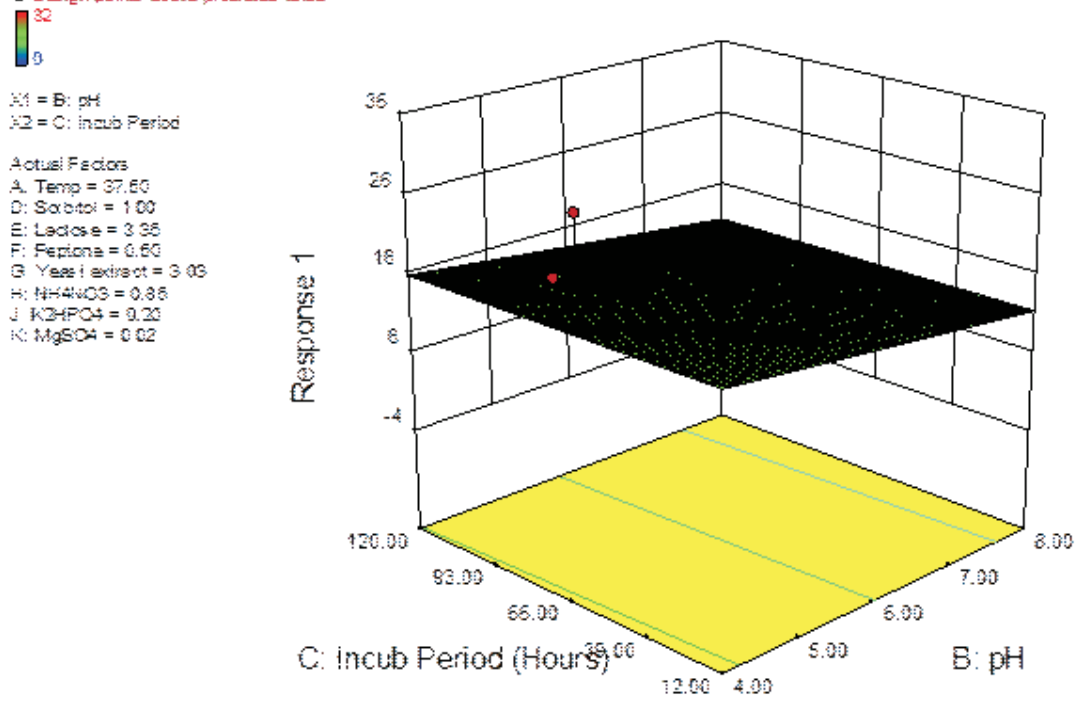


Fig 40 Effect of pH and Incubation period (hrs) on Bacteriocin production (%).

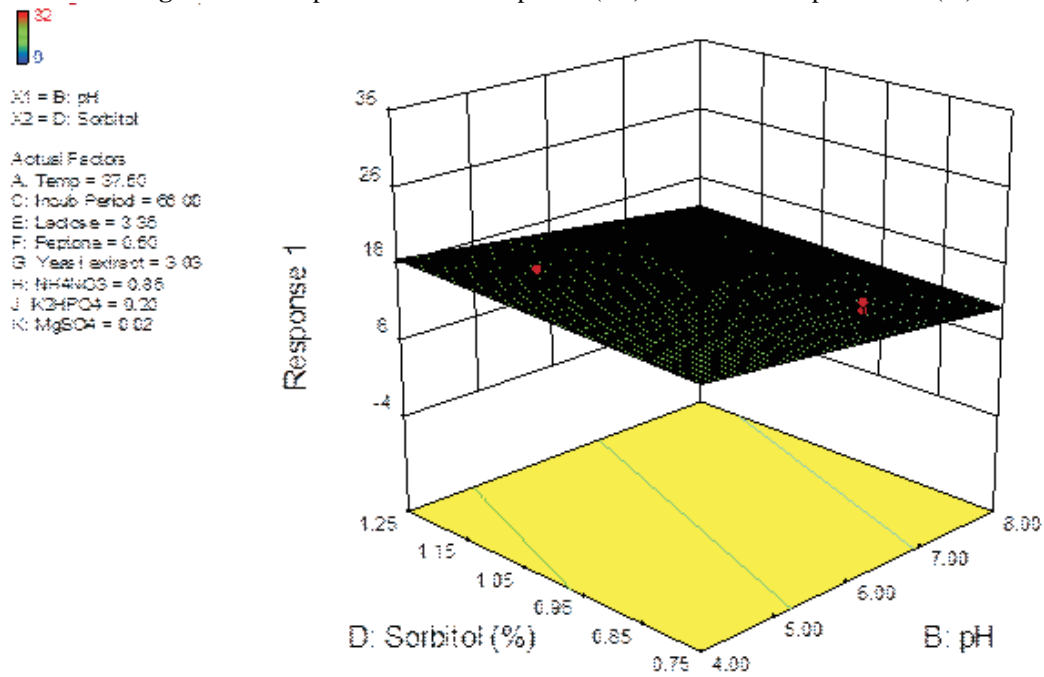


Fig 41 Effect of pH and Sorbitol (%) on Bacteriocin production (%)

■ 32
■ 0
 X1 = B: pH
 X2 = E: Lactose
 Actual Factors
 A: Temp = 37.50
 C: Incub. Period = 68.00
 D: Sorbitol = 1.00
 F: Peptone = 0.50
 G: Yeast extract = 0.05
 H: NH₄NO₃ = 0.35
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

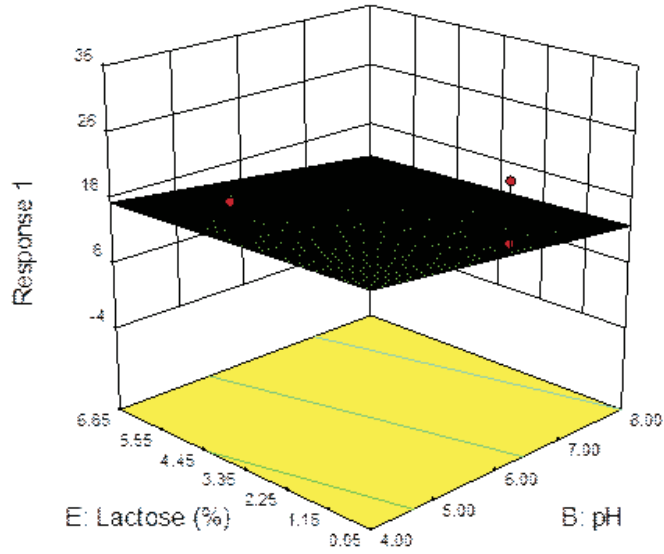


Fig 42 Effect of pH and Lactose (%) on Bacteriocin production (%)

■ 32
■ 0
 X1 = B: pH
 X2 = F: Peptone
 Actual Factors
 A: Temp = 37.50
 C: Incub. Period = 68.00
 D: Sorbitol = 1.00
 E: Lactose = 3.35
 G: Yeast extract = 0.05
 H: NH₄NO₃ = 0.35
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

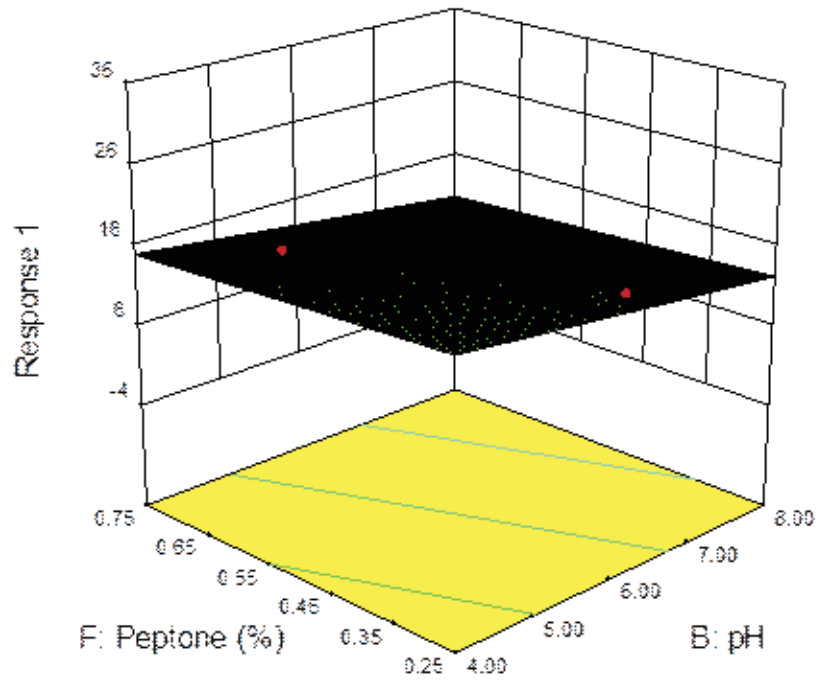



Fig 43 Effect of pH and Peptone (%) on Bacteriocin production (%)


 X1 = B: pH
 X2 = G: Yeast extract
 Actual Factors
 A: Temp = 37.50
 C: Incub Period = 68.00
 D: Sorbitol = 1.00
 E: Lactose = 3.35
 F: Peptone = 0.50
 H: NH4NO3 = 0.85
 J: K2HPO4 = 0.20
 K: MgSO4 = 0.02

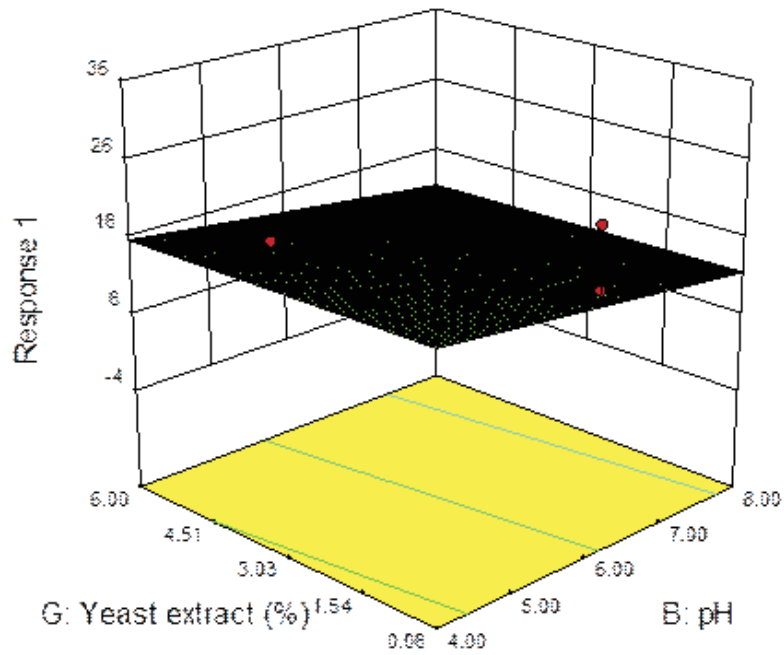



Fig 44 Effect of Yeast extract (%) and pH on Bacteriocin production (%)


 X1 = B: pH
 X2 = H: NH4NO3
 Actual Factors
 A: Temp = 37.50
 C: Incub Period = 68.00
 D: Sorbitol = 1.00
 E: Lactose = 3.35
 F: Peptone = 0.50
 G: Yeast extract = 3.03
 J: K2HPO4 = 0.20
 K: MgSO4 = 0.02

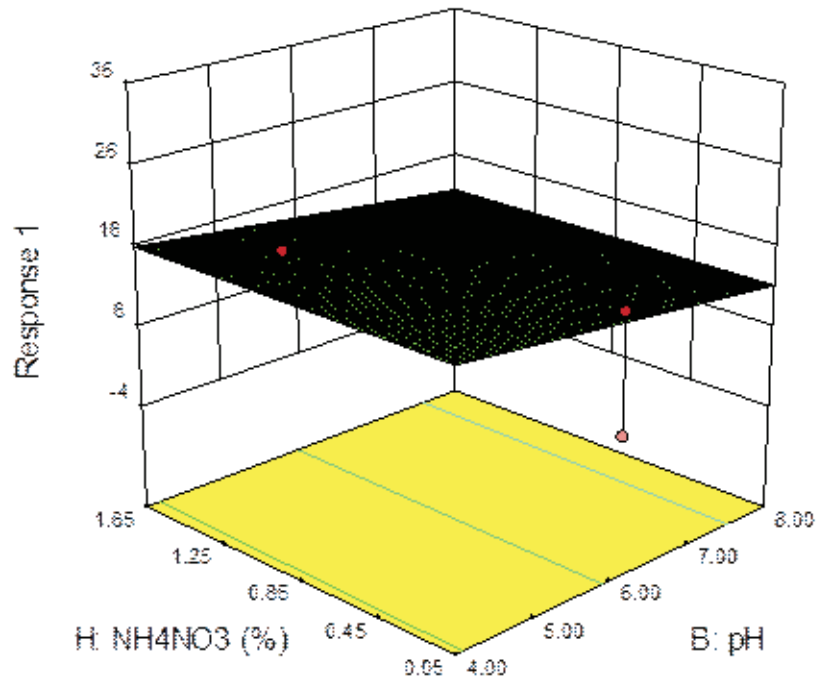


Fig 45 Effect of NH₄NO₃ (%) and pH on Bacteriocin production (%).

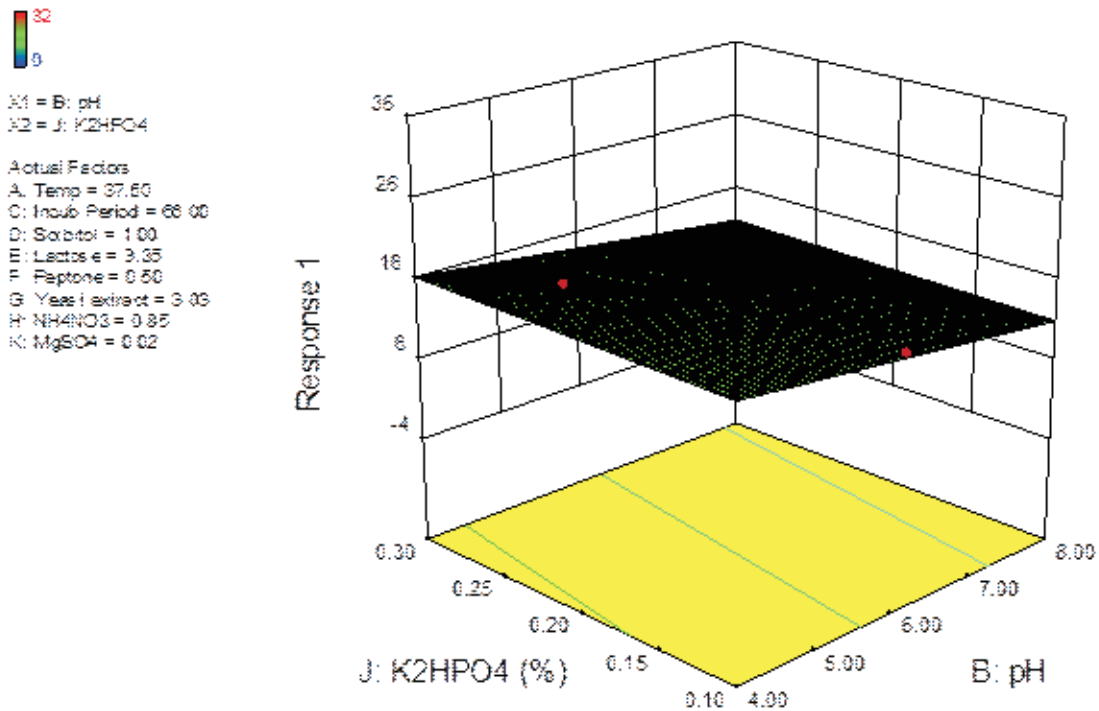


Fig 46 Effect of K₂HPO₄ (%) and pH on Bacteriocin production (%)

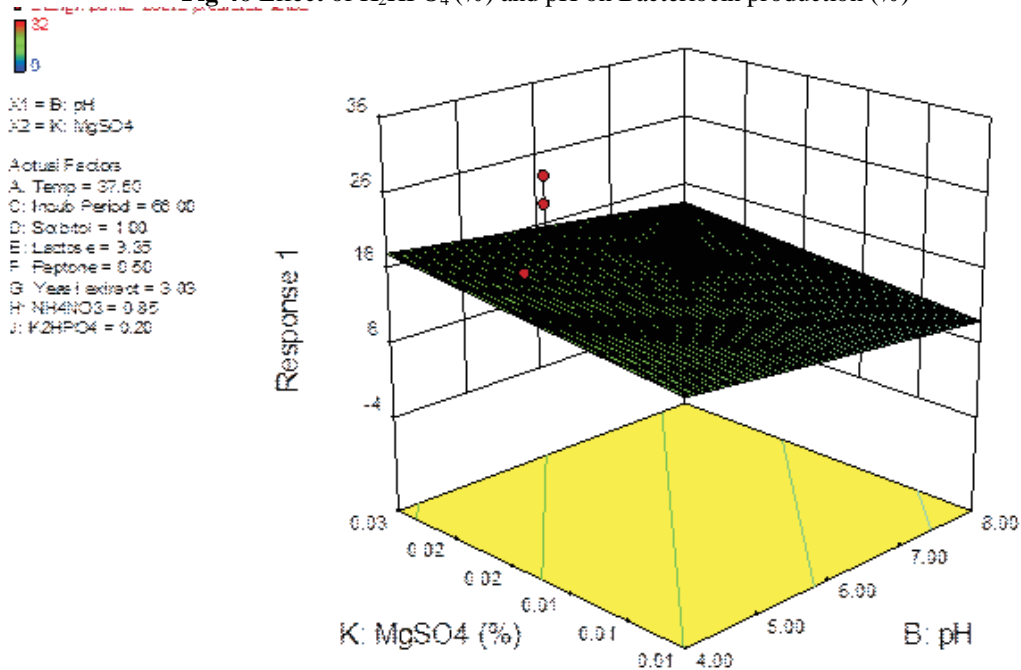


Fig 47 Effect of MgSO₄ (%) and pH on Bacteriocin production (%)

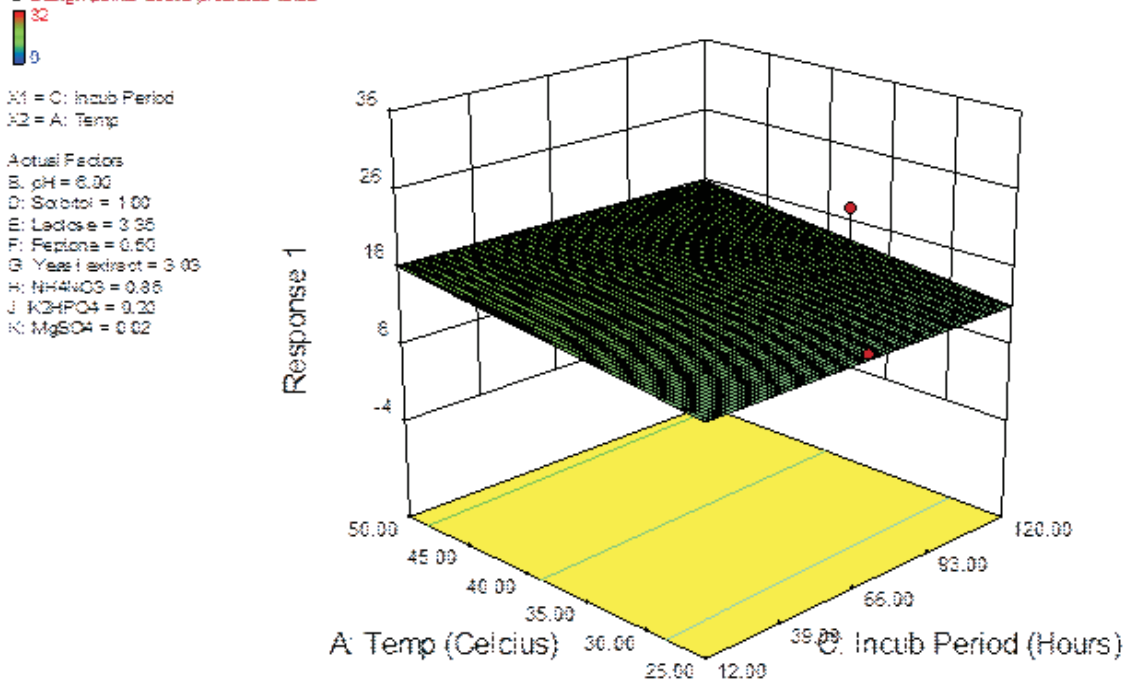


Fig 48 Effect of Incubation period (hrs) and Incubation temperature (°C) on Bacteriocin production (%)

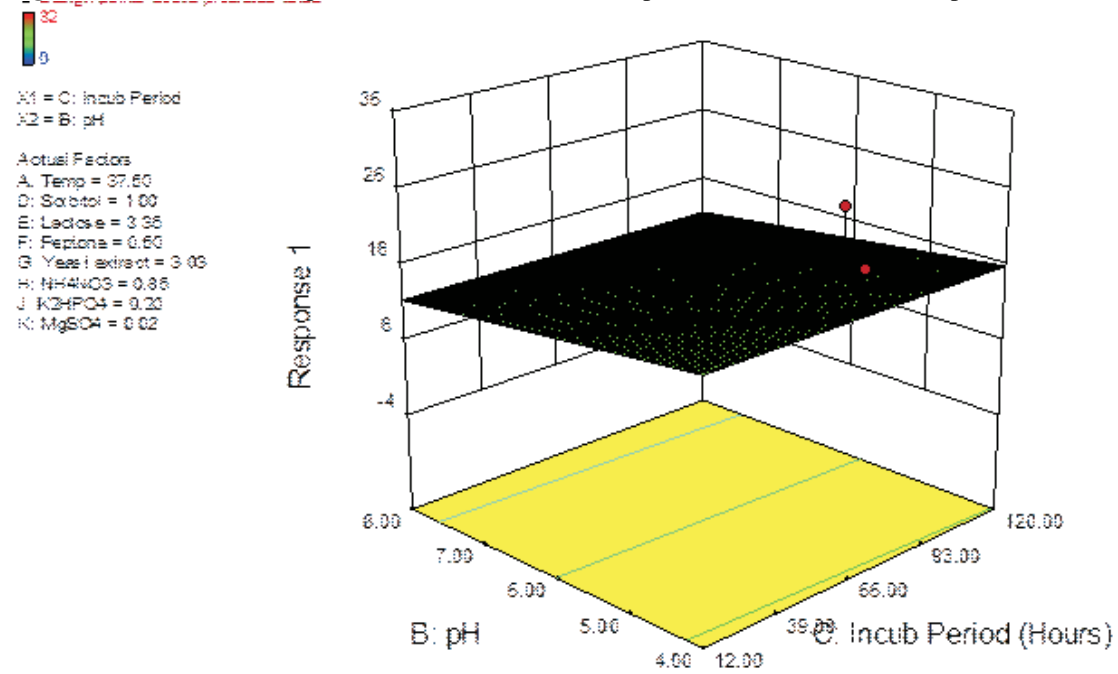



Fig 49 Effect of Incubation period (hrs) and pH on Bacteriocin production (%)



 X1 = C: Incub Period
 X2 = D: Sorbitol

 Actual Factors
 A: Temp = 37.50
 B: pH = 6.00
 E: Lactose = 3.35
 F: Peptone = 0.50
 G: Yeas l extract = 3.00
 H: NH4NO3 = 0.85
 J: K2HPO4 = 0.20
 K: MgSO4 = 0.02

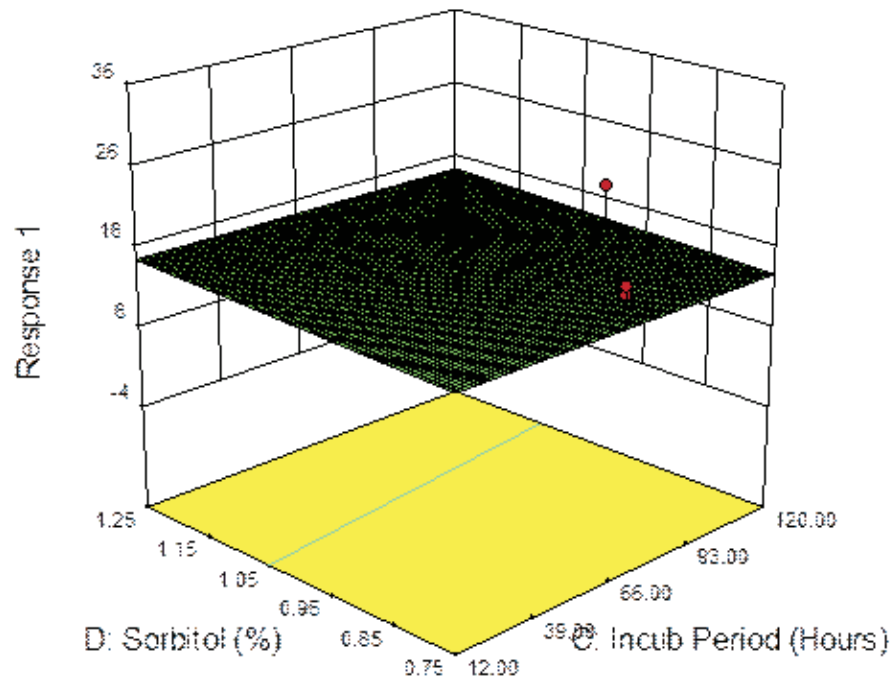



Fig 50 Effect of Sorbitol (%) and Incubation period (hrs) on Bacteriocin production (%)



 X1 = C: Incub Period
 X2 = E: Lactose

 Actual Factors
 A: Temp = 37.50
 B: pH = 6.00
 D: Sorbitol = 1.00
 F: Peptone = 0.50
 G: Yeas l extract = 3.00
 H: NH4NO3 = 0.85
 J: K2HPO4 = 0.20
 K: MgSO4 = 0.02

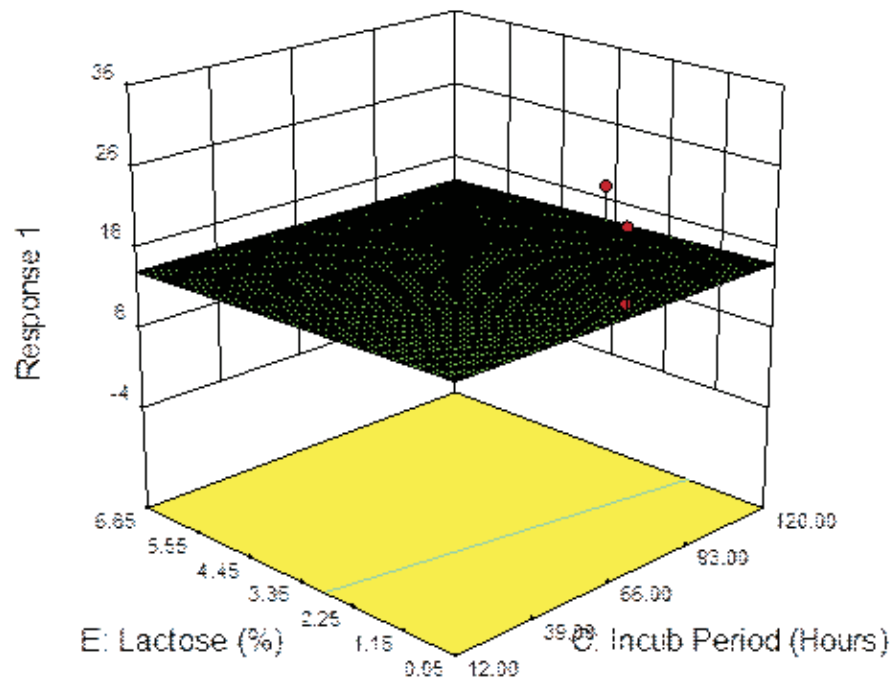


Fig 51 Effect of Lactose (%) and Incubation period (hrs) on Bacteriocin production (%)

■ 32
■ 0
 X1 = C: Inzub Period
 X2 = F: Peptone
 Actual Factors
 A: Temp = 37.50
 B: pH = 8.00
 D: Sorbitol = 1.00
 E: Lactose = 3.35
 G: Yeast extract = 0.03
 H: NH₄NO₃ = 0.85
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

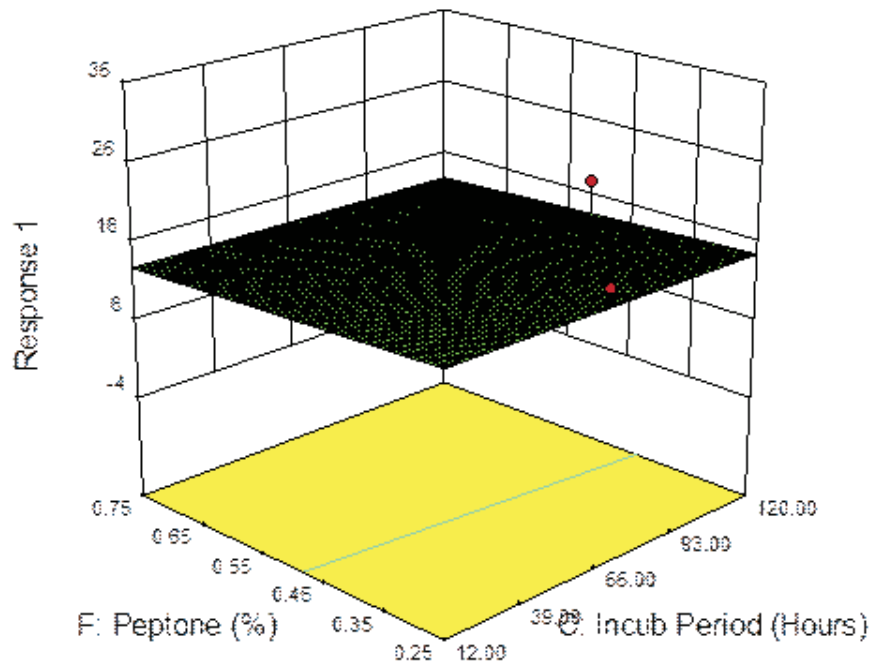


Fig 52 Effect of Peptone (%) and Incubation period (hrs) on Bacteriocin production (%)

■ 32
■ 0
 X1 = C: Inzub Period
 X2 = G: Yeast extract
 Actual Factors
 A: Temp = 37.50
 B: pH = 8.00
 D: Sorbitol = 1.00
 E: Lactose = 3.35
 F: Peptone = 0.50
 H: NH₄NO₃ = 0.85
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

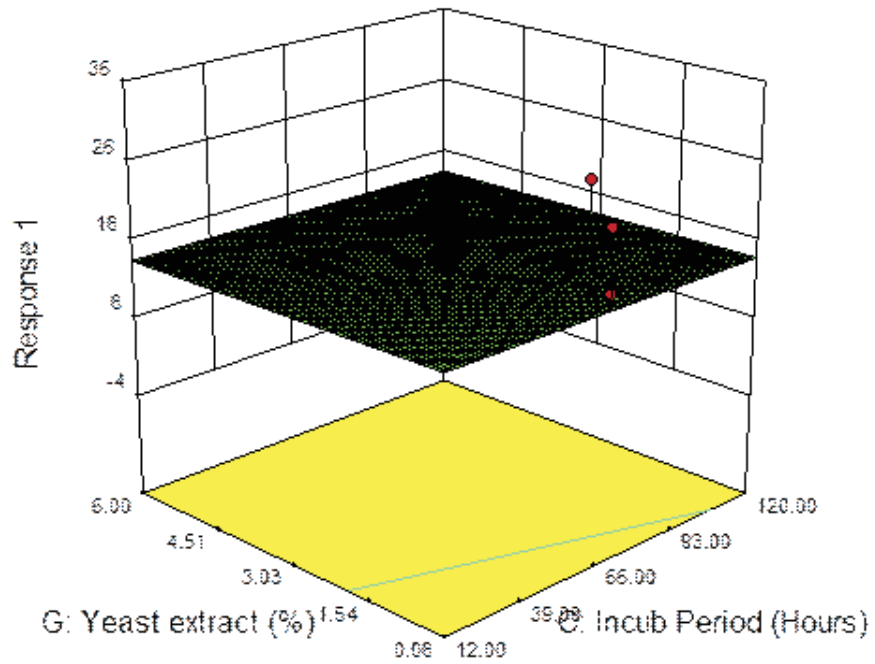


Fig 53 Effect of Yeast extract (%) and Incubation period (hrs) on Bacteriocin production (%)

32
0

X1 = C: Incub Period
X2 = H: NH4NO3

Actual Factors
A: Temp = 37.50
B: pH = 8.00
D: Sorbitol = 1.00
E: Lactose = 3.35
F: Peptone = 0.50
G: Yeast extract = 3.00
J: K2HPO4 = 0.20
K: MgSO4 = 0.02

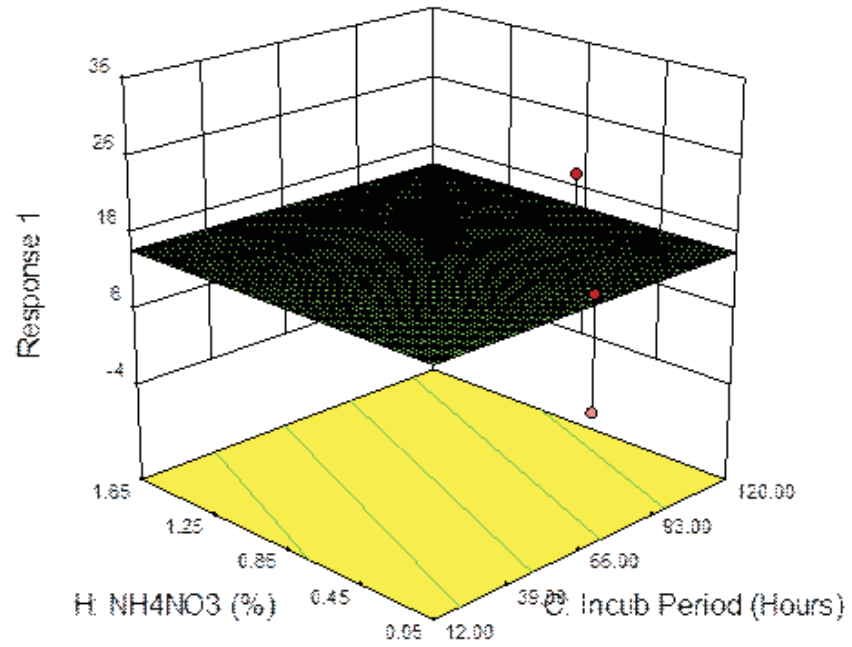


Fig 54 Effect of NH₄NO₃ (%) and Incubation period (hrs) on Bacteriocin production (%)

32
0

X1 = C: Incub Period
X2 = J: K2HPO4

Actual Factors
A: Temp = 37.50
B: pH = 8.00
D: Sorbitol = 1.00
E: Lactose = 3.35
F: Peptone = 0.50
G: Yeast extract = 3.00
H: NH4NO3 = 0.85
K: MgSO4 = 0.02

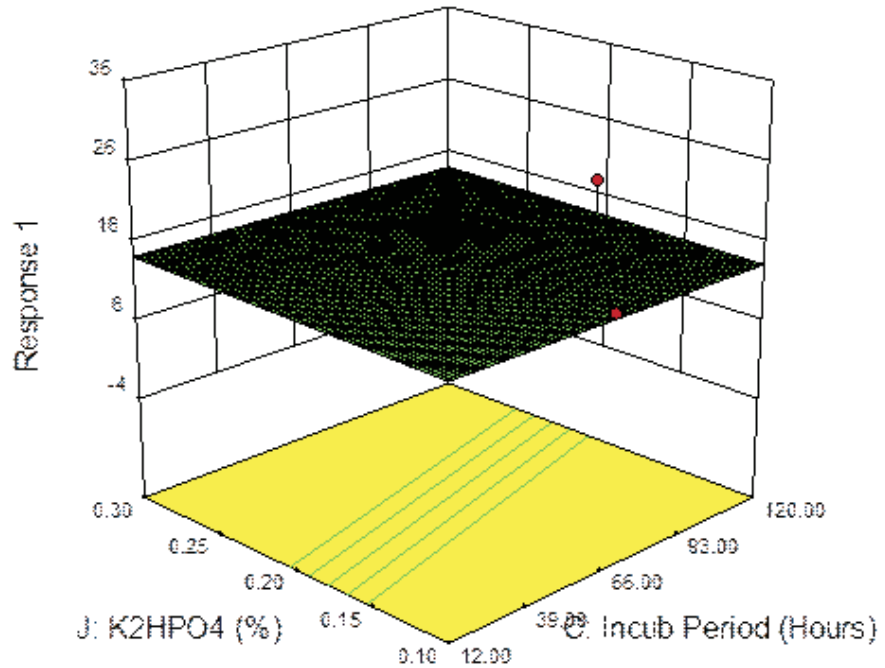


Fig 55 Effect of K₂HPO₄ (%) and Incubation period (hrs) on Bacteriocin production (%)

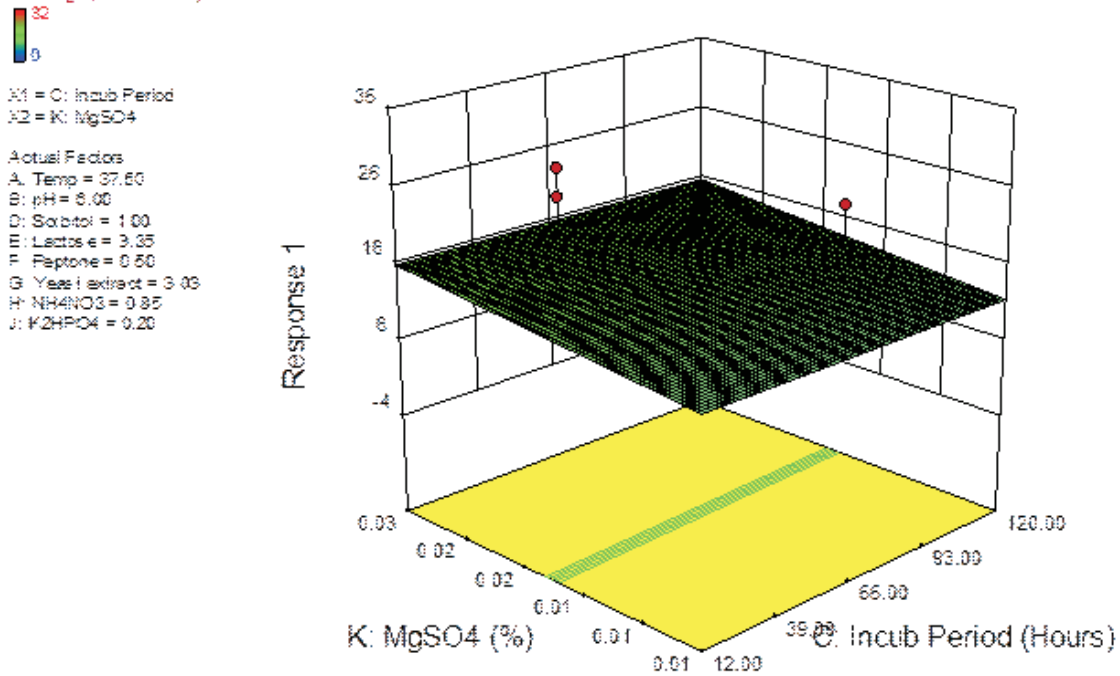


Fig 56 Effect of MgSO₄ and Incubation period (hrs) on Bacteriocin production (%)

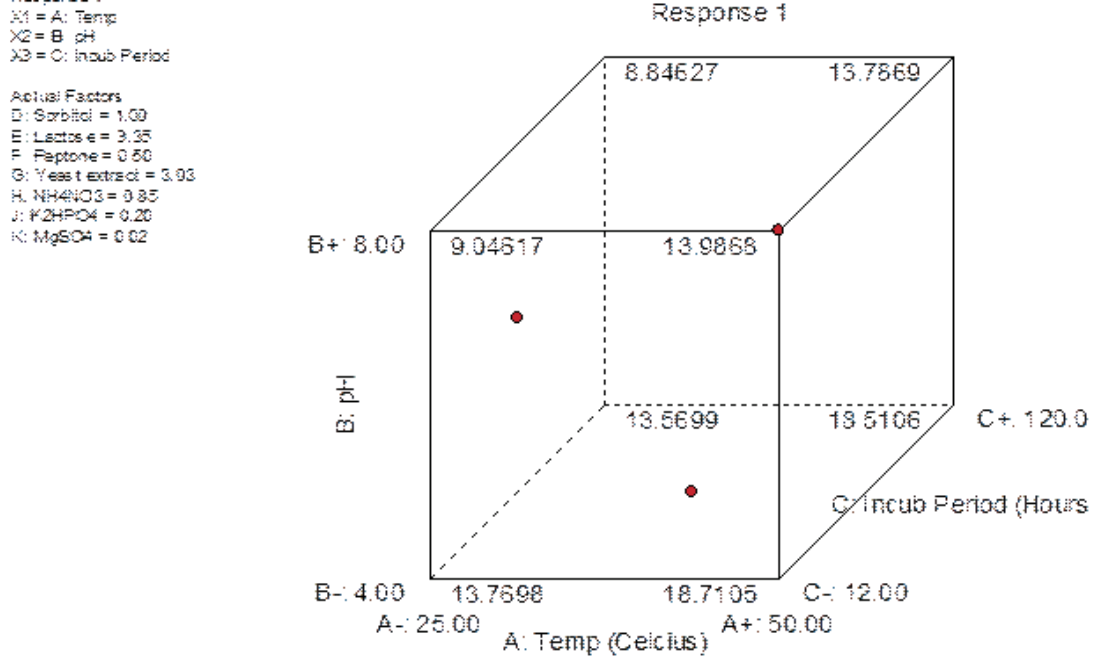


Fig 57 Effect of Incubation temperature (°C), pH and Incubation period (hrs) on Bacteriocin production (%)

Response 1
 X1 = A: Temp
 X2 = D: Sorbitol
 X3 = C: Incub Period

Actual Factors
 B: pH = 6.00
 E: Lactose = 3.35
 F: Peptone = 0.50
 G: Yeast extract = 3.03
 H: NH₄NO₃ = 0.85
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

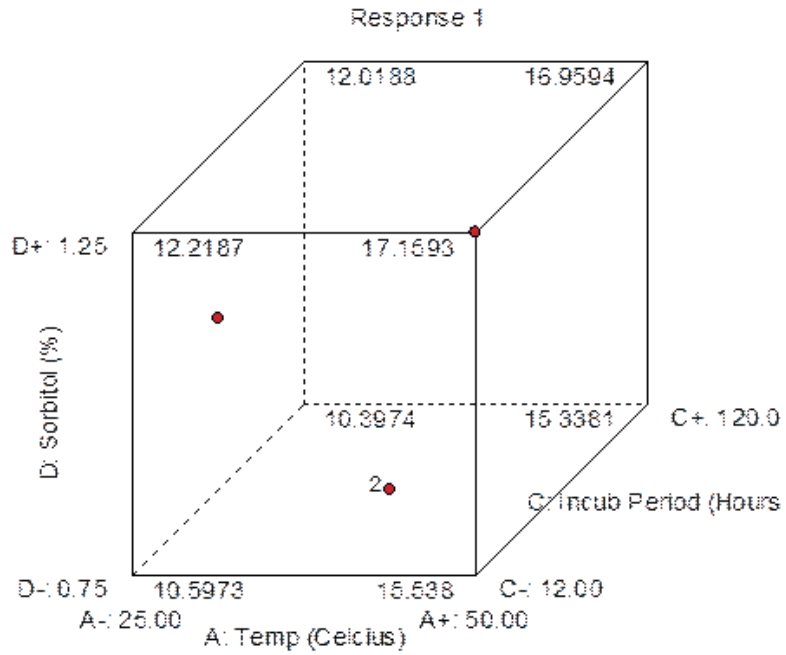


Fig 58 Effect of Incubation temperature (°C), Sorbitol and Incubation period (hrs) on Bacteriocin production (%)

X1 = A: Temp
 X2 = F: Peptone
 X3 = C: Incub Period

Actual Factors
 B: pH = 6.00
 D: Sorbitol = 1.00
 E: Lactose = 3.35
 G: Yeast extract = 3.03
 H: NH₄NO₃ = 0.85
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

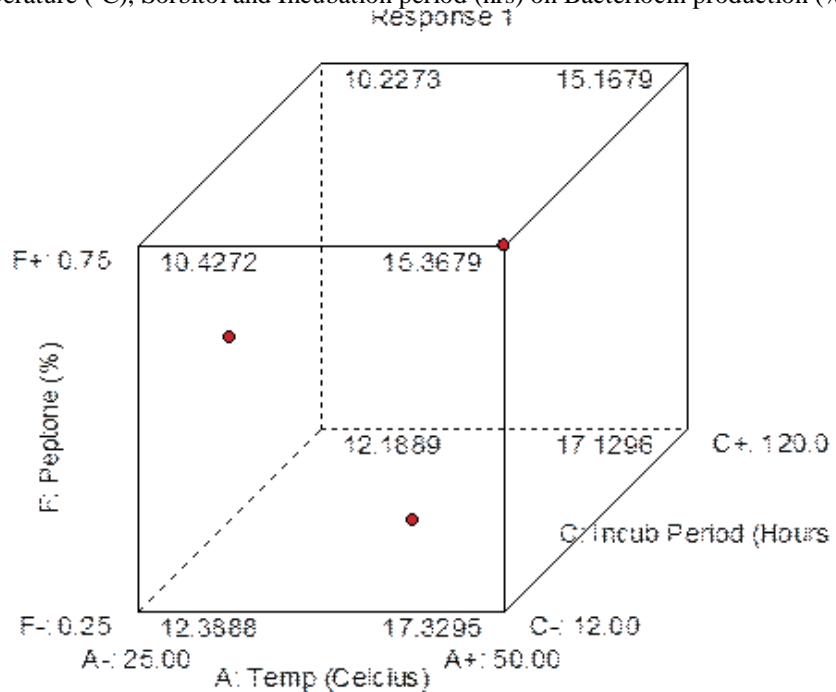


Fig 59 Effect of Incubation temperature (°C), Peptone and Incubation period (hrs) on Bacteriocin production (%)

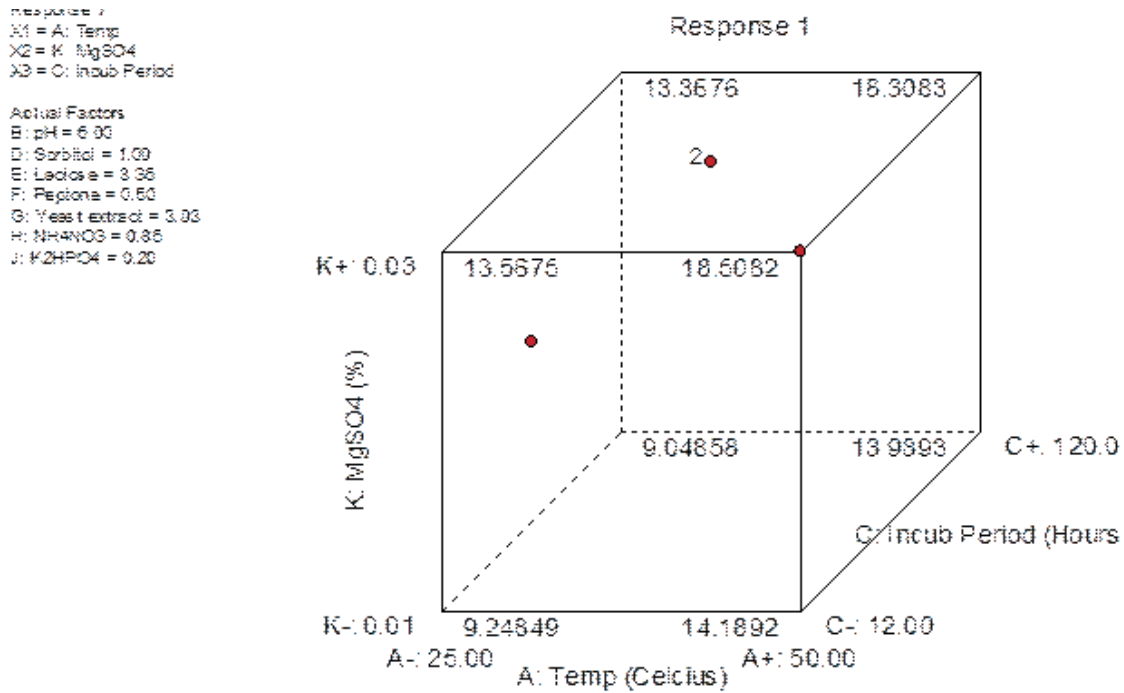


Fig 60 Effect of Incubation temperature (°C), MgSO₄ and Incubation period (hrs) on Bacteriocin production (%)

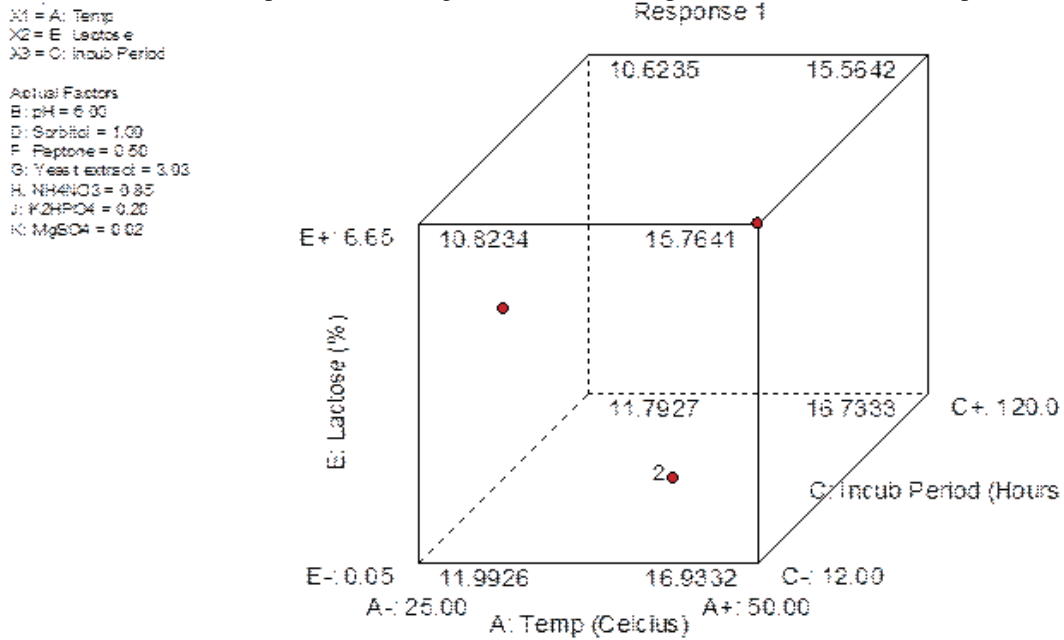


Fig 61 Effect of Incubation temperature (°C), Lactose (%) and Incubation period (hrs) on Bacteriocin production (%)

Response 1
 X1 = A: Temp
 X2 = G: Yeast extract
 X3 = C: Incub Period

Actual Factors
 B: pH = 6.00
 D: Sorbitol = 1.00
 E: Lactose = 3.36
 F: Peptone = 0.50
 H: NH₄NO₃ = 0.85
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

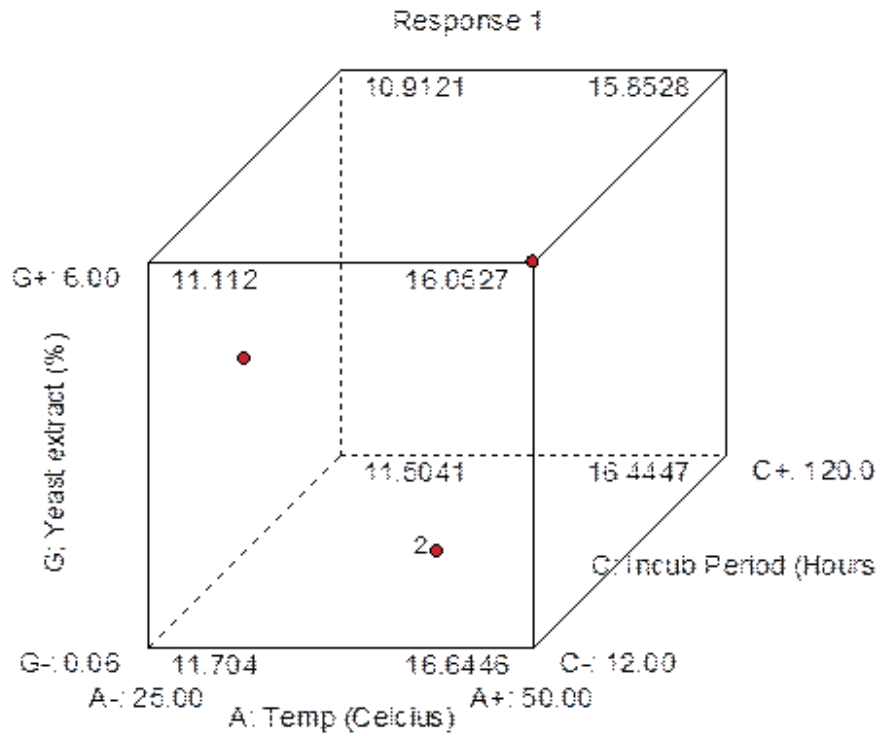


Fig 62 Effect of Incubation temperature (°C), Yeast extract (%) and Incubation period (hrs) on Bacteriocin production (%)

Response 1
 X1 = A: Temp
 X2 = H: NH₄NO₃
 X3 = C: Incub Period

Actual Factors
 B: pH = 6.00
 D: Sorbitol = 1.00
 E: Lactose = 3.36
 F: Peptone = 0.50
 G: Yeast extract = 3.00
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

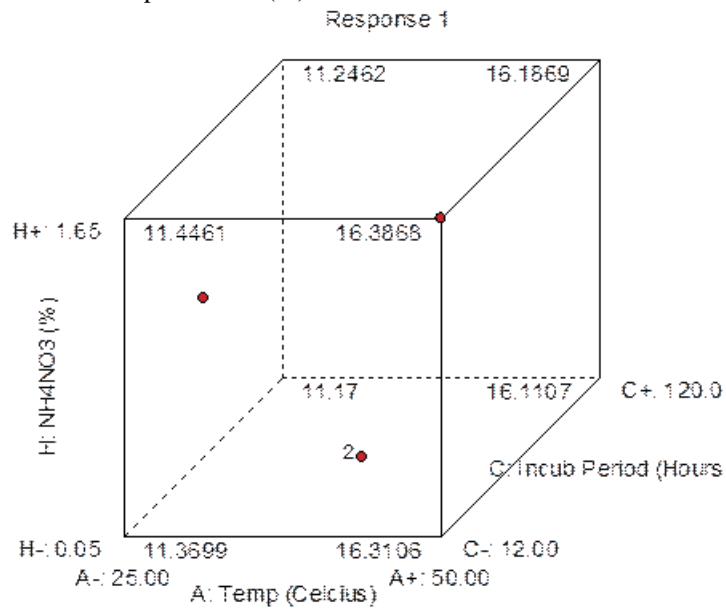


Fig 63 Effect of Incubation temperature (°C), NH₄NO₃ (%) and Incubation period (hrs) on Bacteriocin production (%)

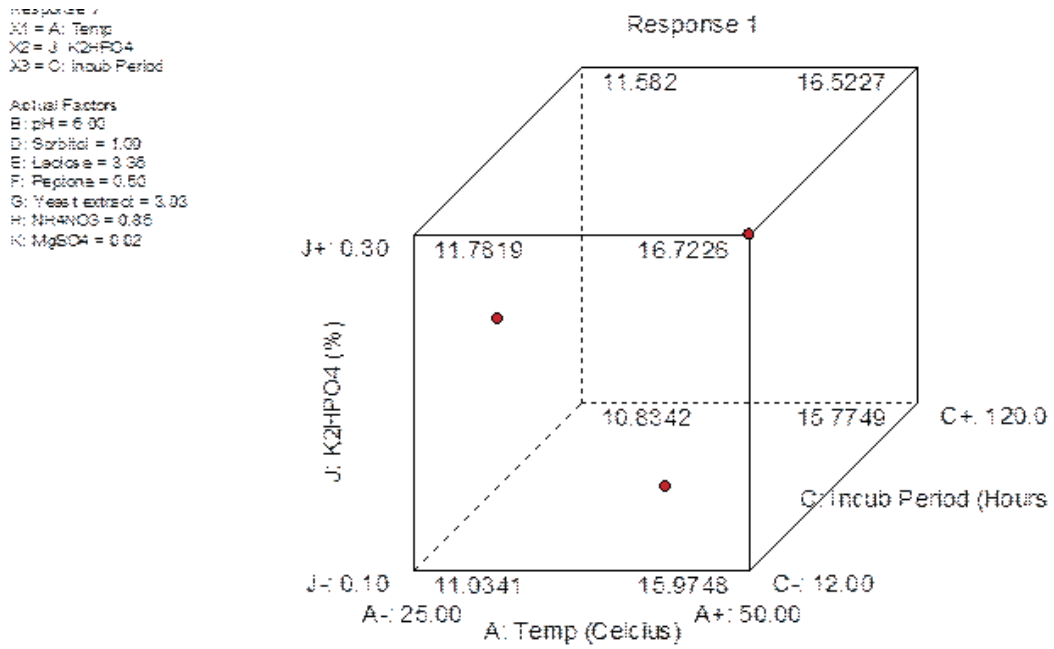


Fig 64 Effect of Incubation temperature (°C), KH₂PO₄ (%) and Incubation period (hrs) on Bacteriocin production (%)

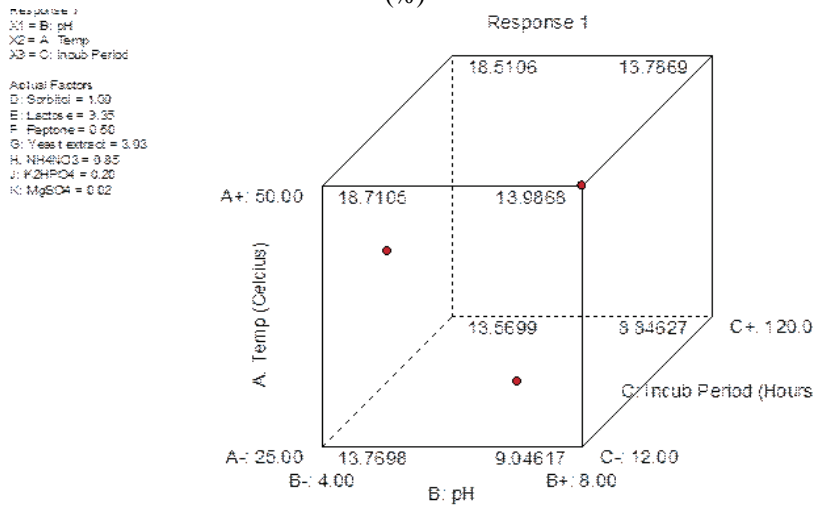


Fig 65 Effect of Incubation time (hrs), Incubation temperature (°C) and pH on Bacteriocin production (%)

Response 1
 X1 = B: pH
 X2 = D: Sorbitol
 X3 = C: Incub Period

Actual Factors
 A: Temp = 37.50
 E: Lactose = 3.35
 F: Peptone = 0.50
 G: Yeast extract = 3.03
 H: NH₄NO₃ = 0.85
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

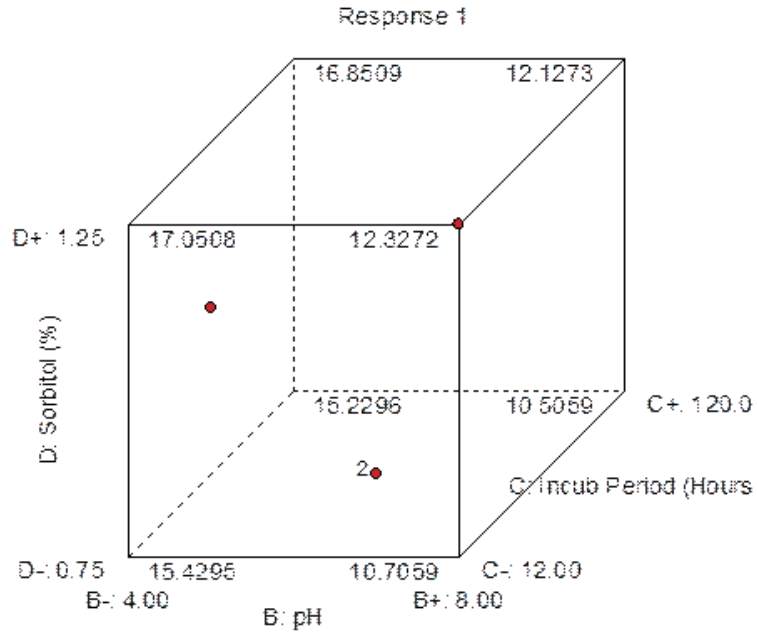


Fig 66 Effect of Incubation time (hrs), Sorbitol (%) and pH on Bacteriocin production (%)

Response 1
 X1 = B: pH
 X2 = F: Peptone
 X3 = C: Incub Period

Actual Factors
 A: Temp = 37.50
 D: Sorbitol = 1.00
 E: Lactose = 3.35
 G: Yeast extract = 3.03
 H: NH₄NO₃ = 0.85
 J: K₂HPO₄ = 0.20
 K: MgSO₄ = 0.02

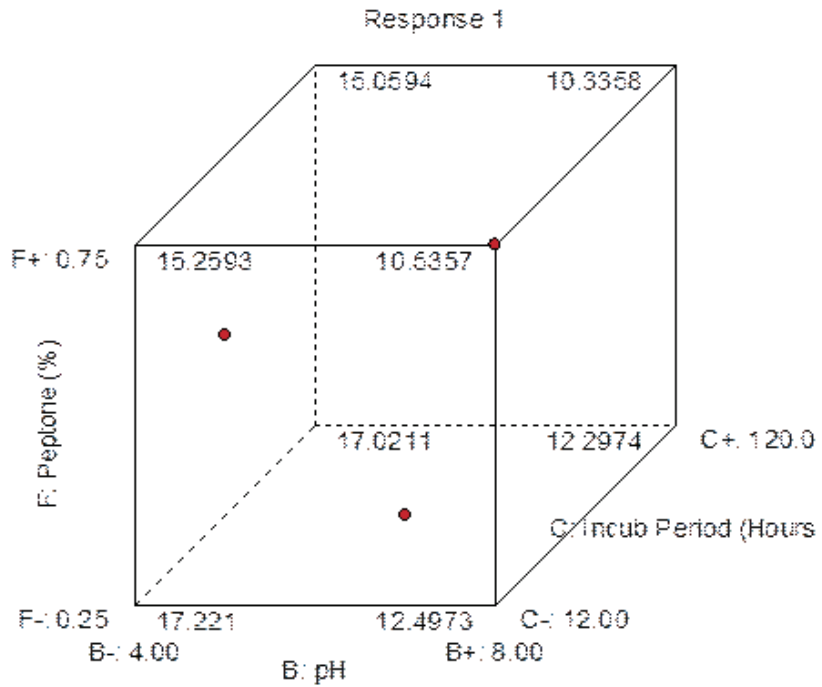


Fig 67 Effect of Incubation time (hrs), Peptone (%) and pH on Bacteriocin production (%)

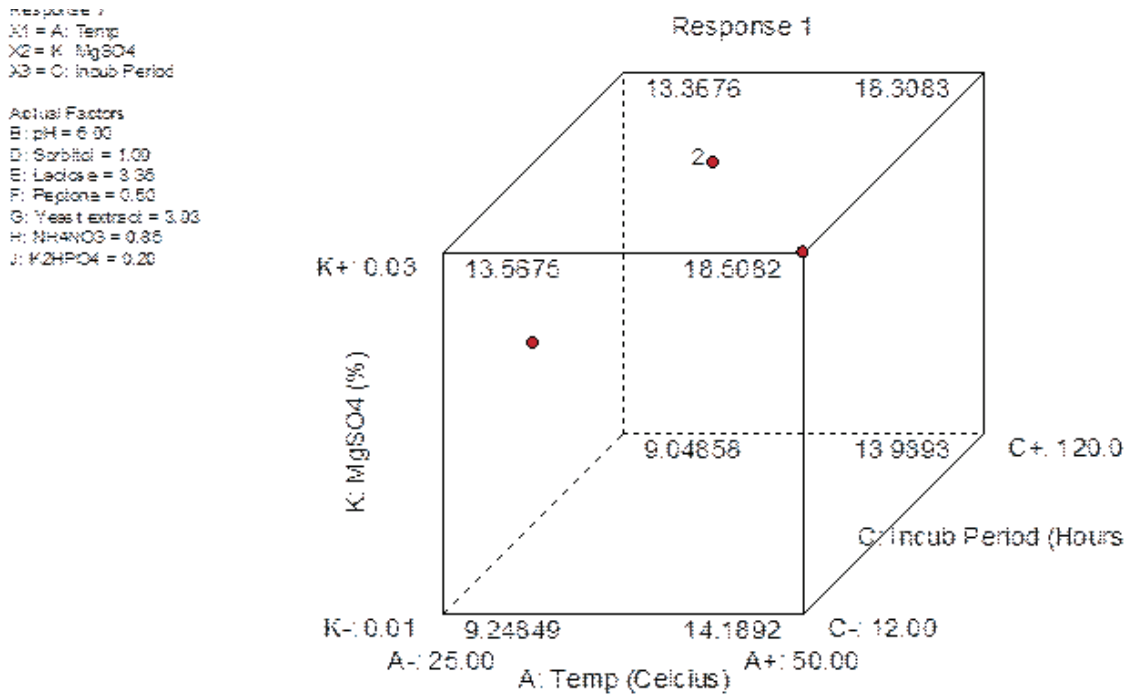


Fig 68 Effect of Incubation time (hrs), MgSO₄ (%) and pH on Bacteriocin production

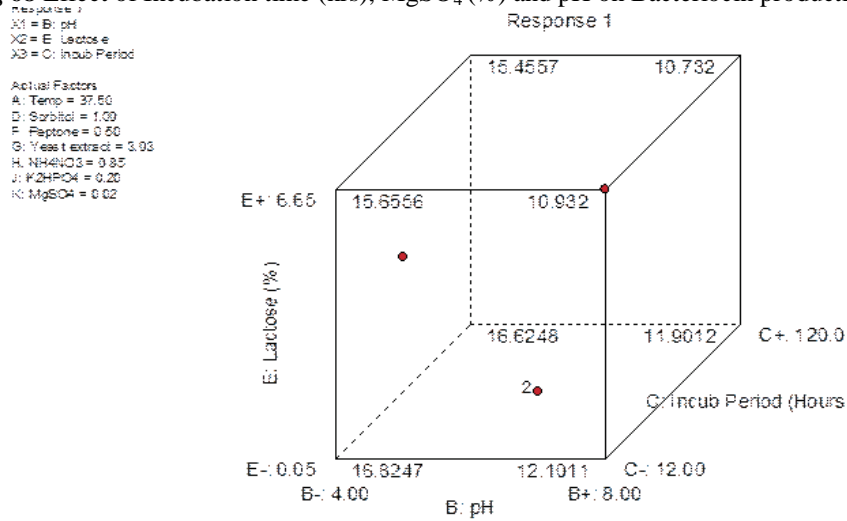


Fig 69 Effect of Incubation time (hrs), Lactose (%) and pH on Bacteriocin production (%)

Response 1
 X1 = B: pH
 X2 = G: Yeast extract
 X3 = C: Incub Period
 Actual Factors
 A: Temp = 37.50
 D: Sorbitol = 1.00
 E: Lactose = 3.36
 F: Peptone = 0.50
 H: NH_4NO_3 = 3.35
 J: K_2HPO_4 = 0.20
 K: MgSO_4 = 0.02

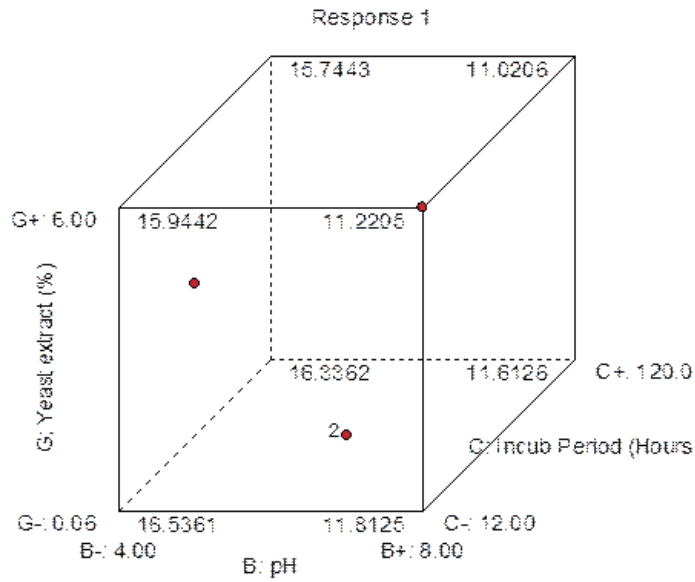


Fig 70 Effect of Incubation time (hrs), Yeast extract (%) and pH on Bacteriocin production (%)

Response 1
 X1 = B: pH
 X2 = H: NH_4NO_3
 X3 = C: Incub Period

Actual Factors
 A: Temp = 37.50
 D: Sorbitol = 1.00
 E: Lactose = 3.36
 F: Peptone = 0.50
 G: Yeast extract = 3.33
 J: K_2HPO_4 = 0.20
 K: MgSO_4 = 0.02

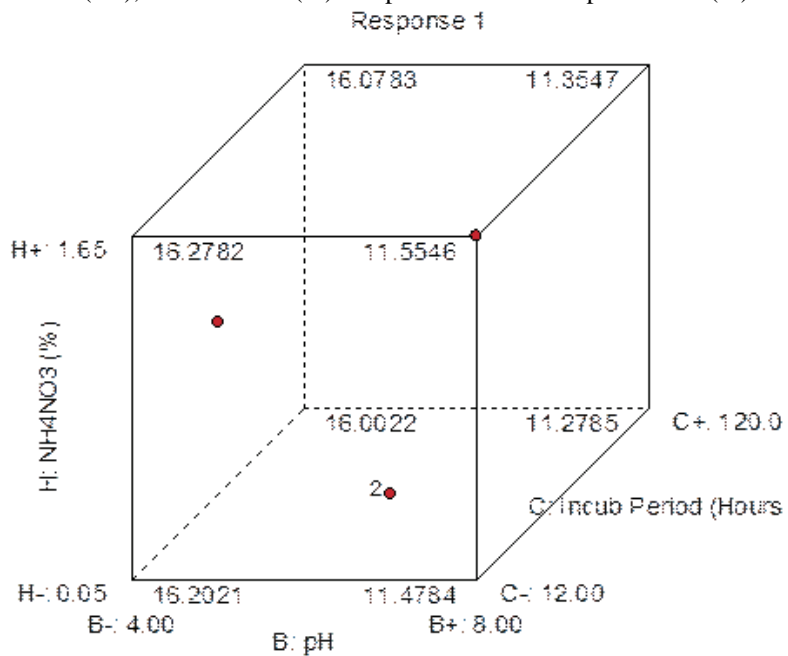


Fig 71 Effect of Incubation time (hrs), NH_4NO_3 (%) and pH on Bacteriocin production (%)

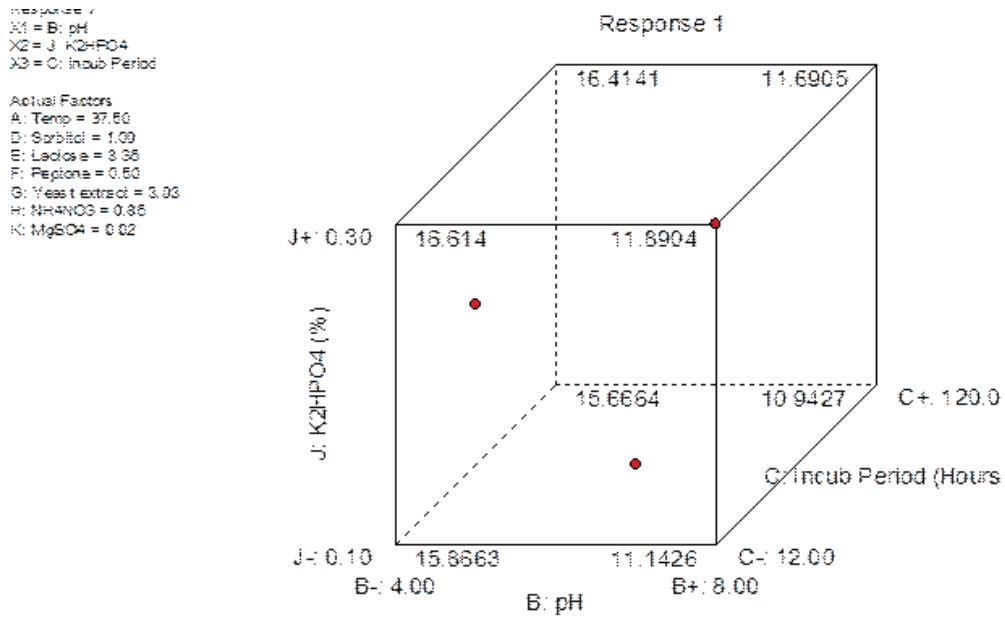


Fig 72 Effect of Incubation time (hrs), K₂HPO₄ (%) and pH on bacteriocin production (%)

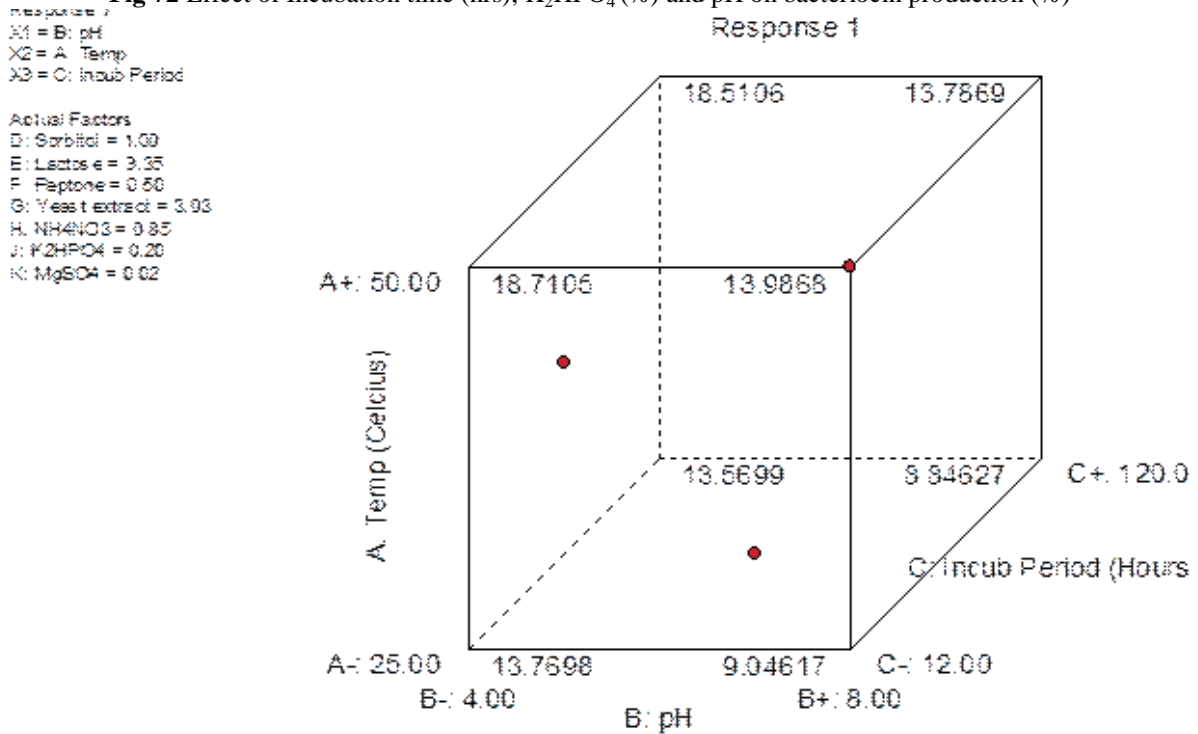


Fig 73 Effect of Incubation time (hrs), Incubation temperature (°C) and pH on Bacteriocin production (%)

Response 1
 X1 = B: pH
 X2 = D: Sorbitol
 X3 = A: Temp

Actual Factors
 C: Inoub. Period = 66.00
 E: Lactose = 3.35
 F: Peptone = 0.50
 G: Yeast extract = 3.03
 H: NH4NO3 = 0.85
 J: K2HPO4 = 0.20
 K: MgSO4 = 0.02

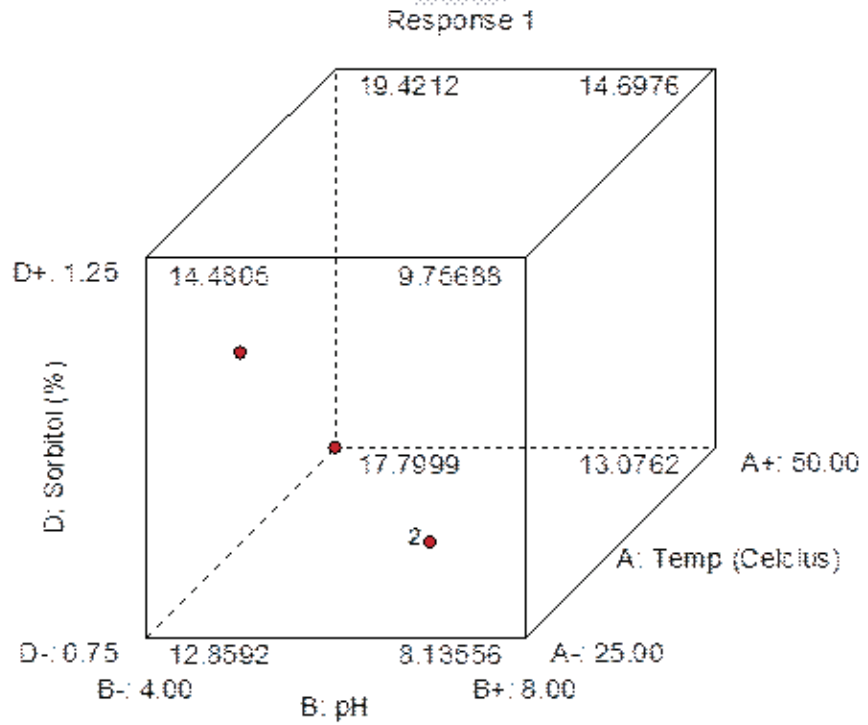


Fig 74 Effect of Incubation time (hrs), Sorbitol (%) and pH on Bacteriocin production (%)

Response 1
 X1 = B: pH
 X2 = E: Lactose
 X3 = A: Temp

Actual Factors
 C: Inoub. Period = 66.00
 D: Sorbitol = 1.00
 F: Peptone = 0.50
 G: Yeast extract = 3.03
 H: NH4NO3 = 0.85
 J: K2HPO4 = 0.20
 K: MgSO4 = 0.02

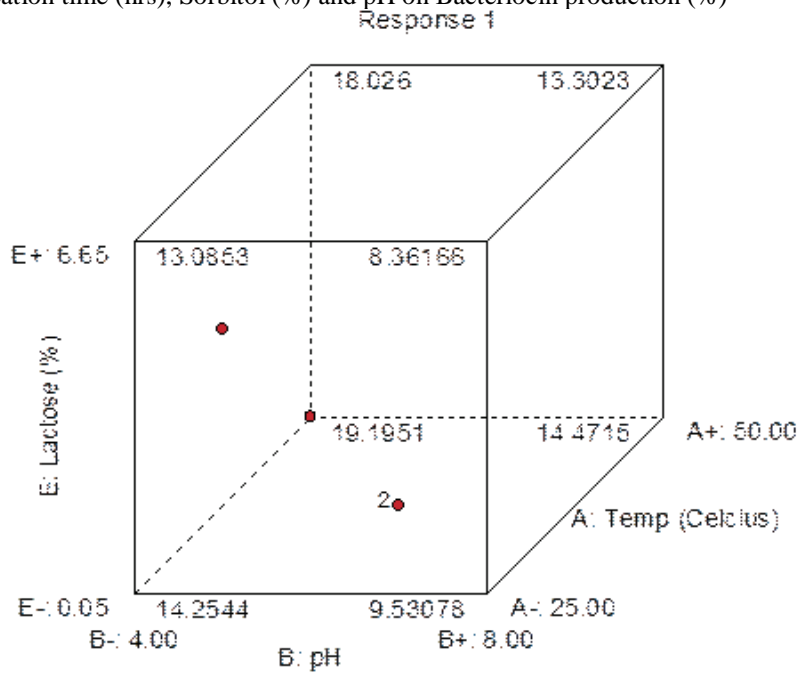


Fig 75 Effect of Incubation time (hrs), Lactose (%) and pH on Bacteriocin production (%)

Response 1
 X1 = B: pH
 X2 = G: Yeast extract
 X3 = A: Temp

Actual Factors
 C: Incub. Period = 66.00
 D: Sorbitol = 1.00
 E: Lactose = 3.36
 F: Peptone = 0.50
 H: NH_4NO_3 = 0.85
 J: K_2HPO_4 = 0.20
 K: MgSO_4 = 0.02

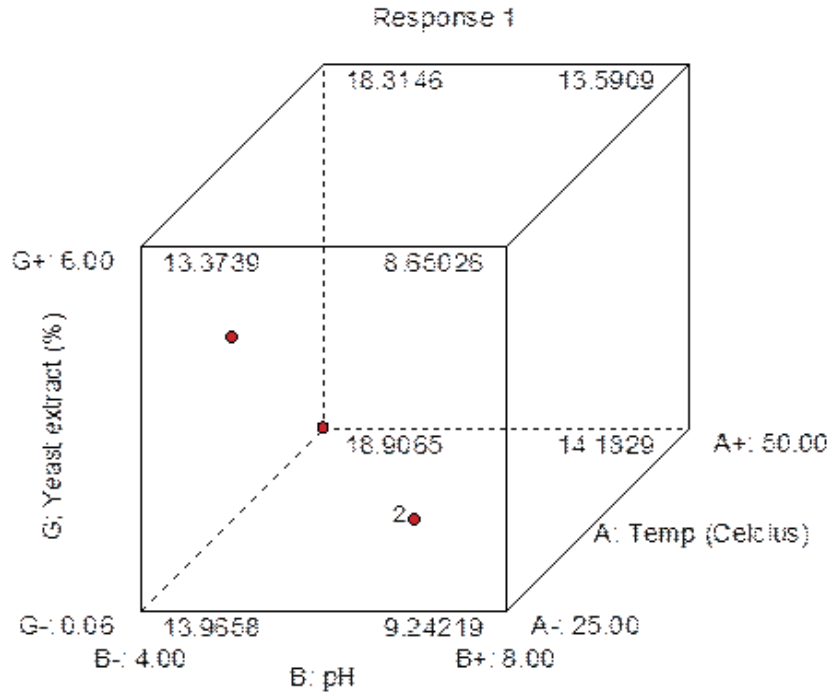


Fig 76 Effect of Incubation time (hrs), Yeast extract (%) and pH on Bacteriocin production (%)

Response 1
 X1 = B: pH
 X2 = H: NH_4NO_3
 X3 = A: Temp

Actual Factors
 C: Incub. Period = 66.00
 D: Sorbitol = 1.00
 E: Lactose = 3.36
 F: Peptone = 0.50
 G: Yeast extract = 3.03
 J: K_2HPO_4 = 0.20
 K: MgSO_4 = 0.02

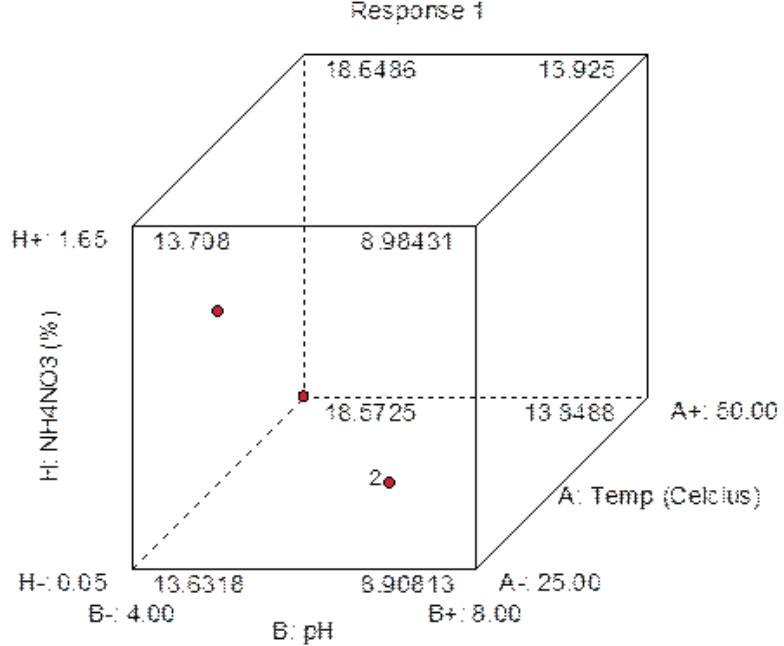


Fig 77 Effect of Incubation time (hrs), NH_4NO_3 (%) and pH on Bacteriocin production (%)

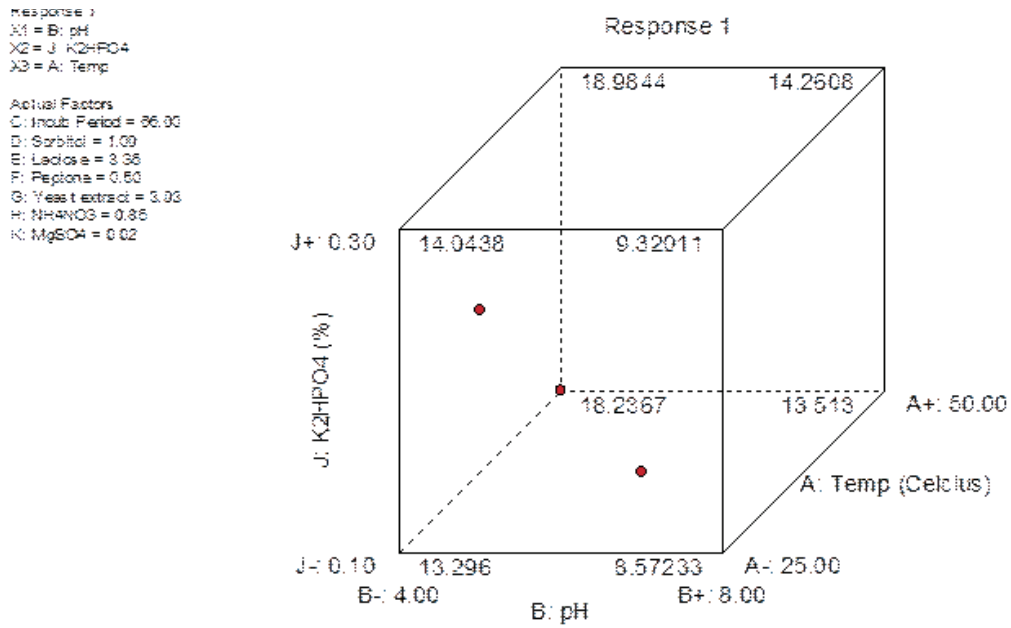


Fig 78 Effect of Incubation time (hrs), K₂HPO₄ (%) and pH on Bacteriocin production (%)

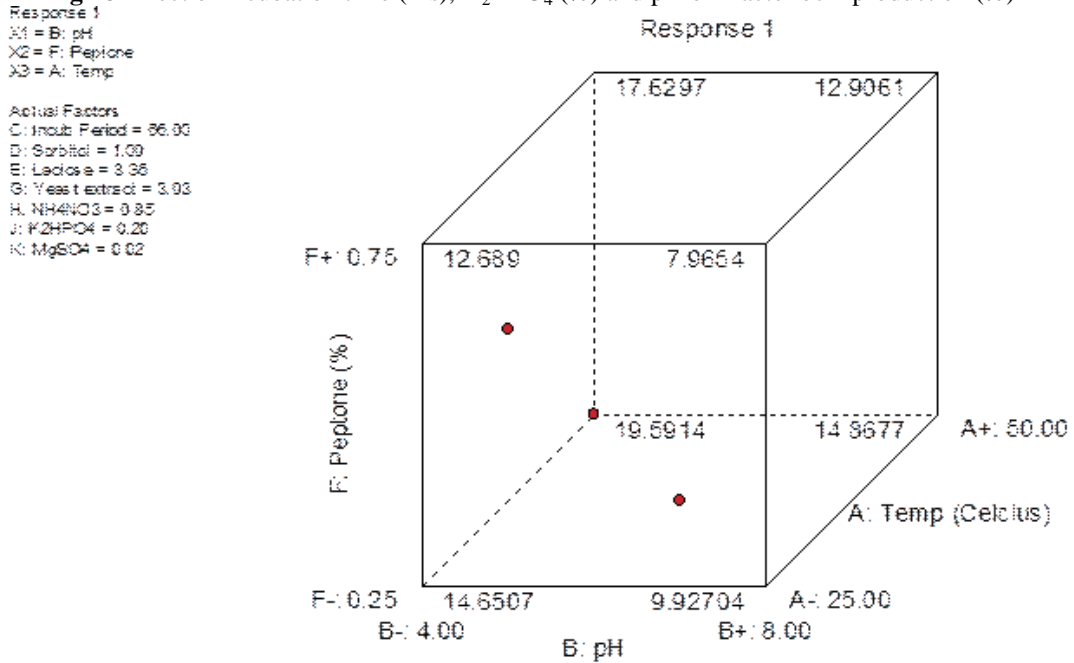


Fig 79 Effect of Incubation temperature (°C), Peptone (%) and pH on Bacteriocin production (%)

X1 = B: pH
 X2 = K: MgSO4
 X3 = A: Temp

 Actual Factors
 C: Inoc. Period = 36.00
 D: Sorbitol = 1.00
 E: Lactose = 3.35
 F: Peptone = 0.50
 G: Yeast extract = 3.00
 H: NH4NO3 = 0.85
 J: K2HPO4 = 0.20

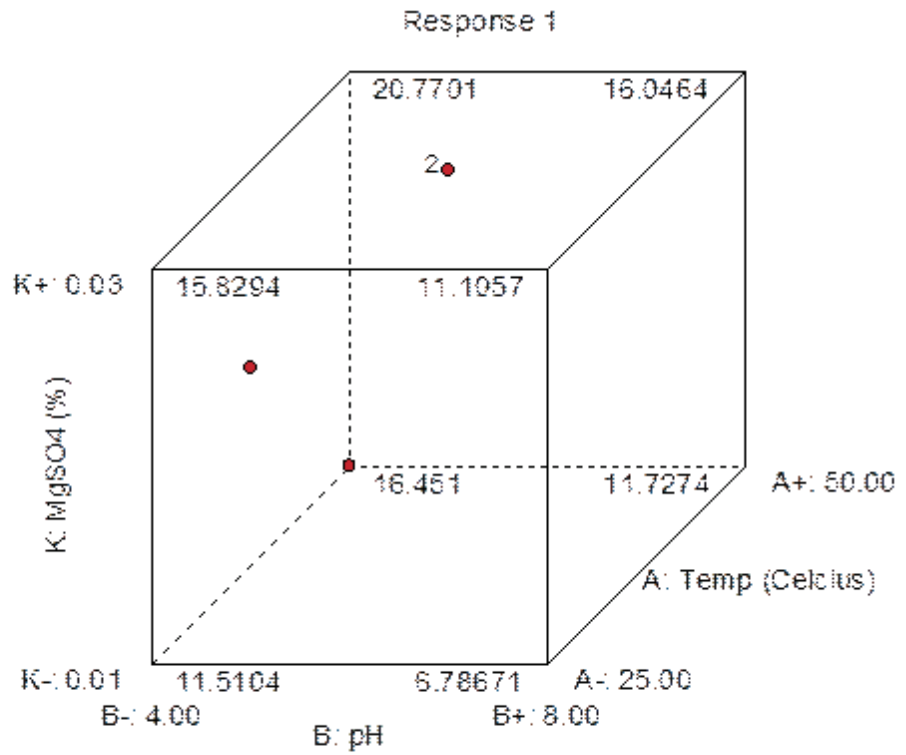
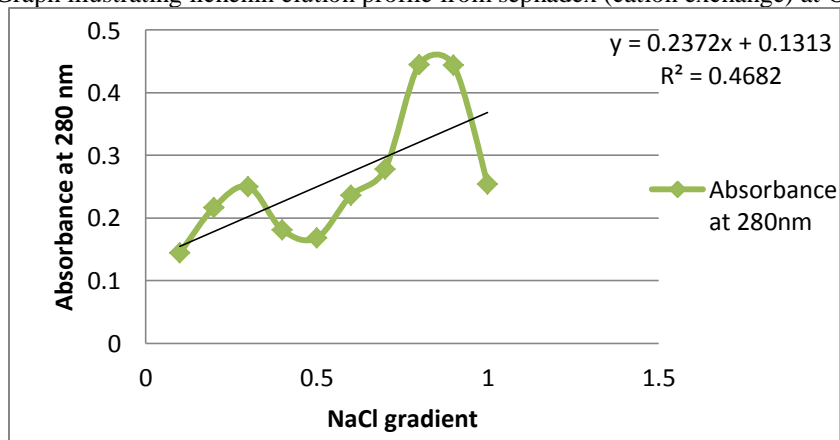


Fig 80 Effect of Incubation temperature (°C), MgSO₄ (%) and pH on Bacteriocin production (%)

Fig 81 Graph illustrating lichenin elution profile from sephadex (cation exchange) at O.D₂₈₀ nm



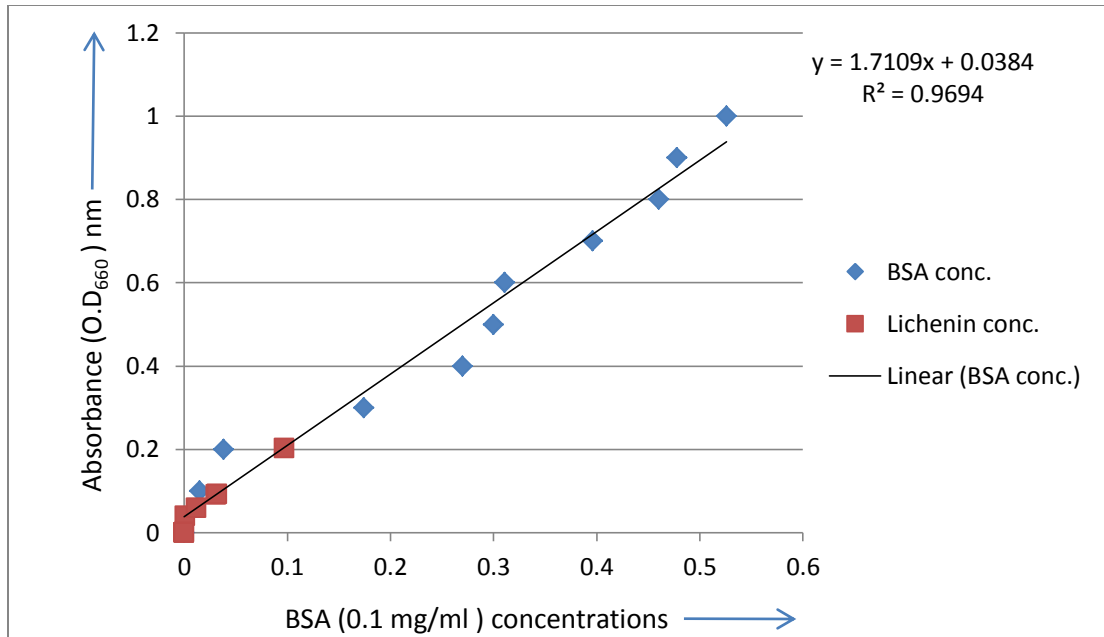


Fig 82 Graph showing Lichenin concentration with respect to BSA standard protein concentration

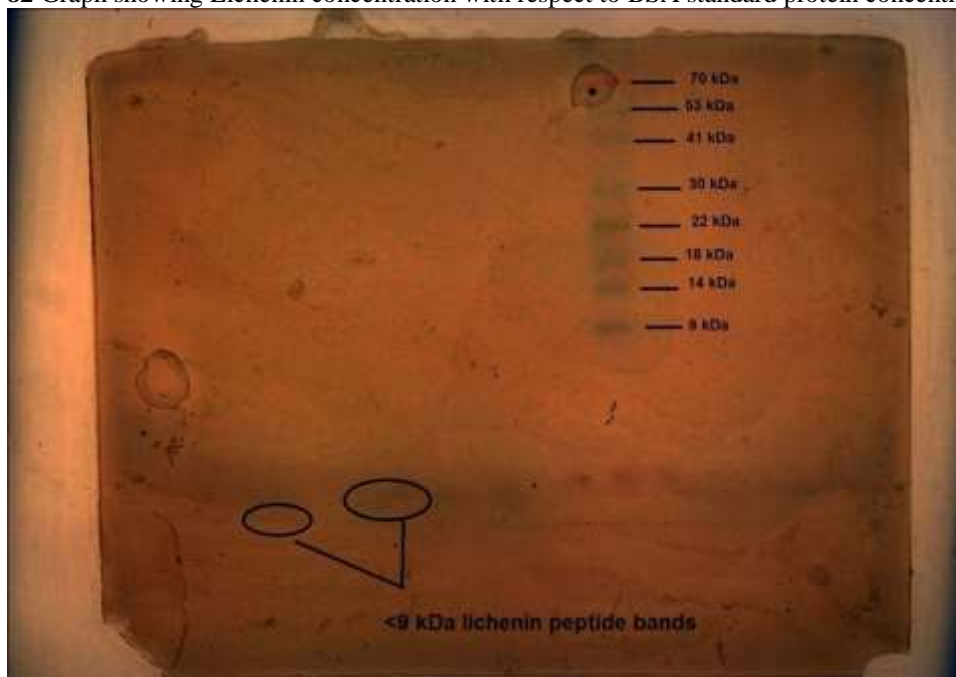


Fig 83 Lichenin (bacteriocin) molecular weight determination by SDS-PAGE revealed < 9 kDa

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