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# Screening, optimization of culture conditions and scale-up for production of the L-Glutaminase by novel isolated *Bacillus sps.* mutant endophyte using response surface methodology

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ABSTRACT

L-Glutaminase is known as the anti-cancer drug to treat anti-leukemic cancer besides its use in food industry for the acrylamide degradation of fried foods. In the present study, submerged fermentation was carried out for the production; statistical based experimental designs were employed to optimize the culture conditions and to maximize the L-Glutaminase activity from novel *Bacillus sps.*, mutant endophyte isolated from *Ocimum tenuiflorum*. Eleven process variables of the medium were examined for their significance using Plackett-Burman design on the production of L-Glutaminase i.e., Galactose, L-Glutamine, $Na_2HPO_4$, $KH_2PO_4$, NaCl, $MgSO_4.H_2O$, Yeast extract, pH, Temperature, RPM, Time were screened and it was observed that activity was 22.49 IU/ml. After Plackett-Burman design experiments, the activity was found to be 2.34 folds increase compared to one variable at a time method. Further, the production was optimized with Response Surface Methodology (RSM) using Central Composite Design (CCD) for significant variables i.e., Galactose (g/L), Time (h), Temperature (T°C) and pH obtained from Plackett-Burman design and the activity was found to be 24.585 IU/ml i.e., 2.56 fold increment in the production compared to unoptimized medium. Experimentation revealed that the isolated novel *Bacillus sps.*, mutants as the potential bacterial source for L-Glutaminase production.

## 1. Introduction

The second most important worldwide disease observed was Cancer (Stewart and Wild, 2014, Society, 2008). Malignant and benign tumours have been reported so far based on the movement of cells within the body (Siegel et al., 2017; Murray and Lopez, 1997b; Cooper and Hausman, 2000). The benign tumour movement is restricted to a particular organ and can be removed by surgery (Murray and Lopez, 1997a; Fidler, 2003). Malignant tumours will spread across the body through blood and lymph (Carmeliet and Jain, 2000). Tobacco, Alcohol, smoking, obesity, ionising radiations, genetic disorders, pathogens and pollutants are some of the causes of cancer (Chambers et al., 2002; Peto, 2001; Zur Hausen, 2002). 15% of cancers are due to viral infections (papilloma virus Epstein bar virus, HIV) (Schottenfeld and Fraumeni, 2006; Parkin, 2006; De Martel et al., 2012). Different type of cancers has been reported-cervical cancer, breast cancer, blood cancer-Acute Lymphocytic Leukemia (ALL), lymphoma, colon cancer etc. depending on the organ they infected (Fitzmaurice et al., 2017; Chua et al., 2017). The most common cancer among women is breast cancer whereas prostate cancer is the common cancer seen in men (Siegel et al., 2017). Environment also plays important role. Stomach cancer is the most common cancer observed in Japan, and less observed in US. Surgery, laser therapy, hormone therapy, radiation therapy, chemotherapy, immunotherapy, biological therapy by small molecules and enzyme therapy are some of the treatments available to diagnose cancer (Forner et al., 2016). Benign tumours can be treated by immunotherapy whereas small molecules will affect the reproductive cells. Breast cancer was the earliest reported cancer by Edwin Smith Papyrus (Lukong, 2017; Denton, 2017). Other types of cancers seen in children are brain tumours and lymphomas.

Based on the origin cancer is categorized into 4 main types:

1. Solid tumours formed across the body are classified as Carcinomas.
   Ex: Skin cancer, Prostate cancer, Lung cancer, etc.
2. Cancer related to the tissue system that connects the organs is termed as Sarcoma
   Ex: Bone cancer

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3. Blood cancer is called leukemia. Based on the type of blood cells many leukemic cancers have been reported.
   Ex: Acute Lymphocytic Leukemia, Chronic Lymphocytic Leukemia, etc.
4. Lymphatic cancers are called lymphomas. Ex: Hodgkin lymphoma.

Fatigue, weight loss, fever, cough, pain are some of the symptoms reported in cancer patients. Colonoscopy, Mammography, Pap test are some of the screening test for detecting cancer (Emery et al., 2014). The stage and type of cancer can be identified by Biopsy. Biopsy was performed to diagnose cancer (Scheel and Holtedahl, 2015).

The most observed cancer among children is Leukemia - Acute Lymphoblastic leukemia, brain tumours and lymphomas (Heim and Mitelman, 2015). Leukemia is the blood cancer which starts in the blood stem cells or bone marrow (Miller et al., 2016). Lymphoid stem cells and myeloid stem cells are formed by the division of blood stem cells. DNA damage of immature blood cells leads to leukemia. Blood stem cells develop into blast cells. Normally, the dead blood cells are replaced with new cells, whereas the mutated blast cells grow continuously and aggregate around the normal cells, restricting their movement and functions (Gill et al., 2014). Leukemia was caused by

a) Genetic factors
b) Smoking
c) Blood disorders
d) Radiation exposure
e) Benzene exposure.

Fever, bleeding, infections are the observed symptoms which can be diagnosed by the bone marrow biopsy. Lymphocytic leukemia is the cancer of lymphoid stem cells and myelogenous leukemia is the cancer of myeloid stem cells. Acute and chronic are the two types of cancer observed in patients (Chang et al., 2017). Acute leukemia is quick and starts suddenly, whereas chronic leukemia develops slowly with the time (Jaffe, 2001).

Based on their quick growth leukemia is classified into 4 types:

a) Acute lymphocytic leukemia (ALL)
b) Acute myelogenous leukemia (AML)
c) Chronic lymphocytic leukemia (CLL)
d) Chronic myelogenous leukemia (CML)

About 6000 cases, who are suffering with ALL has been treated annually. Stem cell therapy, radiation therapy, biological therapy used in combination or individually to treat ALL (Furnari et al., 2007). In this study enzymatic treatment by L-Glutaminase was studied. In recent days L-Glutaminase has gained importance, because of its commercial importance in both therapeutic and food industry (Balagurunathan et al., 2010). L-Glutaminase is an amidohydrolase which is used in combination with chemotherapy to treat cancer (Nandakumar et al., 2003). This enzyme will break the peptide bonds in the presence of water to form glutamic acid (Kashyap et al., 2002). As L-Glutamine acts as the energy source for the growth of cancer cells, an external supply of L-Glutaminase leads to death of cancer cells due to energy starvation (Sabu et al., 2000; El-Sayed, 2009). The antiretroviral nature of this enzyme is used to treat HIV (Roberts and Mcgregor, 1991; Erdmann et al., 2007).

In food industry, L-Glutaminase is used to reduce the acrylamide formation due to high temperatures (Nandakumar et al., 2003). The glutamic acid formed was used as flavour enhancer of fermented foods (Zhao et al., 2016; Kitamoto, 2002). It plays an important role in nitrogen metabolism and ammonia detoxification (Binod et al., 2017). It is also used to synthesize theanine (L-glutamyl ethyl amide) in pharma industry (Zhang et al., 2010). The biosensor application of L-Glutaminase is used to monitor the L-Glutamine levels of hybridoma cells (Mehrotra, 2016; Moser et al., 1995).

L-Glutaminase is ubiquitously produced by plants, *E. coli, Pseudomonas, Bacillus sps*, and others. Two types of Glutaminases have been reported in *E. coli* based on their cellular nature. Kidney type L-Glutaminase is membrane bound whereas liver type L-Glutaminase is extracellular (Vijayan et al., 2017). Both types have applications in medicinal industry to treat cancer.

In the current research, L-Glutaminase production was reported from UV treated *Bacillus sps*. Strain improvement of the microbe was performed by keeping the *in house Bacillus sps* under UV for 3 min followed by treatment with Ethidium bromide. Strain improvement resulted in the increased production which is having high specificity towards L-Glutamine. Production studies were carried out using M-9 media. At first, OVAT (one variable at a time) studies were performed to enhance the enzyme activity. In OVAT studies, the interactions between the variables cannot be studied. Hence, statistical optimization methodology studies were employed to identify the factors influencing on the enzyme activity, as well as to study how the factors are interacting for maximization of L-Glutaminase production. Plackett-Burman studies were employed for 11 components (both media and physical components) to identify the significant variables whose probability is $< 0.05$. The interactions of the significant variables were studied by employing central composite design.

## 2. Materials and methods

### 2.1. Organism

In the current study, *in house* mutated *Bacillus sps* an endophyte isolated from *Ocimum tenuiflorum* was chosen for L-Glutaminase production.

### 2.2. Strain improvement

Improving the metabolic properties of a strain having specific importance by using science and technology is called strain improvement. *Bacillus sps* was chosen in this study, because of high rate of multiplication with genetic stability, economically feasible substrates can be used for production with less time (Parekh et al., 2000). Strain improvement can be done by:

a) Mutation: Sudden heritable change caused by mutagen is called induced mutation. Mutagens can be either physical or chemical.
b) Recombination

In this study, strain improvement was done by UV treatment. UV treatment induces pyrimidine dimerization showing medium effect, followed by treatment with Ethidium bromide. In order to find whether the mutant is able to produce L-Glutaminase, qualitative estimation studies were performed.

### 2.3. Rapid plate assay

#### 2.3.1. Qualitative estimation

Qualitative estimation of L-Glutaminase was studied by rapid plate assay (Balagurunathan et al., 2010). Rapid Plate assay was performed using M-9 media with phenol red indicator. M-9 media was autoclaved and the pH of media was adjusted to 7.0. At acidic/neutral pH, the colour of M-9 media will be yellow. If the mutant is able to produce L-Glutaminase, the media will turn to pink (increase in alkalinity due to the breakdown of L-Glutamine). Control plates were not inoculated with *Bacillus sps.* The plates were incubated in static incubator at 37 °C for 48 h and the pink zone diameter around the microbe was analysed.

#### 2.3.2. Quantitative estimation

Quantitative estimation of L-Glutaminase was carried out by Nesslerization (Campbell et al., 1967). This assay estimates the amount

of ammonia released. One unit of enzyme degrades one unit of L-Asparagine. After the addition of Nessler's reagent to the enzyme mixture, a colour change from clear yellow to brown precipitate was observed (Wade et al., 1971).

### 2.4. OVAT optimization

#### 2.4.1. Pre-inoculum production

The organism was inoculated into 100 ml of autoclaved M-9 media and kept for incubation in orbital shaker for 24 h to revive the microbe. 1 ml from the revived culture was added to production medium and optimization studies were performed. Blind optimization by varying one variable at a time was performed to enhance the production of L-Glutaminase in a traditional way. The effect of Time, Temperature, pH, RPM, Carbon, Nitrogen and L-Glutamine on L-Glutaminase was studied. All the experiments were carried out in triplicates.

#### 2.4.2. Physical variable optimization

*2.4.2.1. Optimization of time.* Submerged fermentation was carried out by incubating the inoculated production media with mutated and wild strain for 40 h separately at 37 °C, 120 RPM. Samples were withdrawn for every 8 h and enzyme activity was estimated by Nesslerization. Uninoculated media will be used as control to measure the optical density (OD) at 426 nm.

*2.4.2.2. Optimization of pH.* pH of the media will show influence on the growth of the microbes. pH-6.0 to 7.0 favours the growth of microorganisms. Five different (100 ml) M-9 media flasks were autoclaved and pH was adjusted from 5.0 to 9.0 with HCL and 2 N NaOH using pH meter which was equilibrated with standards. Microorganism was inoculated and the sample was withdrawn at regular intervals of 8 h and activity was estimated.

*2.4.2.3. Optimization of temperature.* Temperature will influence the enzyme activity and production. At high temperature the enzyme gets deactivated vice versa it will be in inactive form at low temperatures. Some microorganism will produce the required by-products at low temperatures and some at high temperatures. Inoculated media was incubated at different temperature ranges from 20–40 °C. The maximum enzyme activity obtained at 48 h was calculated and the obtained best temperature was fixed and further optimization studies were carried.

*2.4.2.4. Optimization of RPM.* RPM is useful to disperse the media components uniformly in the media. Low RPM doesn't mix media components whereas higher RPM damages the obtained products and bacterial cells. 100–150 RPM was set in three different shakers and optimization experimentation was carried out. The optimum ranges of physical variables were fixed from physical parameter optimization.

#### 2.4.3. Media optimization

Different types of media used for the same microbe favours production of different by-products due to different metabolic pathways. The concentration of the variables will also influence the production. Higher and lower concentration of the components will inhibit the production. The media components optimized were as follows:

*2.4.3.1. Carbon optimization.* Monosaccharides, Disaccharides and Polysaccharides-Glucose, Maltose, Galactose, Starch, Cellulose, Tri Sodium Citrate (TSC), Xylose, Lactose are the 8 carbon sources used in this study. Each carbon source was substituted and its effect on the enzyme production was studied. The best carbon source which is able to produce increased production was taken and its concentration was varied to find the optimum concentration required by the microbe.

*2.4.3.2. Nitrogen source optimization.* Nitrogen sources help the microorganisms to synthesize proteins required for the growth. The Nitrogen sources supplemented in this study were Potassium nitrate, Yeast extract, Ammonium sulphate, Urea and L-Glutamine. L-Glutamine acts as both inducer and nitrogen supplement. Best nitrogen source obtained concentration was varied to find the inhibition effects and optimum concentration required.

*2.4.3.3. Inducer optimization.* L-Glutamine acts as Inducer for the production of L-Glutaminase. L-Glutamine is the non-essential neutral polar amino acid involved in protein synthesis. Increased or decreased concentration will affect the production. The concentration was varied from 1 g/L to 9 g/L to find the optimum concentration required for maximum production of L-Glutaminase.

### 2.5. Statistical optimization

Classical optimization methods consume more time. Hence, statistical optimization techniques were employed to study the non-linear biological reactions by employing mathematical model to generate appropriate results (Bezerra et al., 2008; Candioti et al., 2014). In this study, Plackett-Burman and RSM methods were employed to maximize the production of L-Glutaminase from *Bacillus sps.* Design Expert software was used to perform statistical optimization experiments (Hox et al., 2017).

#### 2.5.1. Plackett-Burman optimization

The interactions between the variables and their influence (Positive or Negative effect) on the enzyme production was studied by using Plackett-Burman design (Reddy et al., 2008). It also helps to minimize the error probability. It generates random experiments based on Plackett-Burman structures. It is a two-level factorial design where the lower and upper limits of the variables were given to generate experiments (Montgomery, 2017). 13 runs were generated using 11 variables with one midpoint. Increase in the number of midpoints will minimize the error. The 11 variables chosen were Galactose, L-Glutamine, $Na_2HPO_4$, $KH_2PO_4$, NaCl, $MgSO_4{\cdot}7H_2O$, Yeast extract, pH, Temperature, RPM and Time. Nesslerization was performed for the experiments generated from the design table and the L-Glutaminase activity was estimated. Regression analysis was performed and significant variables identified were further optimized by using Response Surface Methodology designs (Asfaram et al., 2015).

#### 2.5.2. RSM optimization

RSM helps to quantify the interactions between the variables to find a desirable solution. Maximization or minimization of the desired solution can be obtained by employing RSM methods (Anderson and Whitcomb, 2016). The most popular, two level factorial face centred Central Composite Design (CCD) was employed to optimize the production. Quadratic equation co-efficients can be quantified from CCD (Maran et al., 2017; Pola et al., 2018a). Each factor was varied at 5 levels (by using factorial (plus and minus), star (plus and minus), and central points). The significant variables obtained from the Plackett-Burman design – Time, Temperature, pH and Galactose were chosen to further optimize. A total of 30 runs were generated for which *in vitro* experimentation was conducted and the results were analysed by regression analysis. 3D surface plots will be generated between any 2 factors vs. L-Glutaminase by keeping the other factors constant (Baş and Boyaci, 2007; Pola et al., 2018b).

## 3. Results

### 3.1. Rapid plate assay

M-9 media (with phenol red indicator) plated with endophytes (Fig. 1). The endophyte which is producing L-Glutaminase will turn the plate into pink. Un inoculated plate serves as control. The endophyte which is not able to produce L-Glutaminase is shown in Fig. 1B. The mutated endophyte producing L-Glutaminase is represented in Fig. 1C.

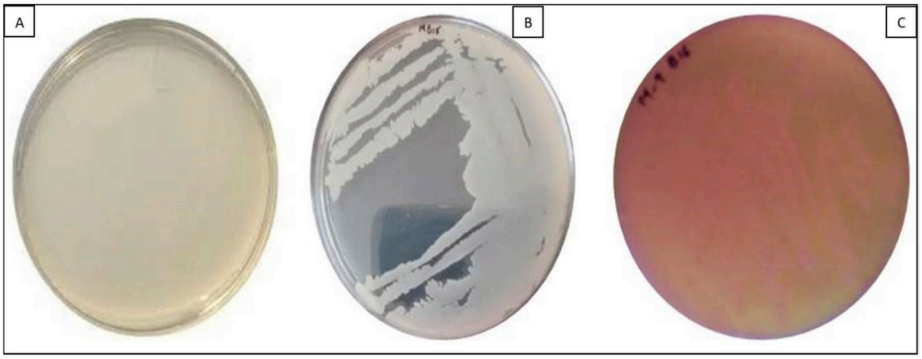


**Fig. 1.** Rapid Plate Assay- A) Control, B) Endophyte not producing L-Glutaminase, C) Endophyte producing L-Glutaminase.

### 3.2. OVAT optimization

#### 3.2.1. Influence of time

At 25 °C, 120 RPM the inoculated media was incubated for 40 h. For every 8 h the sample was taken and centrifuged for 15 min and filtered. As the enzyme produced was extra-cellular, the supernatant will be used as crude enzyme for the estimation of L-Glutaminase. The enzyme activities obtained from wild type and mutant *Bacillus sps*. were shown in the below Table 1. The maximum enzyme activity was estimated at 32 h. 5.73 and 9.59 IU/ml were the maximum enzyme activities obtained at 32 h for wild type and mutant sps. From this data, we can infer that the mutant is able to produce higher amounts of L-Glutaminase (Fig. 2).

**Table 1**
Influence of Time (h) on L-Glutaminase from wild type and mutant type.

| Time h | L-Glutaminase Activity IU/ml | |
|---|---|---|
| | Wild Type | Mutant |
| 0 | 0.042 | 0.049 |
| 8 | 0.39 | 0.63 |
| 16 | 1.82 | 2.4 |
| 24 | 3.08 | 4.75 |
| 32 | 5.73 | 9.59 |
| 40 | 4.185 | 5.583 |

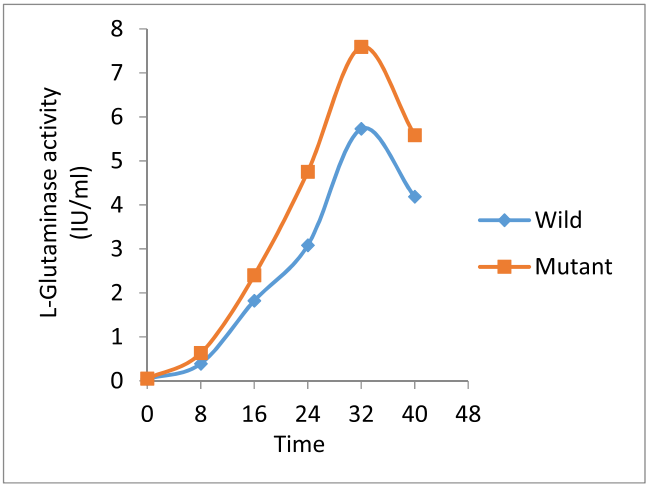


**Fig. 2.** Influence of Time (h) on L-Glutaminase from Wild type and Mutant type.

#### 3.2.2. Influence of carbon sources

After the addition of different carbon sources individually into 8 different production media (Table 2). It was observed that the Galactose gave the highest yield (Fig. 3) and Cellulose gave the lowest yield. The optimal concentration of Galactose for enzyme synthesis was determined to be 1.5 g/L (Table 3) (Fig. 4).

#### 3.2.3. Influence of nitrogen sources

Substitution of different nitrogen sources (Table 4) into the production media influenced the production of L-Glutaminase (Fig. 5). Yeast extract (YE) (Table 5) has shown the highest yield and optimum concentration was found to be 2 g/L (Fig. 6).

#### 3.2.4. Influence of pH

The media was subjected to different pH (5–9) (Table 6). The maximum enzyme production was found at pH 7 and it was observed as 16.425 IU/ml. Increase or decrease in the pH of the medium resulted in decreased enzyme production (Fig. 7).

#### 3.2.5. Influence of RPM

Variation in RPM (Table 7) in either way resulted in decrease of L-Glutaminase production. The optimum RPM was found to be 125 (Fig. 8).

#### 3.2.6. Influence of temperature

The inoculated media was subjected to five different temperatures 20 °C, 25 °C, 30 °C, 35 °C, 40 °C. When the temperatures are changed above or below, the enzyme activity was found to be lowered due to production of some other products. Temperature 30 °C was found to be optimum (Fig. 9) and the enzyme activity was found to be 20.859 IU/ml. The enzyme activities obtained at 32 h for different temperatures were shown in the below Table 8.

#### 3.2.7. Influence of inducer

L-Glutamine acts as inducer, helps to trigger the microbe to synthesize L-Glutaminase. Low and high concentrations of L-Glutamine in the media resulted low production of L-Glutaminase. L-Glutamine concentration was varied from 1 g/L to 9 g/L (Table 9). Highest activity observed was 20.85 IU/ml at 6 g/L concentration of L-Glutamine (Fig. 10).

The potential of isolated novel mutant strain for L-Glutaminase production was analysed under submerged fermentation with different process variables and medium constituents. Maximum production was noticed at pH 7, 30 °C, 125 RPM, Galactose (1.5 g/L) L-Glutamine (6 g/L) and Yeast extract (2 g/L).

### 3.3. Statistical optimization

#### 3.3.1. Plackett-Burman design

13 experiments obtained from the design table (Table 10) was

**Table 2**
Influence of different carbon sources (2 g/L).

| Time h | L-Glutaminase activity IU/ml | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Glucose | Maltose | Galactose | Starch | Cellulose | TSC | Xylose | Lactose |
| 0 | 0.049 | 0.049 | 0.045 | 0.049 | 0.049 | 0.049 | 0.049 | 0.049 |
| 8 | 0.631 | 0.229 | 0.062 | 1.066 | 0.731 | 0.229 | 0.564 | 0.731 |
| 16 | 2.404 | 1.4 | 1.702 | 5.081 | 1.066 | 0.463 | 4.295 | 5.951 |
| 24 | 4.747 | 1.752 | 8.16 | 6.922 | 3.074 | 2.772 | 6.202 | 3.241 |
| 32 | 9.591 | 3.274 | 10.573 | 4.077 | 2.137 | 1.049 | 1.367 | 1.902 |
| 40 | 5.583 | 1.25 | 1.986 | 3.408 | 0.731 | 0.731 | 0.112 | 1.568 |

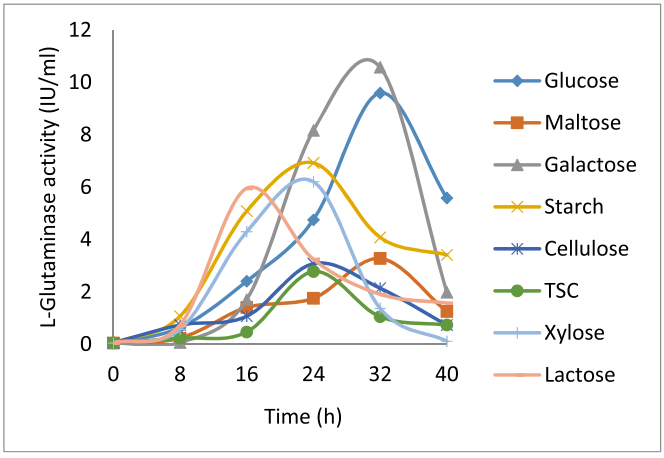


**Fig. 3.** Influence of different carbon sources (2 g/L).

**Table 3**
Influence of different concentration of Galactose.

| Time h | L-Glutaminase Activity IU/ml | | | | |
|---|---|---|---|---|---|
| | Galactose 0.5 g/L | Galactose 1 g/L | Galactose 1.5 g/L | Galactose 2 g/L | Galactose 2.5 g/L |
| 0 | 0.045 | 0.045 | 0.045 | 0.045 | 0.045 |
| 8 | 0.397 | 1.802 | 0.564 | 0.062 | 0.815 |
| 16 | 0.815 | 2.086 | 9.431 | 1.702 | 1.116 |
| 24 | 5.182 | 4.529 | 13.782 | 8.16 | 4.663 |
| 32 | 9.833 | 10.362 | 15.137 | 10.573 | 8.913 |
| 40 | 9.348 | 0.012 | 9.348 | 1.986 | 4.011 |

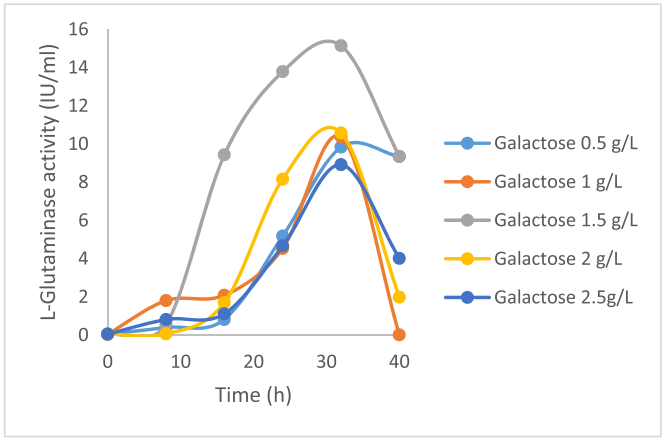


**Fig. 4.** Influence of different concentration of Galactose.

**Table 4**
Influence of different nitrogen sources (2 g/L).

| Time h | L-Glutaminase Activity IU/ml | | | | |
|---|---|---|---|---|---|
| | Yeast extract | Ammonium sulphate | Potassium nitrate | Urea | L-Glutamine |
| 0 | 0.045 | 0.03 | 0.02 | 0.04 | 0.01 |
| 8 | 9.063 | 0.330 | 0.229 | 3.525 | 1.601 |
| 16 | 9.482 | 0.798 | 0.564 | 3.776 | 2.873 |
| 24 | 10.101 | 1.233 | 0.731 | 4.261 | 3.241 |
| 32 | 15.137 | 1.400 | 2.756 | 6.738 | 5.382 |
| 40 | 7.223 | 0.229 | 1.267 | 5.416 | 0.223 |

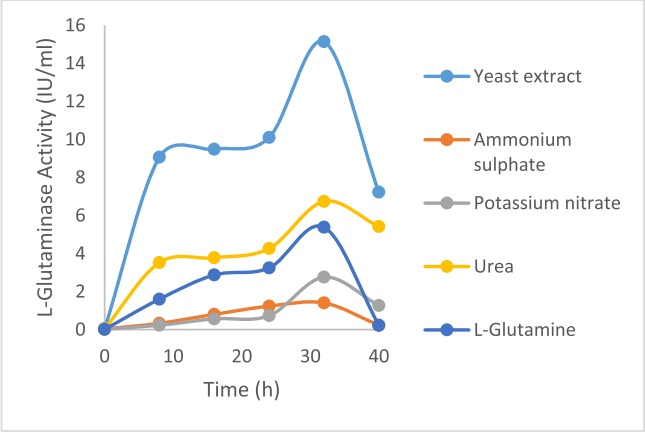


**Fig. 5.** Influence of different nitrogen sources (2 g/L).

**Table 5**
Influence of different yeast extract concentration.

| Time h | L-Glutaminase Activity IU/ml | | | | |
|---|---|---|---|---|---|
| | Yeast extract 0.5 g/L | Yeast extract 1 g/L | Yeast extract 1.5 g/L | Yeast extract 2 g/L | Yeast extract 2.5 g/L |
| 0 | 0.045 | 0.045 | 0.045 | 0.045 | 0.045 |
| 8 | 0.397 | 1.802 | 0.564 | 9.063 | 0.815 |
| 16 | 0.815 | 2.086 | 9.431 | 9.482 | 1.116 |
| 24 | 5.182 | 4.529 | 9.782 | 10.101 | 4.663 |
| 32 | 9.833 | 4.362 | 12.137 | 15.137 | 8.913 |
| 40 | 9.348 | 0.012 | 9.348 | 7.223 | 4.011 |

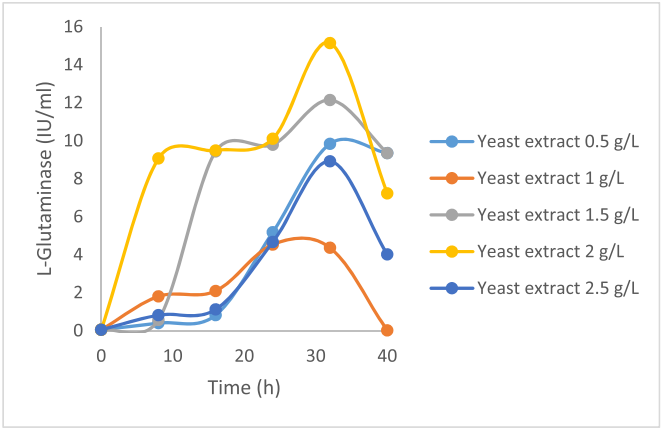


**Fig. 6.** Influence of different yeast extract concentration.

**Table 6**
Influence of different pH.

| pH | L-Glutaminase Activity IU/ml |
|---|---|
| 5 | 8.11 |
| 6 | 11.54 |
| 7 | 16.425 |
| 8 | 15.02 |
| 9 | 14.284 |

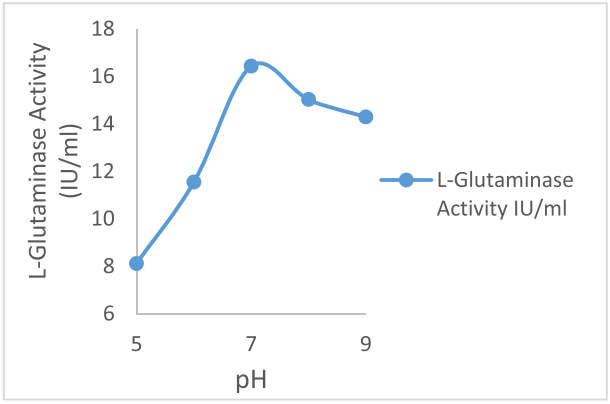


**Fig. 7.** Influence of different pH.

**Table 7**
Influence of different RPM.

| RPM | L-Glutaminase Activity IU/ml |
|---|---|
| 100 | 10.67 |
| 125 | 20.524 |
| 150 | 11.205 |

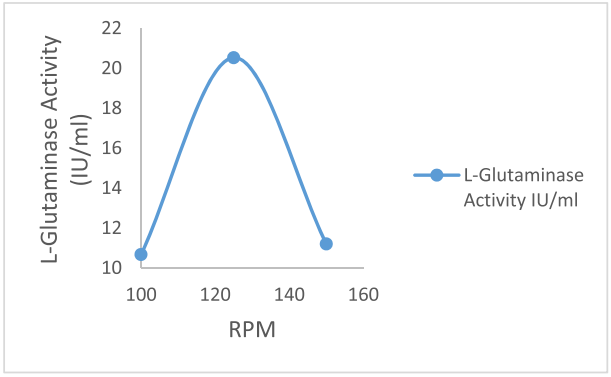


**Fig. 8.** Influence of RPM.

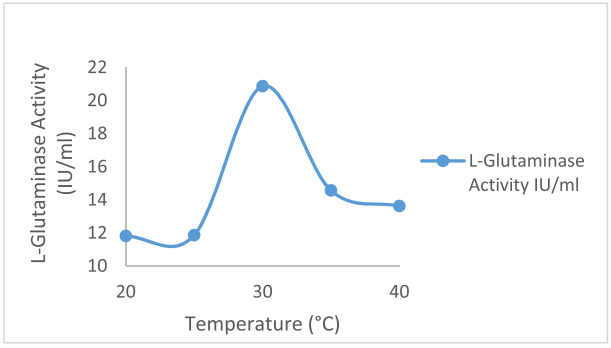


**Fig. 9.** Influence of different temperatures.

**Table 8**
Influence of different Temperatures.

| Temperature °C | L-Glutaminase Activity IU/ml |
|---|---|
| 20 | 11.807 |
| 25 | 11.858 |
| 30 | 20.859 |
| 35 | 14.561 |
| 40 | 13.614 |

**Table 9**
Influence of inducer.

| Time h | L-Glutaminase Activity IU/ml | | | |
|---|---|---|---|---|
| | L-Glutamine 1 g/L | L-Glutamine 3 g/L | L-Glutamine 6 g/L | L-Glutamine 9 g/L |
| 0 | 0.03 | 0.023 | 0.03 | 0.012 |
| 8 | 1.23 | 2.31 | 0.98 | 3.12 |
| 16 | 1.87 | 3.11 | 3.45 | 7.56 |
| 24 | 3.24 | 6.02 | 12.45 | 14.34 |
| 32 | 5.56 | 7.59 | 20.85 | 19.48 |
| 40 | 2.56 | 5.21 | 15.11 | 12.01 |

conducted in triplicates and the average response obtained were tabulated in the design table and ANOVA regression analysis was employed to identify the factors influencing on the L-Glutaminase Activity. In the Design summary Table 11, the lower and upper limits supplied for the variables was represented. Mean and standard deviation was calculated by the algorithm for each variable. The minimum and maximum activities observed from Table 10 were 2.03 IU/ml and 25.38 IU/ml respectively. 12.5 is the ratio observed with maximum and minimum activities hence, square root transformation was applied for further regression analysis. The average mean and standard deviation observed from factorial analysis were 17.592 and 6.524 respectively.

From the factorial model, ANOVA analysis was performed by using partial sum of squares algorithm (Table 12). The model obtained was significant with F value of 34.9. Significant variables has the probability value < 0.05, A, C, F, G, H, J, K, L are the significant variables obtained from analysis. Curvature is the average distance between the average central and average factorial points. It was

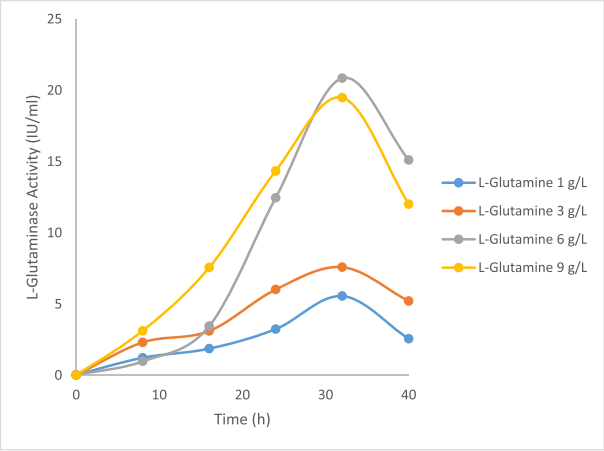


**Fig. 10.** Influence of inducer.

found to be 9.74. Standard deviation, mean and adequate precision values obtained from ANOVA analysis were 0.20, 4.08, 19.50 respectively. The polynomial equation obtained from ANOVA analysis was shown below:

Final Equation in Terms of Actual Factors

Sqrt(L-Glutaminase) = -5.76 + 0.62 × Galactose +0.24 × $Na_2HPO_4$ +0.73 × $MgSO_4{\cdot}7H_2O$ +0.29 × Yeast Extract +0.18 × pH + 0.038 × Temperature + 9.337E-003 × RPM +0.088 × Time

### 3.3.2. RSM optimization results

The 30 experiments generated from the RSM-CCD design table (Table 13) were performed in triplicates and the enzyme activity was recorded. Quadratic model generated the polynomial equation with Standard deviation of 6.254 and Mean of 17.39 was observed. The maximum L-Glutaminase activity observed was 25.43 (Run 12) shown in Table 13. No transformation is required as the ratio between minimum and maximum observed was 8.37.

Significant quadratic model with Mean square 79.69 and F-value of 20.66 was observed. The lack of fit is not significant with probability value of 0.0971 (Table 14). The significant terms obtained from RSM are A, D, AB, AC, BD, $A^2$, $B^2$, $D^2$ whose probability is < 0.05. The $R^2$ value obtained from RSM analysis was 0.972. The polynomial equation generated fro L-Glutaminase from RSM was shown below:

Final Equation in Terms of Actual Factors

L-Glutaminase = - 91.74 + 0.35 × Galactose+13.3 × pH +0.66 × Temperature+2.96 × Time +0.70 × Galactose*pH +0.20 × Galactose*Temperature+0.086 × Galactose*Time-0.024 × pH*Temperature-0.124 × pH*Time-6.98E-003 × Temperature* Time-3.04 × Galactose^2–0.68 × pH^2–0.0112 × Temperature^2– 0.025 × Time^2

From numerical optimization (Table 15), the optimal values

**Table 10**
Plackett-Burman design.

| Run | Galactose (g/L) | L-Glutamine (g/L) | $Na_2HPO_4$ (g/L) | $KH_2PO_4$ (g/L) | NaCl (g/L) | $MgSO_4.7H_2O$ (g/L) | Yeast extract (g/L) | pH | Temperature (°C) | RPM | Time (h) | L-Glutaminase (IU/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 7 | 7 | 4 | 0.1 | 0.1 | 1 | 8 | 25 | 150 | 40 | 18.5784 |
| 2 | 2 | 7 | 5 | 2 | 0.1 | 0.9 | 1 | 8 | 35 | 100 | 40 | 24.37 |
| 3 | 2 | 7 | 7 | 2 | 0.1 | 0.1 | 3 | 6 | 35 | 150 | 24 | 16.903 |
| 4 | 1 | 5 | 5 | 2 | 0.1 | 0.1 | 1 | 6 | 25 | 100 | 24 | 2.03 |
| 5 | 2 | 5 | 5 | 2 | 0.9 | 0.1 | 3 | 8 | 25 | 150 | 40 | 25.38 |
| 6 | 1 | 7 | 7 | 2 | 0.9 | 0.9 | 3 | 6 | 25 | 100 | 40 | 22.91 |
| 7 | 1 | 5 | 5 | 4 | 0.1 | 0.9 | 3 | 6 | 35 | 150 | 40 | 23.6887 |
| 8 | 1 | 7 | 5 | 4 | 0.9 | 0.1 | 3 | 8 | 35 | 100 | 24 | 9.37 |
| 9 | 1 | 5 | 7 | 2 | 0.9 | 0.9 | 1 | 8 | 35 | 150 | 24 | 14.9917 |
| 10 | 2 | 5 | 7 | 4 | 0.9 | 0.1 | 1 | 6 | 35 | 100 | 40 | 20.158 |
| 11 | 1.5 | 6 | 6 | 3 | 0.5 | 0.5 | 2 | 7 | 30 | 125 | 32 | 21.996 |
| 12 | 2 | 5 | 7 | 4 | 0.1 | 0.9 | 3 | 8 | 25 | 100 | 24 | 16.7743 |
| 13 | 2 | 7 | 5 | 4 | 0.9 | 0.9 | 1 | 6 | 25 | 150 | 24 | 11.54 |

**Table 11**
Design summary of Plackett-Burman design.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Design Summary** | | | | | | | | | | | |
| | **Study Type** | Factorial | | **Runs** | 13 | | | | | | |
| | **Initial Design** | Plackett Burman | | **Blocks** | No Blocks | | | | | | |
| | **Center Points** | 1 | | | | | | | | | |
| | **Design Model** | Main effects | | | | | | | | | |
| | **Factor** | **Name** | **Units** | **Type** | **Low Actual** | **High Actual** | **Low Coded** | **High Coded** | **Mean** | **Std. Dev.** | |
| | A | Galactose | gm | Numeric | 1 | 2 | -1 | 1 | 1.5 | 0.48 | |
| | B | L-Glutamine | gm | Numeric | 5 | 7 | -1 | 1 | 6 | 0.96 | |
| | C | Na2HPO4 | gm | Numeric | 5 | 7 | -1 | 1 | 6 | 0.96 | |
| | D | KH2PO4 | gm | Numeric | 2 | 4 | -1 | 1 | 3 | 0.96 | |
| | E | NaCl | gm | Numeric | 0.1 | 0.9 | -1 | 1 | 0.5 | 0.38 | |
| | F | MgSO4 | gm | Numeric | 0.1 | 0.9 | -1 | 1 | 0.5 | 0.38 | |
| | G | Yeast | gm | Numeric | 1 | 3 | -1 | 1 | 2 | 0.96 | |
| | H | pH | | Numeric | 6 | 8 | -1 | 1 | 7 | 0.96 | |
| | J | Temp | C | Numeric | 25 | 35 | -1 | 1 | 30 | 4.84 | |
| | K | Rpm | | Numeric | 100 | 150 | -1 | 1 | 125 | 24.0 | |
| | L | Time | H | Numeric | 24 | 40 | -1 | 1 | 32 | 7.6 | |
| **Response** | **Name** | **Units** | **Obs** | **Analysis** | **Minimum** | **Maximum** | **Mean** | **Std. Dev.** | **Ratio** | **Trans** | **Model** |
| Y1 | L-Glutaminase | IU/ml | 13 | Factorial | 2.03 | 25.38 | 17.592 | 6.524 | 12.502 | Square root | RMain effects |

**Table 12**
ANOVA analysis of Plackett-Burman.

| Response | 1 | L-Glutaminase | | | | | |
|---|---|---|---|---|---|---|---|
| | Transform: | Square root | Constant: | 0 | | | |
| | ANOVA for selected factorial model | | | | | | |
| | Analysis of variance table [Partial sum of squares - Type III] | | | | | | |
| | | Sum of | | Mean | F | p-value | |
| | Source | Squares | df | Square | Value | Prob > F | |
| | Model | 11.44 | 8 | 1.43 | 34.9 | 0.0071 | significant |
| | *A-Galactose* | *1.17* | *1* | *1.17* | *28.55* | *0.0128* | |
| | *C-Na2HPO4* | *0.72* | *1* | *0.72* | *17.59* | *0.0247* | |
| | *F-MgSO4* | *1.03* | *1* | *1.03* | *25.2* | *0.0152* | |
| | *G-Yeast* | *1.04* | *1* | *1.04* | *25.33* | *0.0151* | |
| | *H-pH* | *0.42* | *1* | *0.42* | *10.18* | *0.0497* | |
| | *J-Temp* | *0.44* | *1* | *0.44* | *10.63* | *0.0471* | |
| | *K-Rpm* | *0.65* | *1* | *0.65* | *15.96* | *0.0281* | |
| | *L-Time* | *5.97* | *1* | *5.97* | *145.78* | *0.0012* | |
| | Curvature | 0.4 | 1 | 0.4 | 9.74 | 0.0524 | not significant |
| | Residual | 0.12 | 3 | 0.041 | | | |
| | Cor Total | 11.96 | 12 | | | | |

**Table 13**
RSM design table.

| Run | Galactose (g/L) | pH | Temperature (°C) | Time (h) | L-Glutaminase (IU/ml) |
|---|---|---|---|---|---|
| 1 | 3 | 5 | 40 | 48 | 21.78 |
| 2 | 1 | 5 | 20 | 48 | 18.67 |
| 3 | 1 | 5 | 20 | 24 | 9.44 |
| 4 | 2 | 7 | 30 | 36 | 22.43 |
| 5 | 1 | 7 | 30 | 36 | 19.78 |
| 6 | 1 | 5 | 40 | 24 | 4.45 |
| 7 | 3 | 5 | 20 | 24 | 4.09 |
| 8 | 2 | 9 | 30 | 36 | 18.97 |
| 9 | 1 | 9 | 40 | 48 | 3.05 |
| 10 | 2 | 7 | 20 | 36 | 24.76 |
| 11 | 3 | 9 | 40 | 48 | 18.87 |
| 12 | 2 | 7 | 30 | 36 | 25.43 |
| 13 | 2 | 7 | 30 | 48 | 21.88 |
| 14 | 1 | 9 | 40 | 24 | 12.08 |
| 15 | 1 | 9 | 20 | 48 | 11.03 |
| 16 | 1 | 9 | 20 | 24 | 11.48 |
| 17 | 3 | 5 | 20 | 48 | 17.74 |
| 18 | 2 | 7 | 40 | 36 | 20.19 |
| 19 | 2 | 7 | 30 | 36 | 22.78 |
| 20 | 1 | 5 | 40 | 48 | 14.89 |
| 21 | 2 | 5 | 30 | 36 | 22.78 |
| 22 | 3 | 7 | 30 | 36 | 21.34 |
| 23 | 3 | 5 | 40 | 24 | 12.76 |
| 24 | 3 | 9 | 20 | 24 | 15.01 |
| 25 | 2 | 7 | 30 | 24 | 17.86 |
| 26 | 3 | 9 | 40 | 24 | 17.54 |
| 27 | 2 | 7 | 30 | 36 | 23.56 |
| 28 | 3 | 9 | 20 | 48 | 17.76 |
| 29 | 2 | 7 | 30 | 36 | 25.12 |
| 30 | 2 | 7 | 30 | 36 | 24.3 |

**Table 14**
RSM ANOVA analysis.

| Response | 1 | L-Glutaminase | | | |
|---|---|---|---|---|---|
| ANOVA for Response Surface Quadratic Model | | | | | |
| Analysis of variance table [Partial sum of squares - Type III] | | | | | |
| Source | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | |
| Model | 1115.68 | 14 | 79.69 | 20.66 | < 0.0001 | significant |
| A-galactose | 98.09 | 1 | 98.09 | 25.43 | 0.0001 | |
| B-pH | 0.04 | 1 | 0.04 | 0.01 | 0.9238 | |
| C-temperature | 1.06 | 1 | 1.06 | 0.28 | 0.6076 | |
| D-Time | 93.21 | 1 | 93.21 | 24.16 | 0.0002 | |
| AB | 31.98 | 1 | 31.98 | 8.29 | 0.0115 | |
| AC | 66.02 | 1 | 66.02 | 17.11 | 0.0009 | |
| AD | 17.14 | 1 | 17.14 | 4.44 | 0.0523 | |
| BC | 3.69 | 1 | 3.69 | 0.96 | 0.3438 | |
| BD | 142.44 | 1 | 142.44 | 36.93 | < 0.0001 | |
| CD | 11.26 | 1 | 11.26 | 2.92 | 0.1082 | |
| A^2 | 23.96 | 1 | 23.96 | 6.21 | 0.0249 | |
| B^2 | 19.26 | 1 | 19.26 | 4.99 | 0.0411 | |
| C^2 | 3.29 | 1 | 3.29 | 0.85 | 0.3706 | |
| D^2 | 36.07 | 1 | 36.07 | 9.35 | 0.0080 | |
| Residual | 57.86 | 15 | 3.86 | | | |
| Lack of Fit | 50.35 | 10 | 5.03 | 3.35 | 0.0971 | not significant |
| Pure Error | 7.51 | 5 | 1.50 | | | |
| Cor Total | 1173.55 | 29 | | | | |

**Table 15**
Numerical optimization table from RSM.

| Number | Galactose (g/L) | pH | Temperature (°C) | Time (h) | L-Glutaminase (IU/ml) |
|---|---|---|---|---|---|
| 1 | 2.52 | 7.19 | 33.4 | 36 | 24.585 |

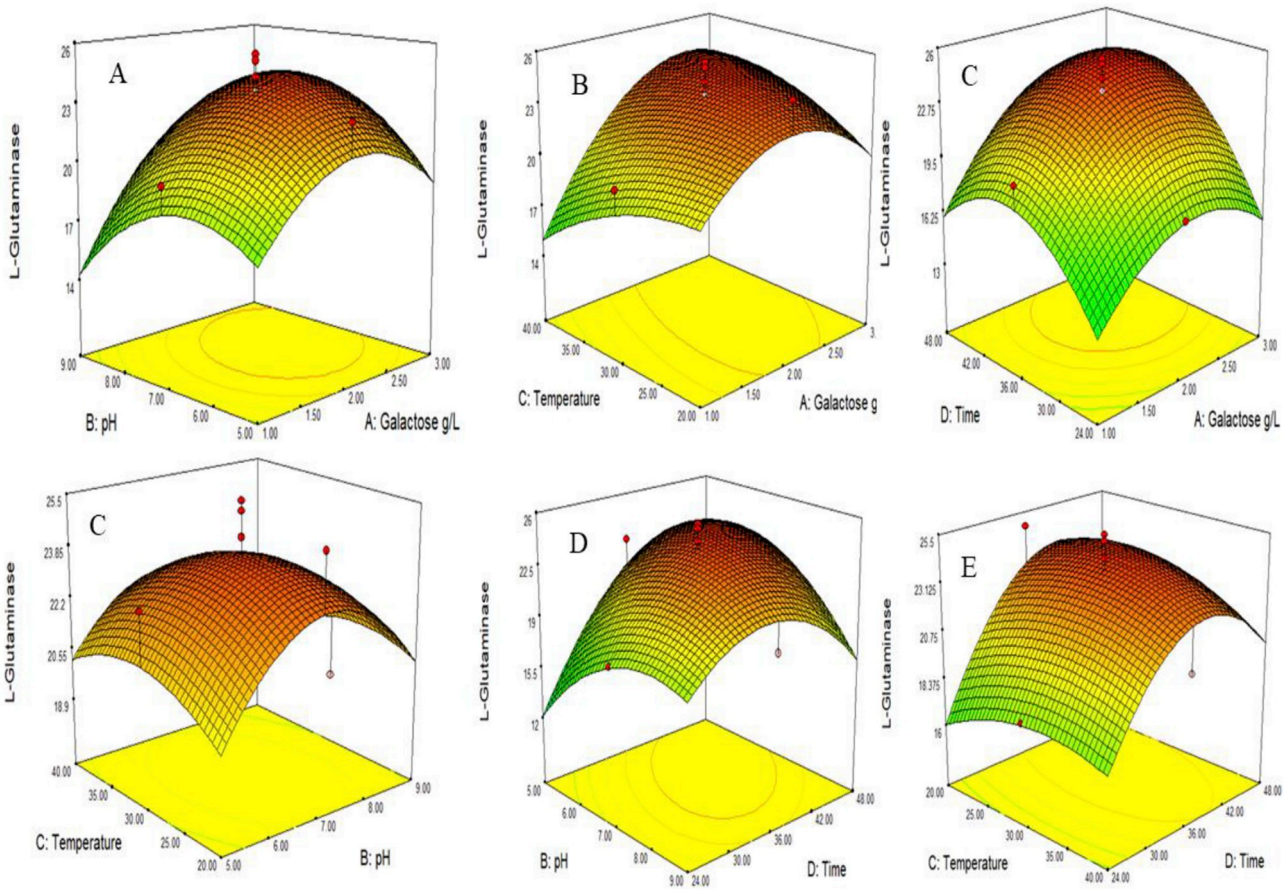


**Fig. 11.** 3D surface plots from RSM ANOVA analysis.

attained for the variables for maximum production of L-Glutaminase from mutated *Bacillus sps.* was shown below:

24.585 IU/ml is the achieved enzyme activity at Time 36 h Temperature 33.4 °C and Galactose 2.52 g/L. The media pH was adjusted to 7.19. The 3D surface plots obtained were shown (Fig. 11) below:

## 4. Conclusion

*In house Bacillus sps* was mutated and it was conformed from Rapid plate assay that it is producing higher amounts of L-Glutaminase in comparison with wild type. Initially, the enzyme activity obtained by performing Nesslerization was 9.59 IU/ml. OVAT optimization resulted in 20.859 IU/ml. Time, Temperature, pH and Galactose were identified as significant variables with positive effect on L-Glutaminase production. These 4 variables were further optimized by RSM-CCD experiments. The maximum activity attained was 24.585 IU/ml at 36 h. 2.56 fold increase in L-Glutaminase activity was observed after employing optimization studies.

## Conflict of interest

Authors have no conflict of interest regarding the publication of paper.

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