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*Research article*

# The nutritional and cultural conditions in shake flask culture for improved production of L-Asparaginase from endophytic fungus *Fusarium* sp. LCJ324: A sequential statistical method

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**Abstract**
L-Asparaginase has a greater demand due to its potential application in the food industry as well as in the pharmaceutical industry. To meet this demand, optimization of nutritional as well as physical conditions is critical for scaling up the production of L-Asparaginase. This study aims to achieve enhanced L-Asparaginase production, a therapeutic enzyme from the endophytic fungus *Fusarium* sp. LCJ324 isolated from *Glycosmis mauritiana* (Lam.) Tanaka belonging to the Rutaceae family. *Fusarium* sp. LCJ324 exhibited L-Asparaginase activity of 8.75 ± 0.55 U $mL^{-1}$ in modified Czapek Dox broth, the highest among all the seven different media tested by submerged fermentation. Different sources and concentrations of carbon, nitrogen and amino acid inducers (nutritional factors) as well as pH and temperature (physical parameters) were optimized to achieve enhanced L-Asparaginase production. Maximum L-Asparaginase activity of 19.94 ± 0.35 U $mL^{-1}$ was obtained at 30º C with dextrose (3 g $L^{-1}$), ammonium sulphate (30 g $L^{-1}$), and L-Asparagine (20 g $L^{-1}$) at pH 6. Response Surface Methodology was employed for statistical optimization of minimum and maximum levels of the selected parameters. The levels of parameters suggested by the response surface methodology for maximum production of L-Asparaginase were similar to the conventional optimization. A 2.29 fold increase in L-Asparaginase production was obtained through conventional and statistical optimization.

**Keywords**
Modified Czapek Dox broth, L-Asparagine, one factor at a time, response surface methodology, submerged fermentation

## Introduction

The enzyme L-Asparaginase (EC 3.5.1.1) catalyses the hydrolysis of L-asparagine to L-aspartic acid and ammonia. Cancerous cells typically require asparagine because they have lost the ability to express the gene encoding asparagine synthetase (Ekpenyong et al., 2021). Thus, these cells depend on external sources of L-asparagine for their persistence. Therefore, the usage of L-Asparaginase as an intravenous injection will reduce L-Asparagine levels and in turn prevents the growth of cancerous cells. As a result, in the treatment of children with acute lymphoblastic leukaemia (ALL), L-Asparaginase is considered as the most effective drug (Nunes et al., 2020). Furthermore, L-Asparaginase is widely

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used to treat other types of cancer such as lymphosarcoma, Hodgkin's disease, reticulosarcoma as well as different types of leukaemia (El-Naggar et al., 2018). L-Asparaginase finds application in food industry namely to reduce the development of acrylamide (carcinogenic substance formed in foods that have undergone heat processing) in starchy foods. L-Asparaginase based biosensors that can quantify L-Asparagine levels finds application in both pharmaceutical as well as food industry (Nunes et al., 2020).

The sources of L-Asparaginase are plants (Bano and Sivaramakrishnan, 1980; Oza et al., 2011; Mohamed et al., 2015) and microbes. The complicated process of obtaining L-Asparaginase from plants coupled with the low yield restricts the plants as a go to source for L-Asparaginase production. Microbial source of L-Asparaginase includes bacteria, actinomycetes and fungi. L-Asparaginase from bacteria *Acetinobacter soli* was reported by Jiao et al. (2020). Alrumman et al. (2019) has reported that *Bacillus licheniformis* was capable of yielding 8.1 U $mL^{-1}$ of L-Asparaginase. There are two clinically approved formulations of bacterial L-Asparaginases (*Erwinia chrysanthemi* and *E. coli*) that are in use for ALL treatments. The bacterial derived (prokaryotic sources) L-Asparaginase has high glutaminase activity and long-term therapeutic use causes unpleasant side effects due to normal cell toxicity (bacterial toxins in the enzyme) and the dissociation of the enzyme from the bloodstream, limits its usage (Ramya et al., 2012; El-Naggar et al., 2014).

L-Asparaginase from fungal sources can have fewer adverse effects as they belong to the eukaryotic type of organisms (Ali et al., 1994; Meghavarnam et al., 2022). Endophytic fungi are capable of producing L-Asparaginase but are less explored. Endophytic fungi isolated from medicinal plants have huge potential for the production of numerous metabolites and enzymes. Production of L-Asparaginase using endophytic fungi can be done by solid state or submerged fermentation. In submerged fermentation, the utilization of substrate by fungi is rapid with the added advantage of having minimal chances for contamination. This method is well-suited for the extraction of extracellular metabolites from microbes (Couto and Sanromán, 2006). The endophytic fungus *Fusarium* sp. LCJ324 produced significant L-Asparaginase of 8.84 U $mL^{-1}$ in submerged fermentation using modified Czapek Dox broth (Udayan and Gnanadoss, 2023). However, to deal with low yields of the enzyme, optimization of the nutritional parameters and physical conditions of the medium is necessary in order to improve the yields. Optimization of the bioprocess can be done by One Factor at a Time (OFAT) method which is a classical method of optimization. OFAT method of optimization is tedious and expensive. However, the development of contemporary mathematical and statistical techniques can assist in media optimization. Response Surface Methodology (RSM) is one such statistical tool that can be used in analyzing different levels of variables, interaction and their impact on product formation. RSM generates an experimental design that reduces the number of experiments and increases the efficiency of the optimization process (Li et al., 2015; Abdollahzadeh et al., 2020). The study was aimed to formulate and optimize the medium for enhanced L-Asparaginase production by the endophytic fungus *Fusarium* sp. LCJ324 using OFAT and RSM methods.

## Materials and methods

*Isolation and characterization of endophytic fungal culture*

The endophytic fungal culture LCJ324 was isolated from *Glycosmis mauritiana* (Lam.) Tanaka belonging to the Rutaceae family (Udayan and Gnanadoss, 2023). LCJ324 exhibited high L-Asparaginase enzyme activity and was subjected to molecular characterization using ITS1 (5'TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5'TCCTCCGCTTATTGATATGC-3′) primer pair. The endophytic fungal culture LCJ324 was identified as *Fusarium* sp. The pure culture was maintained on PDA slants at 4°C.

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*Selection of medium for L-Asparaginase production*

The endophytic fungus *Fusarium* sp. LCJ324 was cultured in seven different media (glucose asparagine broth, asparagine dextrose salts broth, glycerol asparagine broth, modified M9 broth, modified M2 broth, modified Czapek Dox broth and inorganic salt starch asparagine broth) to identify the medium that best supports production of L-Asparaginase. The shake flask method of culturing was followed in this experiment to identify the medium that supports maximum production of L-Asparaginase. A hundred mL of each media was prepared in a separate 250 mL conical flask, sterilized and supplemented with streptomycin (100 µg $mL^{-1}$). Three mycelial discs (5 mm) of four days old *Fusarium* sp. LCJ324 maintained in PDA were inoculated into the flasks under aseptic conditions and incubated at 120 rpm in an orbital shaker (REMI RIS-24BL) for 8 days. The L-Asparaginase activity was evaluated every 24 h using the supernatant obtained by centrifuging 0.5 mL of crude culture filtrate.

*L-Asparaginase assay*

L-Asparaginase assay was done by Nesslerization method described by Imada et al. (1973). Culture filtrate was pipetted out daily to make a reaction mixture (2.5 mL) that contained 500 µl of each of the following culture filtrate, 0.5 M Tris buffer, 0.04 M L-asparagine and distilled water. The reaction mixture was incubated for about 30 min followed by the addition of 500 µl of Trichloroacetic acid. The reaction mixture was then centrifuged at 10,000 rpm for 5 min. A hundred µl of supernatant was pipetted out from the reaction solution to which distilled water (3700 µl) and Nesseler's reagent (200 µl) was added and incubated for 20 min. The brownish yellow colour developed was read at 450 nm using an UV visible spectrophotometer (Elico). At 37 ± 2 °C, one unit of asparaginase is defined as the enzyme quantity required to catalyze the creation of 1 µmol of $NH_4$/min.

$$\text{Units mL}^{-1}\text{ enzyme} = \frac{(\mu\text{M of ammonia liberated})\,(\text{Volume of reaction solution})}{(\text{Volume of supernatant})\,(\text{Incubation Time})\,(\text{Volume of crude enzyme})}$$

Volume of reaction solution - 2.5 mL
Volume of supernatant - 0.1 mL
Incubation Time - 30 min
Volume of crude enzyme - 0.5 mL

*Studies for optimizing production parameters of L-Asparaginase enzyme*

Optimization studies are necessary to evaluate the various factors and conditions to improve enzyme production. The following factors such as carbon source, nitrogen source, amino acid inducer and culture conditions like pH and temperature were optimized by following one factor at a time method. Optimization was carried out by varying any one factor or condition while keeping other variables as constant. Modified Czapek Dox broth medium (g $L^{-1}$): [Glucose (2), L-Asparagine (10), $KH_2PO_4$ (1.52), $MgSO_4.7H_2O$ (0.52), KCL (0.52), $Cu(NO_3).3H_2O$ (0.001), $ZnSO_4.7H_2O$ (0.001), $FeSO_4.7H_2O$ (0.001)] (Jenila and Gnanadoss, 2018) was used for the optimization of L-Asparaginase production using *Fusarium* sp. LCJ324. The optimized parameter was used in the subsequent studies.

*Optimization of carbon source and its concentration*

Effect of addition of different carbon sources such as lactose, sucrose, dextrose, maltose and galactose at 2 g $L^{-1}$ on L-Asparaginase production by *Fusarium* sp. LCJ324 was studied by substituting the carbon source in the modified Czapek Dox broth medium. The standard medium was used as a control. The best carbon source identified was further studied to find the optimum concentration for L-Asparaginase production. The concentration of the best carbon source was varied from 1 to 6 g $L^{-1}$. L-Asparaginase activity and protein activity were determined every day.

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*Optimization of nitrogen source and its concentration*

Effect of different nitrogen sources on L-Asparaginase production by *Fusarium* sp. LCJ324 was studied. Peptone, potassium nitrate, urea, ammonium sulphate and yeast extract are the different nitrogen sources used in this study at a concentration of 10 g $L^{-1}$. The standard medium was used as a control. The best nitrogen source was further scrutinized to find the optimum concentration. The concentration of the nitrogen source was varied from 5 to 30 g $L^{-1}$.

*Optimization of amino acid inducer sources and its concentration*

Effect of different amino acid sources such as L-Asparagine, L-Arginine, L-Tryptophan, L-Tyrosine and L-Glutamic acid on L-Asparaginase production by *Fusarium* sp. LCJ324 was studied at a concentration of 10 g $L^{-1}$. After the selection of the best amino acid source, the optimal concentration for the enhanced L-Asparaginase production was studied by varying the concentrations of L-Asparagine from 5 to 30 g $L^{-1}$.

*Optimization of initial pH*

Effect of initial pH of the medium on the production of L-Asparaginase by *Fusarium* sp. LCJ324 was studied by varying the pH of the modified Czapek Dox broth in the range of 4 to 9. The pH of the medium was varied using 0.1N HCL and 0.1N NaOH.

*Optimization of temperature*

The effect of different temperatures on L-Asparaginase production was studied. The inoculated flasks were incubated in varying temperatures ranging from 28 ºC to 36 ºC.

*Optimization of L-Asparaginase production using RSM*

RSM is a statistical tool that is employed to optimize the output when more than two factors are involved. Central composite design (CCD) is the widely used tool in RSM to design experiments. After the selection of major parameters and their ranges, levels were coded for the study using Face Centre Central Composite Design (FCCCD). The parameters such as dextrose, ammonium sulphate, L-Asparagine and pH were studied. Three different levels (-1, 0, 1) of the four parameters were coded in the software Design Expert (version 13, Stat-Ease, Inc., Minneapolis, MN).

Thirty experiments were generated by varying the concentrations of each of the selected four parameters. The experiments were carried out and their response (enzyme activity) was noted. A polynomial equation was generated that is in accordance with the data as well as related to all the variables in the experiment. The polynomial equation generated using the four factors for L-Asparaginase production is:

$$Y= \beta_0 + \beta_1X_1+ \beta_2X_2+ \beta_3X_3+ \beta_4X_4+ \beta_{11}X_1^2+ \beta_{22}X_2^2+ \beta_{33}X_3^2+ + \beta_{44}X_4^2+ \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{14}X_1X_4+ \beta_{23}X_2X_3+ \beta_{24}X_2X_4+ \beta_{34}X_3X_4$$

In the above equation, Y represents the L-Asparaginase (predicted response) of the model; $\beta_0$ represents intercept; $\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$ represent linear coefficient; $\beta_{11}$, $\beta_{22}$, $\beta_{33}$, $\beta_{44}$ represent squared coefficients; $\beta_{12}$, $\beta_{13}$, $\beta_{14}$, $\beta_{23}$, $\beta_{24}$, $\beta_{34}$ represents interaction coefficients. Analysis of variance (ANOVA) was used as the statistical method to look into the significance and suitability of the model. The Design Expert 13.0 software was employed in handling all of the three-dimensional and counter plotting, as well as the statistical analysis.

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*Comparison of L-Asparaginase production using original and optimized medium*

L-Asparginase production by *Fusarium* sp. LCJ324 using the original and optimized medium was studied. *Fusarium* sp. LCJ324 was inoculated and cultured for 8 days in separate flasks containing the original and optimized medium. The L-Asparaginase activity was determined every day.

## Results and Discussion

*Isolation and Characterization of Endophytic fungal culture*

The endophytic fungal LCJ324 isolated from *Glycosmis mauritiana* (Lam.) Tanaka was identified as *Fusarium* sp. LCJ324 using ITS sequence. *Fusarium* sp. LCJ324 showed high L-Asparaginase activity in screening medium modified Czapek Dox. The obtained ITS sequence was submitted to Genbank and the accession number MZ646108 was obtained. Similarly, Hatamzadeh et al. (2020) performed screening for L-Asparaginase enzyme from endophytic fungal isolates in modified Czapek Dox medium.

*Selection of medium for L-Asparaginase production*

The L-Asparaginase production was tested using the seven different media for 8 days and the production varied in all the media. Modified Czapek Dox broth medium showed the highest L-Asparaginase activity of 8.70 ± 0.52 U $mL^{-1}$ on the 5$^{th}$ day when compared to other media. The lowest L-Asparaginase activity of 3.89 ± 0.25 U $mL^{-1}$ was noted in inorganic salt starch asparagine broth. Intermediate L-Asparaginase activity was observed in modified M9 broth (8.12 ± 0.35 U $mL^{-1}$), glycerol asparagine broth (7.48 ± 0.44 U $mL^{-1}$), asparagine dextrose salts broth (7.14 ± 0.23 U $mL^{-1}$), modified M2 broth (6.63 ± 0.29 U $mL^{-1}$) and glucose asparagine broth (6.73 ± 0.35 U $mL^{-1}$).

Submerged fermentation for L-Asparaginase production was investigated by Elshafei and El-Ghonemy (2015) from *Trichoderma viridae* Pers and was found to be 14.23 ± 0.87 U $mL^{-1}$. Jenila and Gnanadoss (2018) studied L-Asparaginase production from five different liquid media. Among them modified Czapek Dox broth showed significant L-Asparaginase activity and was subsequently subjected to conventional optimization. L-Asparaginase from *Fusarium equiseti* (Corda) Sacc. under submerged fermentation produced 40.78 U $mL^{-1}$ of enzyme after conventional optimization of modified Czapek Dox broth (El-Gendy et al., 2021). L-Asparaginase production from endophytic *Lasiodiplodia theobromae* (Pat.) Grifton & Maubl. was studied in modified Czapek Dox broth (Balbool and Azeem, 2020).

*Optimization of carbon source and its concentration*

The effect of different carbon sources on L-Asparaginase production such as lactose, sucrose, dextrose, maltose and galactose was studied. Dextrose yielded higher L-Asparaginase activity of 11.90 ± 0.57 U $mL^{-1}$ when compared to other carbon sources on the 5$^{th}$ day (Table 1). The effect of different concentrations of dextrose on L-Asparaginase activity was studied, 3 g $L^{-1}$ of dextrose increased L-Asparaginase activity to 13.20 ± 0.57 U $mL^{-1}$ (Fig. 1).

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**Table 1 -** Effect of different nutritional parameters on the production of L-Asparaginase.

| Source | L-Asparaginase activity U $mL^{-1}$ |
|---|---|
| **Carbon source (2 g $L^{-1}$)** | |
| Lactose | 9.81 ± 0.52 |
| Sucrose | 9.36 ± 0.29 |
| Dextrose | **11.90 ± 0.57** |
| Maltose | 9.02 ± 0.52 |
| Galactose | 9.07 ± 0.52 |
| **Nitrogen source (10 g $L^{-1}$)** | |
| Peptone | 5.64 ± 0.29 |
| Potassium nitrate | 3.58 ± 0.17 |
| Urea | 5.83 ± 0.40 |
| Ammonium sulphate | **14.70 ± 0.52** |
| Yeast extract | 5.95 ± 0.52 |
| **Amino acid inducer (10 g $L^{-1}$)** | |
| L-Asparagine | **18.80 ± 0.58** |
| L-Arginine | 16.00 ± 0.58 |
| L-Trytophan | 17.30 ± 0.64 |
| L-Tyrosine | 16.50 ± 0.58 |
| L-Glutamic acid | 15.00 ± 0.69 |

Values are mean ±SEM
Values differ ($p<0.05$) as analyzed by one-way ANOVA

The primary structural and functional element in microbial cells is carbon. Although fungi may use a wide range of carbon molecules, most of them prefer simple sugars (Uzma et al., 2016). Among 9 different carbon sources tested, sucrose and molasses increased L-Asparaginase activity for *Aspergillus candidus* P. Micheli ex Haller (Ekpenyong et al., 2021). Two g $L^{-1}$ of glucose was used as the carbon source for enhanced production of L-Asparaginase (6.21 U) using *Aspergillus terreus* Thom (Costa-silva et al., 2019). Two g $L^{-1}$ of sucrose was found to be best carbon souce for enhancing L-Asparaginase production (0.166 U $mL^{-1}$) by *Fusarium solani* (Mart.) Sacc. (Uzma et al., 2016). Study on optimization of nutritional parameters revealed that 2 g $L^{-1}$ of glucose as best carbon source for increasing L-Asparaginase production by *Fusarium proliferatum* (Matsuch.) Nirenberg (Yap et al., 2021).

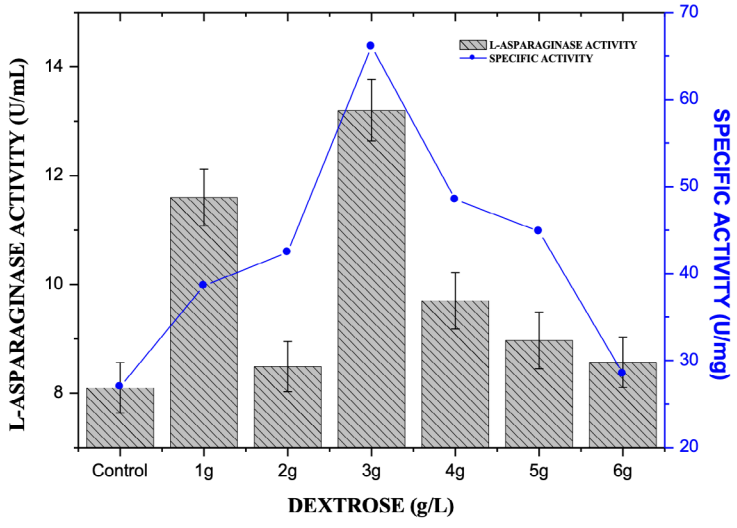


**Fig. 1 -** Effect of dextrose concentrations on the production of L-Asparaginase by *Fusarium* sp. LCJ324 on the 5th day.

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*Optimization of nitrogen source and its concentration*

The effect of different nitrogen sources on L-Asparaginase production in modified Czapek Dox broth was studied. Ammonium sulphate as nitrogen source yielded better L-Asparaginase production by *Fusarium* sp. LCJ324 (14.70 ± 0.52 U $mL^{-1}$) than other nitrogen sources (Table 1). Different concentration of ammonium sulphate was tested for optimum production of L-Asparaginase and 30 g $L^{-1}$ of ammonium sulphate resulted in enhancing the L-Asparaginase activity to 16.30 ± 0.64 U $mL^{-1}$ (Fig. 2).

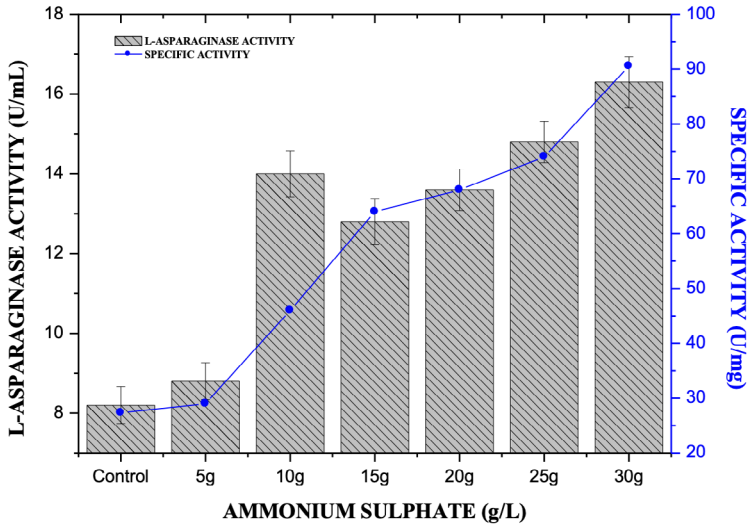


**Fig. 2 -** Effect of ammonium sulphate concentrations on the L-Asparaginase production by *Fusarium* sp. LCJ324 on the 5th day.

Nitrogen as a limiting nutrient is crucial for the fungi's metabolism as well as for the formation of L-Asparaginase (El-Hadi et al., 2017). Ammonium sulphate as nitrogen source for *Fusarium* sp. at a concentration of 20 g $L^{-1}$ increased the L-Asparaginase activity to 13.69 U $mL^{-1}$ (Jenila and Gnanadoss, 2018). Yeast extract, proline, ammonium chloride, asparagine and sodium nitrate were examined as different nitrogen sources to enhance L-Asparaginase activity. Ammonium chloride as nitrogen source for *Fereydounia khargensis* significantly increased the L-Asparaginase activity to 51.7 U $mL^{-1}$ from 13.6 U $mL^{-1}$ (Fazeli et al., 2021). Ammonium nitrate and sodium nitrate as nitrogen source demonstrated increased L-Asparaginase activity for *Fusarium solani* (Uzma et al., 2016).

*Optimization of amino acid inducer source and its concentration*

The effect of different amino acid inducer sources on L-Asparaginase production was studied. The addition of L-Asparagine as amino acid inducer yielded better L-Asparaginase activity of 18.80 ± 0.58 U $mL^{-1}$ (Table 1). Subsequently, the effect of different concentrations of L-Asparagine was studied. Maximum L-Asparaginase activity of 19.10 ± 0.69 U $mL^{-1}$ was observed at 20 g $L^{-1}$ of L-Asparagine (Fig. 3).

One g $L^{-1}$ of L-Asparagine was used as an inducer for increasing L-Asparaginase activity of *Trichoderma viridae* to 223 U $g^{-1}$ while other inducers such as L-aspartic acid and L-glutamic acid resulted in reduced L-Asparaginase activity (Elshafei and El-Ghonemy, 2015). The final composition of the optimized medium for increased production of L-Asparaginase using *Talaromyces pinophilus* (Hedge.) Samson, Yilmaz, Frisrad & Seifert devised by Krishnapura and Belur (2020) had 17.2 g $L^{-1}$ of L-Asparagine as an inducer.

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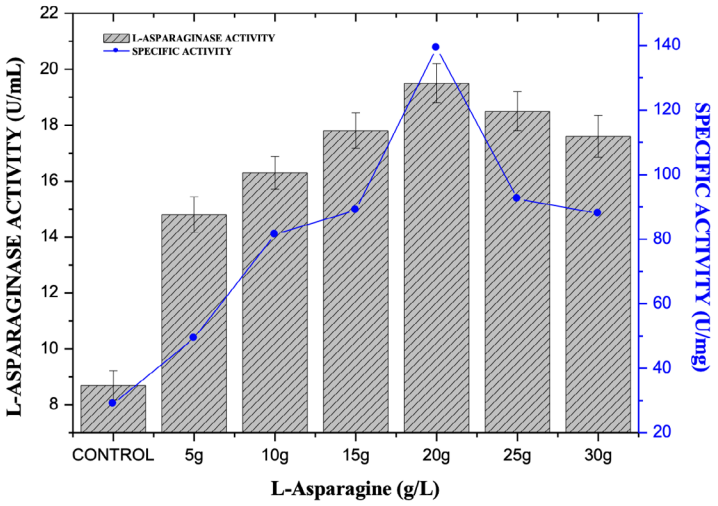


**Fig. 3** - Effect of L-Asparagine concentrations on the L-Asparaginase production by *Fusarium* sp. LCJ324 on the 5th day.

*Optimization of initial pH*

The effect of different initial pH in the medium for L-Asparaginase production was studied since pH is an important physical parameter for increased enzyme production. In the present study, the optimum L-Asparaginase activity of 19.30 ± 0.23 U $mL^{-1}$ was achieved at initial pH 6 (Fig. 4).

Similarly, maximum L-Asparaginase activity at pH 7 was reported by Hosamani and Kaliwal (2011), Chandrasekhar (2012), and Uzma et al. (2016). pH 6.5 was found to be favourable for L-Asparaginase enzyme production for *Talaromyces pinophilus*. An increase in pH led to a decrease in enzyme activity (Krishnapura and Belur, 2020). Maximum L-Asparaginase enzyme yield was obtained from *Aspergillus niger* P. Micheli ex Haller at pH 6.5 (Mishra, 2006). pH 7 was identified as the optimum pH for increased L-Asparaginase production (32.25 U $mL^{-1}$) by *Aspergillus terreus* (Kalyanasundaram et al., 2015). In another study *Aspergillus terreus*, a marine derived endophytic fungus yielded maximum L-Asparaginase activity at pH 6 (Farag et al., 2015). pH of the production medium plays a vital role in affecting the enzyme production both positively and negatively.

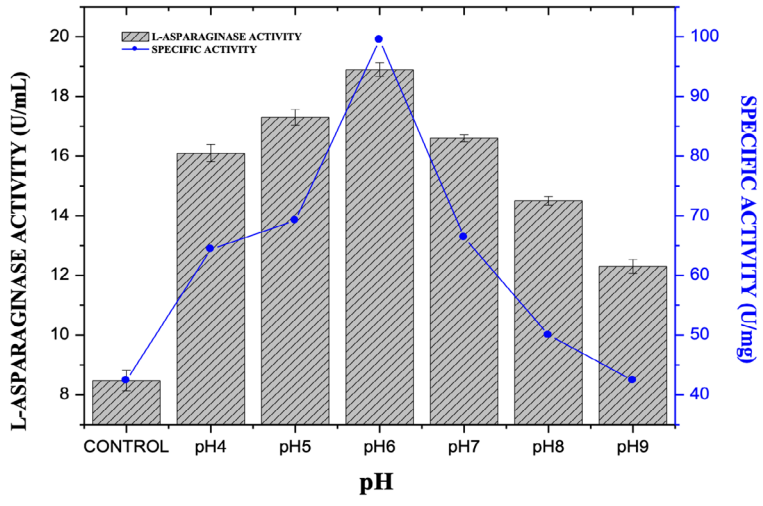


**Fig. 4** - Effect of pH on L-Asparaginase production by *Fusarium* sp. LCJ324 on the 5th day.

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*Optimization of temperature*

The suitable incubation temperature of *Fusarium* sp. LCJ324 was studied for enhancing L-Asparaginase production. A temperature of 30°C was found to be the optimum temperature for increased L-Asparaginase activity of 19.94 ± 0.346 U $mL^{-1}$ (Fig. 5). Higher temperatures resulted in a decrease in enzyme production.

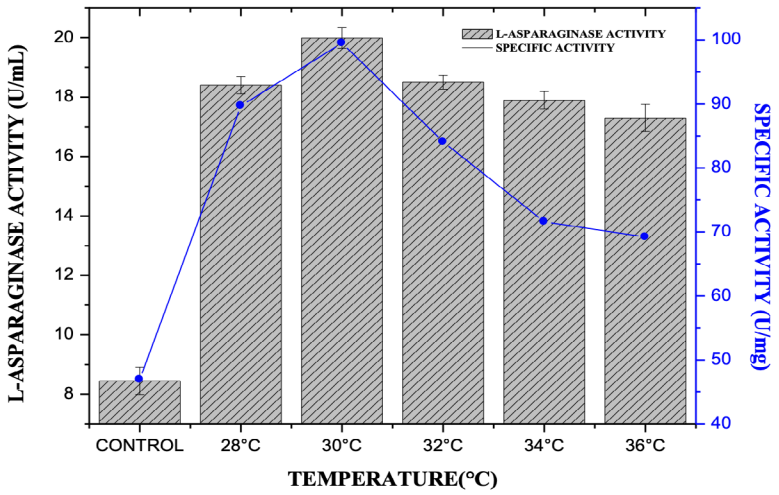


**Fig. 5 -** Effect of temperature on L-Asparaginase production by *Fusarium* sp. LCJ324 on the 5th day.

An incubation temperature of 30°C was reported as the optimum temperature for the L-Asparaginase production from *Aspergillus* sp. (Pundir et al., 2020; Priya and Subhashini, 2022) and *Fusarium proliferatum* (Yap et al., 2021). An incubation temperature of 29 °C was found to be the best condition for L-Asparaginase production using *Aspergillus terreus* (Da Rocha et al., 2019). Incubation temperature is a crucial factor for enzyme production as it influences both growth of the organisms as well as the rate of the chemical reaction in the medium (Shah et al., 2014).

*Optimization of L-Asparaginase production using RSM*

RSM was employed in finding the influence of the four major parameters (dextrose, ammonium sulphate, L-Asparagine and pH) on the L-Asparaginase production. Using FCCCD 30 trials were generated. The trial runs were conducted in triplicates and the results were analyzed using RSM. Table 2 displays the design matrix and the accompanying outcomes of the observed and predicted responses for L-Asparaginase production.

The analysis of variance (ANOVA) of the model yielded *P*-value of <0.0001 which indicates that the model is significant. The $R^2$ value of 0.9594 was obtained from this study indicating a good connection between the observed and predicted responses. Statistical optimization for enhancing L-Asparaginase production from *Aspergillus candidus* was done using RSM and $R^2$ value of 0.9874 was reported (Ekpenyong et al., 2021). Similarly, $R^2$ values of 0.9488 and 0.9679 were reported for RSM mediated statistical optimization of L-Asparaginase production from *Penicillium lilacinum* Thom and *Cladosporium tenuissimum* Cooke respectively (Hamed et al., 2021; Vimal and Kumar, 2022). The F and P value of lack of fit was found to be 2.44 and 0.1682 respectively. Indicating lack of fit was insignificant and the model is very effective.

The obtained polynomial equation for L-Asparaginase production is as follows:

$$Y = 19.34 + 0.5244X_1 + 0.5517X_2 + 0.4872X_3 - 0.0478X_4 - 0.0319X_1X_2 + 0.0169X_1X_3 + 0.3906X_1X_4 + 0.1106X_2X_3 + 0.4194X_2X_4 + 0.0731X_3X_4 - 0.7011X_1^2 - 0.5161X_2^2 - 0.7061X_3^2 - 1.68\ X_4^2$$

where, Y represents the L-Asparaginase activity, $X_1$, $X_2$, $X_3$ and $X_4$ represents dextrose, ammonium sulphate, L-Asparagine and pH respectively.

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**Table 2 -** Observed and predicted values with factors in coded values for L-Asparaginase production using FCCCD.

| Run | Dextrose | Ammonium sulphate | L-Asparagine | pH | L-Asparaginase activity U $mL^{-1}$ | |
|---|---|---|---|---|---|---|
| | | | | | Actual value | Predicted value |
| 1 | 0 | 0 | 1 | 0 | 18.84 | 19.12 |
| 2 | 0 | 1 | 0 | 0 | 19.18 | 19.38 |
| 3 | -1 | 1 | -1 | -1 | 14.70 | 15.31 |
| 4 | 1 | -1 | 1 | 1 | 16.88 | 16.13 |
| 5 | -1 | 0 | 0 | 0 | 17.77 | 18.11 |
| 6 | 0 | 0 | 0 | 0 | 19.96 | 19.34 |
| 7 | 1 | -1 | -1 | 1 | 14.86 | 15.20 |
| 8 | -1 | -1 | -1 | 1 | 13.20 | 13.34 |
| 9 | 0 | 0 | 0 | 0 | 19.40 | 19.34 |
| 10 | 1 | 1 | 1 | -1 | 16.84 | 16.56 |
| 11 | -1 | -1 | 1 | -1 | 15.69 | 15.77 |
| 12 | 1 | 1 | -1 | -1 | 15.46 | 15.48 |
| 13 | 1 | 0 | 0 | 0 | 18.96 | 19.16 |
| 14 | -1 | 1 | 1 | 1 | 16.35 | 16.43 |
| 15 | 0 | 0 | 0 | 0 | 19.46 | 19.34 |
| 16 | 1 | 1 | 1 | 1 | 17.80 | 18.23 |
| 17 | -1 | -1 | -1 | -1 | 15.62 | 15.20 |
| 18 | 0 | 0 | 0 | 0 | 19.99 | 19.34 |
| 19 | 0 | 0 | -1 | 0 | 17.88 | 18.15 |
| 20 | 0 | -1 | 0 | 0 | 17.92 | 18.27 |
| 21 | 0 | 0 | 0 | 1 | 17.12 | 17.61 |
| 22 | 0 | 0 | 0 | -1 | 17.65 | 17.71 |
| 23 | -1 | -1 | 1 | 1 | 14.21 | 14.20 |
| 24 | -1 | 1 | -1 | 1 | 15.61 | 15.12 |
| 25 | 0 | 0 | 0 | 0 | 19.82 | 19.34 |
| 26 | 1 | -1 | 1 | -1 | 15.64 | 16.14 |
| 27 | 1 | 1 | -1 | 1 | 17.08 | 16.85 |
| 28 | 1 | -1 | -1 | -1 | 15.72 | 15.50 |
| 29 | 0 | 0 | 0 | 0 | 19.05 | 19.34 |
| 30 | -1 | 1 | 1 | -1 | 16.65 | 16.32 |

Four components were combined into pairs for the construction of three-dimensional response plots to predict the L-Asparaginase production for various values of the investigated variables and examine the interaction between them (Fig. 6). Since the observed and predicted values of L-Asparaginase production were found to be in close agreement, the model was validated successfully.

The results of the present study indicate that both OFAT and RSM evaluation yielded the same optimum condition for increased L-Asparaginase production. The results coincide with the results reported by Yap et al. (2021). Optimization of culture conditions for better production of L-Asparaginase has been reported using *Fusarium* sp. (Jenila and Gnanadoss, 2018), *Fusarium solani* (As-Suhbani and Bhosale, 2020), *Talaromyces pinophilus* (Krishnapura and Belur, 2020) and *Aspergillus candidus* (Ekpenyong et al., 2021).

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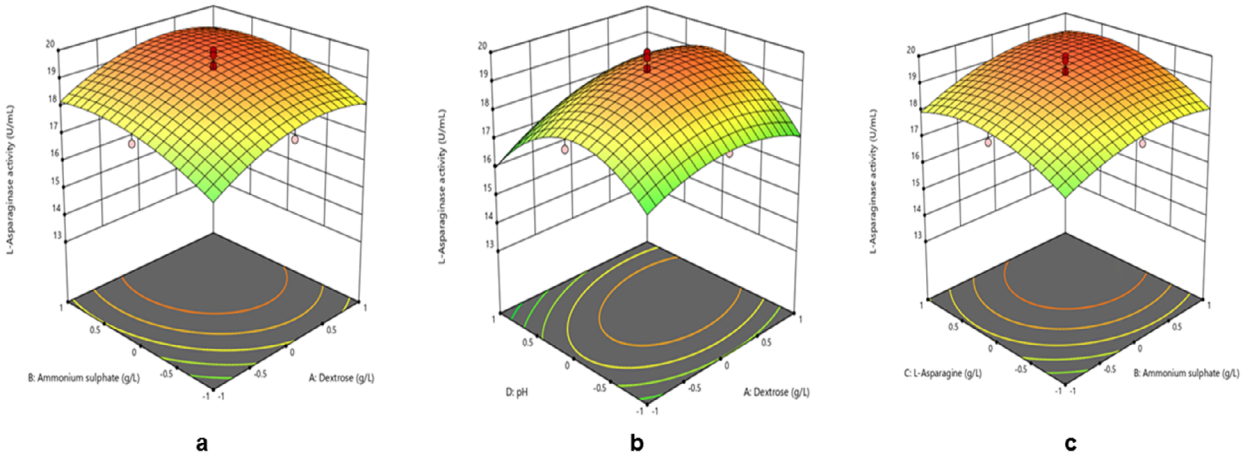


**Fig. 6 -** Three dimensional plots from RSM showing the interaction between variables a) Dextrose and ammonium sulphate b) Dextrose and pH c) Ammonium sulphate and L-Asparagine.

*Comparison of L-Asparaginase production using original and optimized medium*

Comparative study of L-Asparaginase production by *Fusarium* sp. LCJ324 in the original and optimized medium was done. L-Asparaginase activity for *Fusarium* sp. LCJ324 was found to be 8.78 ± 0.52 U $mL^{-1}$ in original medium and 19.95 ± 0.59 U $mL^{-1}$ in optimized medium (g $L^{-1}$; Dextrose (3), Ammonium sulphate (30), L-Asparagine (2), $KH_2PO_4$ (1.52), $MgSO_4.7H_2O$ (0.52), KCL (0.52), $Cu(NO_3).3H_2O$ (0.001), $ZnSO_4.7H_2O$ (0.001), $FeSO_4.7H_2O$ (0.001). A 2.29-fold increase in L-Asparaginase production was obtained in optimized medium in comparison to the original medium.

## Conclusion

This study has revealed that *Fusarium* sp. LCJ324, an endophytic fungus isolated from *Glycosmis mauritiana* was capable of producing L-Asparaginase, a highly intriguing enzyme with significant applications in the food and pharmaceutical industries. To increase the yield of L-Asparaginase, the best medium was formulated by comparing seven different media. Modified Czapek Dox broth medium formulation was selected and taken up for optimization to achieve maximum production of L-Asparaginase. The main components that were optimized are carbon, nitrogen, amino acid inducer sources, pH and temperature. By combining the application of conventional and statistical methods of optimization, the selection of suitable medium components at correct levels maximized the L-Asparaginase production. This resulted in 2.29-fold increase in the L-Asparaginase yield from 8.7 to 19.99 U $mL^{-1}$. To conclude, given the paucity of studies on L-Asparaginase production from endophytic fungi, the findings of the current study open up fresh avenues for designing novel methods for the synthesis of this enzyme, with the potential to yield L-Asparaginase with superior properties to L-Asparaginase produced by bacteria that are available on the market currently.

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