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Optimizing the culture conditions for L-Asparaginase production from endophytic fungus *Curvularia* sp. LCJ413 through conventional and statistical approach

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Optimization

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RSM

ABSTRACT

L-Asparaginase (L-ASNase) is a crucial anti-tumour drug used to cure acute lymphocytic leukaemia. The current study aimed to enhance the production medium for the endophytic fungus *Curvularia* sp. LCJ413 that showed significant L-ASNase activity. L-ASNase production from *Curvularia* sp. LCJ413 was examined in six different media to select an appropriate liquid medium. Among the various media tested, Modified Czapek Dox broth (MCDB) exhibited the maximum L-ASNase activity (8.81 ± 0.52 U/mL). Physical (pH and temperature) and nutritional (carbon, nitrogen, inducer, and their concentrations) parameters were also optimized to boost L-ASNase production. Results of the study suggested a temperature of 28°C at pH 7 with 2 g/L maltose, 10 g/L L-Asparagine, and 25 g/L ammonium sulphate as the optimal carbon, inducer, and nitrogen source resulted in a high L-ASNase activity of 18.9 ± 0.40 U/mL. The statistical enhancement of L-ASNase by Response Surface Methodology (RSM) produced 20.11 U/mL of L-ASNase, which was 2.2 fold higher than the non-optimized medium. This is the first study on L-ASNase production from the endophytic *Curvularia* sp. LCJ413 isolated from *Vitex negundo* medicinal plant. Continuous fermentation with the medium composition provided in the study can produce L-ASNase on a large scale.

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1 Introduction

L-ASNase is an amidase enzyme that catalyzes the breakdown of L-Asparagine into L-Aspartate and ammonia. For more than four decades, this enzyme has been used to treat leukaemia and lymphoid system carcinomas (da Cunha et al. 2018). Aside from its medicinal properties, L-ASNase is widely utilized in the food sector to combat acrylamide (human carcinogen) formed once starch-rich foods are processed at extreme temperatures (Muneer et al. 2020). L-ASNases occurs in a broad spectrum of microorganisms, animals, and plants. Microbes are used for L-ASNase production because they are easier to process upstream and downstream than all other sources (Jia et al. 2021). Currently available L-ASNase products are made by bacteria and often induce toxicity and anaphylactic responses in patients (Moguel et al. 2020). As a result, L-ASNase synthesis by eukaryotic organisms received a lot of attention. The side effects of fungal L-ASNases are mild compared to bacterial L-ASNases (Meghavarnam et al. 2022).

L-ASNases from fungi have gained prominence as they are extracellular, simple to extract, and can be processed downstream (da Cunha et al. 2019). Plant-derived endophytic fungi have the ability to synthesize various exogenous enzymes such as proteases, lipases, xylanases, amylases, chitinases, cellulases, laccases, and asparaginases (Raghav et al. 2022). However, their use in enzyme synthesis for the food, pharmaceutical, and biotechnological industries and human welfare is limited (Mishra et al. 2019). Several strategies for producing L-ASNase from fungal endophytes have been investigated, such as solid-state (Supriya et al. 2015; Krishnapura and Belur 2020; Singh and Sao 2021) and submerged fermentations (Uzma et al. 2016; Jenila and Gnanadoss 2018; Priya and Subashini 2022). Sarquis et al. (2004) reported that submerged fermentation is a highly efficient method that demands less energy and poses little risk of contamination.

The extracellular synthesis of L-ASNase primarily relies on the enhancement of culture parameters to produce it in large quantities using a low-cost method. L-ASNase production is based mainly on optimizing nitrogen, carbon sources, and other parameters such as inoculum size, pH, and temperature (Moubasher et al. 2022). Enhancement of bioprocess using one factor at a time (OFAT) is an accepted technique. However, there are still a few drawbacks, such as increased time consumption and experimental runs, and a lack of information about the interaction between the process variables (Abdel-Fattah and Olama 2002). The utilization of a statistical approach for monitoring and optimizing process parameters is ideally suited to analyze the interactive impact of factors on the desired outcome (Ghosh et al. 2013). RSM is a statistical approach for optimizing organism growth conditions to enhance total biomass and metabolite synthesis (Abhini et al. 2022).

Many studies on L-ASNase production from numerical microbes have been reported, but L-ASNase production from endophytic fungi has rarely been examined. In this regard, this study was to select an appropriate liquid medium and enhance its culture parameters for the production of L-ASNase by the endophytic *Curvularia* sp. LCJ413 through conventional (OFAT) and statistical (RSM) methods.

2 Materials and Methods

2.1 Isolation and screening of *Curvularia* sp. LCJ413 for L-ASNase activity

The endophytic *Curvularia* sp. LCJ413 was isolated from *Vitex negundo* L. medicinal plant collected from Loyola College, Chennai (Kathiravan and Gnanadoss 2022). *Curvularia* sp. LCJ413 exhibited high L-ASNase activity on modified Czapek Dox agar medium added with phenol red (2.5 %) as substrate. Later, the isolate was cultured on the potato dextrose agar slants for further experiments at 4 ˚C. This suggests that large-scale production of the L-ASNase enzyme may be feasible.

2.2 Selection of suitable medium for L-ASNase Production

Submerged fermentation requires a liquid medium for L-ASNase production. Hence, L-ASNase production was investigated in six different basal media namely modified Czapek Dox broth (Gulati et al. 1997), modified M9 medium (Moorthy et al. 2010), modified ISP-2 broth (Mangamuri et al. 2017), Asparagine dextrose salts broth (El-Naggar et al. 2015), Glucose asparagine broth (Patro et al. 2014) and Glycerol asparagine broth (Palaniappan et al. 2013) respectively. For this, 100 mL of different media was prepared in separate conical flasks and autoclaved at 121ºC for 15 min. To avoid bacterial contamination streptomycin (100 µg/mL) was added to each liquid medium. Under sterile conditions, three mycelial discs (5mm) of *Curvularia* sp. LCJ413 cultured in different liquid media and maintained for about 8 days at 120 rpm. After every 24 hours, 0.5 mL of culture filtrate was pipetted and centrifuged for 10 min at 10,000 rpm. After centrifugation, supernatants were collected to determine the L-ASNase activity.

2.3 L-ASNase activity

Imada et al. (1973) proposed that the volume of NH_3 released from L-Asparagine was used to determine L-ASNase activity using Nessler's reagent. In brief, the reaction mixture consisting of culture filtrate (0.5 mL), 0.5 M tris HCl buffer (0.5 mL), 0.04 M L-Asparagine (0.5 mL), and distilled water (0.5 mL) was incubated for 30 min. Following incubation, 1.5 M trichloroacetic acid (0.5 mL) was added to terminate the reaction. Then the reaction solution was spun at 10,000 rpm for 15 minutes. A collection of 0.1 mL supernatant followed by 3.7 mL distilled water and 0.2 mL

Nessler's reagent were mixed and incubated for about 20 minutes. Absorbance was recorded at 450 nm. One unit of asparaginase is the quantity of enzyme necessary to produce 1 µmol of NH_3 per minute under specific conditions. The L-ASNase activity was determined using the formula below (Balbool and Abdel-Azeem 2020).

$$\text{Enzyme activity } \left(\frac{\text{U}}{\text{mL}}\right) = \frac{NH_3 \text{ liberated } (\mu M) * \text{Reaction mixture (initial volume)}}{\text{Reaction mixture (final volume)} * \text{Volume of enzyme used} * \text{time of incubation}}$$

2.4 Protein estimation

Protein concentrations were determined using the method by Lowry et al. (1951) using bovine serum albumin as standard. Briefly, 1000 µL of the crude enzyme (diluted) was added to 5 mL of alkaline copper solution and maintained at room temperature for 10 minutes. Then the reaction solution was mixed with Folin's phenol reagent (0.5 mL) followed by 30 minutes of incubation. After incubation, the intensity of the formed blue colour was measured at 660 nm.

2.5 Optimization studies for L-ASNase production by submerged fermentation

The primary objective of medium optimization is to investigate the operational parameters that enhance enzyme production. Inducer, nitrogen, and carbon sources are important factors when optimizing the medium to improve L-ASNase production. They are also essential for synthesizing key nutrients in a liquid medium for organism development. The nutritional parameters were optimized by OFAT (Jenila and Gnanadoss 2018). The L-ASNase production from endophytic *Curvularia* sp. LCJ413 was examined in MCDB consisting of 2 g/L glucose,1.52 g/L KH_2PO_4, 10 g/L L-Asparagine, 0.52 g/L $MgSO_4$, 0.52 g/L KCl, 0.0001 g/L of $FeSO_4$, $CuNO_3$, $ZnSO_4$ and distilled water (1L).

2.5.1 Influence of carbon source and its concentration on L-ASNase production

The influence of various carbon sources on L-ASNase production from *Curvularia* sp. LCJ413 was investigated in MCDB. Lactose, dextrose, maltose, sucrose, and galactose were used as carbon sources. In separate sets of experiments, the original carbon source in the medium (glucose) was replaced by various carbon sources at 2 g/L concentration, while the standard MCDB medium was treated as a control. After selecting the optimal carbon source, the concentration of the selected carbon source was optimized (1-6 g/L). Protein and L-ASNase activities were measured. The optimal carbon source was chosen for subsequent experiments.

2.5.2 Influence of nitrogen source and its concentration on L-ASNase production

The L-ASNase production from *Curvularia* sp. LCJ413 was examined using different organic (yeast extract, urea, and peptone) and inorganic (ammonium sulphate and potassium nitrate) nitrogen sources at 10 g/L concentration in MCDB and the standard MCDB medium was employed as control. After selecting the optimal nitrogen source, the concentration of the chosen nitrogen source was optimized at various concentrations (5 to 30 g/L).

2.5.3 Influence of inducer and its concentration on L-ASNase production

Various inducers influence on L-ASNase production from *Curvularia* sp. LCJ413 was investigated. In separate experiments, MCDB was treated with inducers such as L-Arginine, L-Tryptophan, L-Tyrosine, L-Asparagine, and L-Glutamic acid at 10 g/L concentration. After selecting the best inducer, the concentration of the inducer was optimized (5 to 30 g/L).

2.5.4 Influence of pH on L-ASNase production

The pH of the MCDB was varied from 4 to 9 for the L-ASNase production from *Curvularia* sp. LCJ413 using 0.1N HCL and 0.1N NaOH. The L-ASNase and protein activities were measured every day.

2.5.5 Influence of temperature on L-ASNase production

Different incubation temperatures (28 ºC to 36 ºC) on L-ASNase production from the isolate *Curvularia* sp. LCJ413 in MCDB was investigated.

2.6 Optimizing the L-ASNase production by RSM

Central Composite Design (CCD) was employed in RSM to investigate the optimized medium parameters for enzyme production. Face Centre Central Composite Design (FCCCD) was adopted to identify the values of critical parameters (maltose, ammonium sulphate, L-Asparagine, and pH) for investigation. The Design Expert 13 software was used to estimate test results. Table 1 shows the four variables investigated at three levels (-1, 0, and +1).

To predict the pure error, 30 trials were designed, and four factors in a minimum and maximum range were investigated using CCD. A polynomial function used to describe the experimental result revealed the response (L-ASNase production).

$$Y= \beta_0 + \beta_1A+ \beta_2B+ \beta_3C+ \beta_4D+ \beta_{11}A^2+ \beta_{22}B^2+ \beta_{33}C^2+ + \beta_{44}D^2+ \beta_{12}AB+ \beta_{13}AC+ \beta_{14}AD+ \beta_{23}BC+ \beta_{24}BD+ \beta_{34}CD$$

Table 1 Experimental range and levels of four independent variables for L-ASNase production from *Curvularia* sp. LCJ413

		Range of levels					
Variables	Symbol	Actual	coded	Actual	coded	Actual	Coded
Maltose (g/L)	A	1	-1	2	0	3	+1
Ammonium sulphate (g/L)	B	20	-1	25	0	30	+1
pH	C	6	-1	7	0	8	+1
L-Asparagine (g/L)	D	5	-1	10	0	15	+1

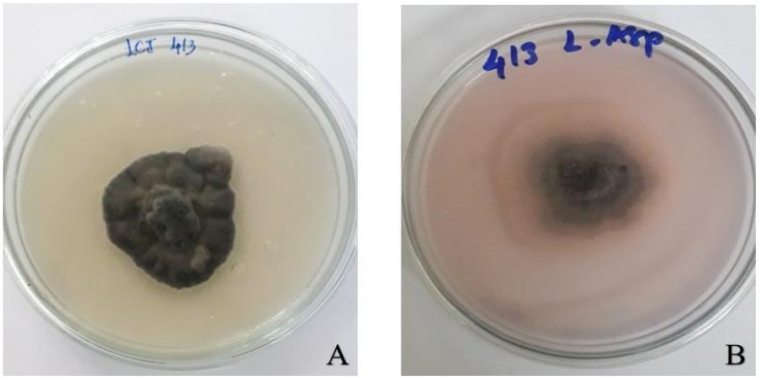

Figure 1 A) Pure culture of endophytic fungus *Curvularia* sp. LCJ413
B) *Curvularia* sp. LCJ413 showing L-ASNase activity on modified Czapek Dox agar

Here, Y - L-ASNase production (predicted response), β_0 – Intercept, Squared coefficients (β_{11}, β_{22}, β_{33}, β_{44}), Linear coefficients (β_1, β_2, β_3, β_4), and Interaction coefficients (β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34})

To support the statistical model, regression analysis was used to determine coefficients and significance levels. The analysis of variance (ANOVA) by design expert 13 was employed for the L-ASNase production in the experimental design. The response surface graphs were computed to determine the ideal parameter levels for increased L-ASNase production.

2.7 Comparative study of original and optimized medium for L-ASNase production

The effectiveness of the improved medium (MCDB) on L-ASNase production from *CurvIularia* sp. LCJ413 was demonstrated by comparing the original (standard MCDB) and optimized medium. *Curvularia* sp. LCJ413 was cultured in 1L of standard MCDB as well as optimized medium and maintained for 8 days as separate experiments, and the L-ASNase activity was estimated every day.

3 Results and Discussion

3.1 Isolation and screening of *Curvularia* sp. LCJ413 for L-ASNase activity

The endophytic *Curvularia* sp. LCJ413 was isolated from *V. negundo* medicinal plant. The culture showed high activity for the L-ASNase enzyme in plate assay (modified Czapek Dox agar) (Figure 1). The 18S rRNA sequence of *Curvularia* sp. LCJ413 was submitted, and the accession number MZ646132 was obtained from Genbank. Jenila and Gnanadoss (2018) reported L-ASNase producing endophytic *Fusarium* sp. from *Adhatoda vasica* medicinal plant. L-ASNase has been revealed to be used therapeutically as an antileukaemic drug, industrially as a biosensor, and in amino acid biosynthesis (Balbool and Abdel-Azeem 2020). Fungal endophytes have the potential to produce a wide array of secondary metabolites and enzymes with a diverse range of biological properties. Nevertheless, endophytic fungi are sparingly exploited as a reservoir of industrially essential enzymes (Correa et al. 2014).

3.2 Selection of Liquid medium for L-ASNase production

The L-ASNase and protein was produced in six different basal media for about 8 days. The L-ASNase production significantly varied across the selected media. Among the six studied media, MCDB showed maximum enzyme production of 8.81 ± 0.52 U/mL, while the lowest L-ASNase production was reported in modified ISP-2 media, i.e., 6.14 ± 0.346 U/mL on the 6th day. The results from this study demonstrated that other media also exhibited L-ASNase activity from *Curvularia* sp. LCJ413 but relatively less enzyme activity when compared with MCDB (Figure 2). Solid-state or submerged fermentation can produce L-ASNase (Doriya and Kumar 2016). The L-ASNase production was improved mainly by the fermentation medium, particularly the nitrogen and carbon sources, as well as physical parameters like

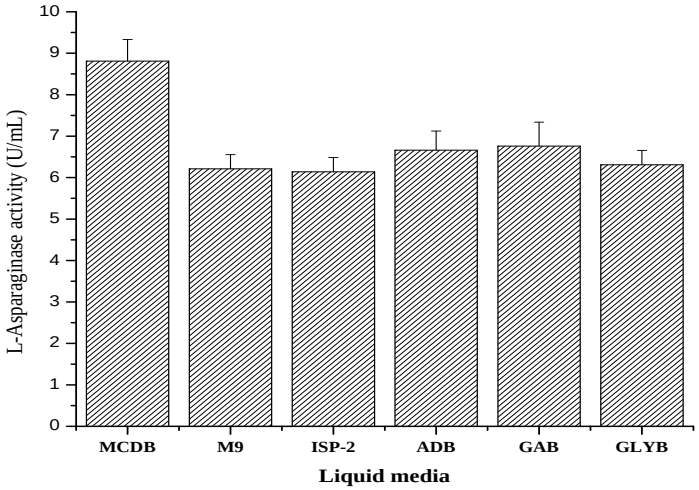

Figure 2 Selection of suitable liquid media for L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day (*Modified Czapek Dox broth – MCDB, M9 - modified M9 medium, ISP-2 – Modified ISP-2 medium, ADB - Asparagine Dextrose Salts Broth, GAB - Glucose Asparagine Broth, GLYB - Glycerol Asparagine Broth)

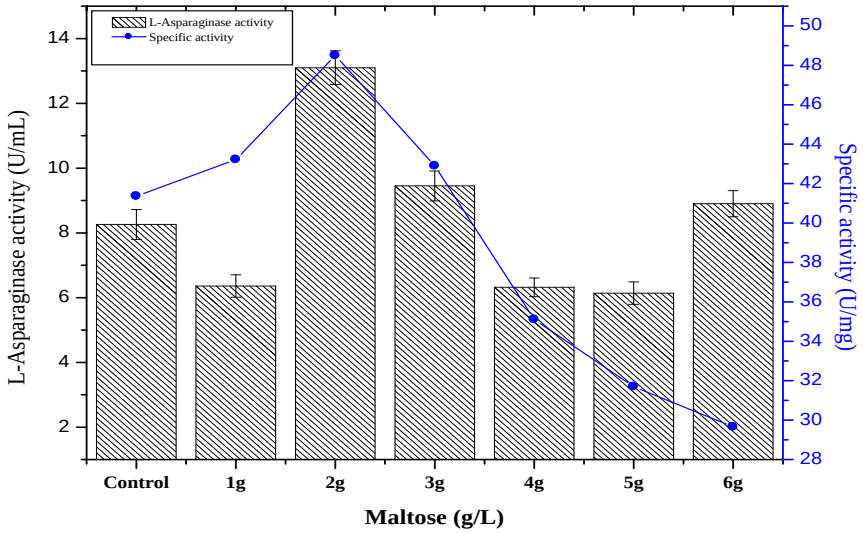

Figure 3 Influence of maltose concentrations on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

inoculum size, incubation time, temperature, and pH (da Cunha et al. 2019). Several other research works on L-ASNase production from different endophytic fungi were reported by Araujo-Magalhaes et al. (2021), Chow and Ting (2021), and Jenila and Gnanadoss (2018).

3.3 Influence of carbon source and its concentration

In this study, glucose was substituted with various carbon sources like maltose, lactose, sucrose, dextrose, and galactose in the fermentation medium. The findings showed that maltose displayed the highest L-ASNase production (11.3 ± 0.231 U/mL) compared to other carbon sources tested on the sixth day of incubation from *Curvularia* sp. LCJ413. Subsequently, the L-ASNase production decreased when galactose was used as a carbon source (4.98 ± 0.115 U/mL) (Table 2). The influence of maltose (1 to 6 g/L) was also investigated in the MCDB. The optimal L-ASNase production was observed at maltose (2 g/L), i.e., 13.1 ± 0.52 U/mL (Figure 3). The increased L-ASNase production from the endophytic fungus *Talaromyces pinophilus* was achieved using starch 10 g/L as a carbon source (Krishnapura and Belur 2016). Incorporating glucose as the carbon source in the MCDB medium resulted in the maximum L-ASNase production by the endophytic *Aspergillus niger* (El-said et al. 2016). *Trichoderma viridae*, a marine soil fungus, exhibited L-ASNase activity of 759.5 U/mL, with maltose as the carbon source (Lincoln et al. 2015).

Table 2 Influence of different carbon, nitrogen, and inducer sources on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

Sources	L-ASNase activity (U/mL)
Carbon source (2 g/L)	
Dextrose	8.45 ± 0.289
Maltose	11.3 ± 0.231
Sucrose	7.98 ± 0.462
Lactose	8.58 ± 0.52
Galactose	4.98 ± 0.115
Nitrogen source (10 g/L)	
Potassium nitrate	3.63 ± 0.231
Peptone	5.95 ± 0.404
Ammonium sulphate	14.5 ± 0.346
Urea	5.15 ± 0.173
Yeast extract	6.06 ± 0.289
Inducer source (10 g/L)	
L-Arginine	14.5 ± 0.462
L-Tryptophan	15 ± 0.115
L-Tyrosine	14.9 ± 0.462
L-Asparagine	17.7 ± 0.52
L-Glutamic acid	14.7 ± 0.173

3.4 Influence of nitrogen source and its concentration

In the MCDB, the effects of different nitrogen sources (potassium nitrate, peptone, urea, yeast extract, and ammonium sulphate) on L-ASNase production were investigated. The results proved that better production of L-ASNase (14.5 ± 0.346 U/mL) was attained with the addition of ammonium sulphate. Potassium nitrate as a nitrogen source yielded the lowest L-ASNase activity in a medium (Table 2). The optimal nitrogen source was found to be ammonium sulphate which was further investigated at various concentrations (5-30 g/L). The optimal L-ASNase production was 16.1 ± 0.289 U/mL at 25 g/L (Figure 4). The results from this study follow the results of Jenila and Gnanadoss (2018), where ammonium sulphate at 20 g/L improved the L-ASNase production by the endophytic fungus *Fusarium* sp. According to Baskar and Renganathan (2009), peptone was utilized as an additional nitrogen source by *Aspergillus terreus* to enhance L-ASNase production. Many reports suggest that L-Asparagine is the sole source of nitrogen for the increased L-ASNase production (Benchamin et al. 2019; Prihanto et al. 2019; Doriya and Kumar 2016; Abbas Ahmed et al. 2015). The nitrogen source is critical for L-ASNase production. Microorganisms can utilize organic or inorganic nitrogen sources (Elshafei and El-Ghonemy 2015).

3.5 Influence of inducer and its concentration

Different amino acid inducers were studied for their effect on L-ASNase production. However, the L-Asparagine with enzyme activity of 17.7 ± 0.52 U/mL served as a favourable inducer source for the L-ASNase production. Subsequently, the inducers such as

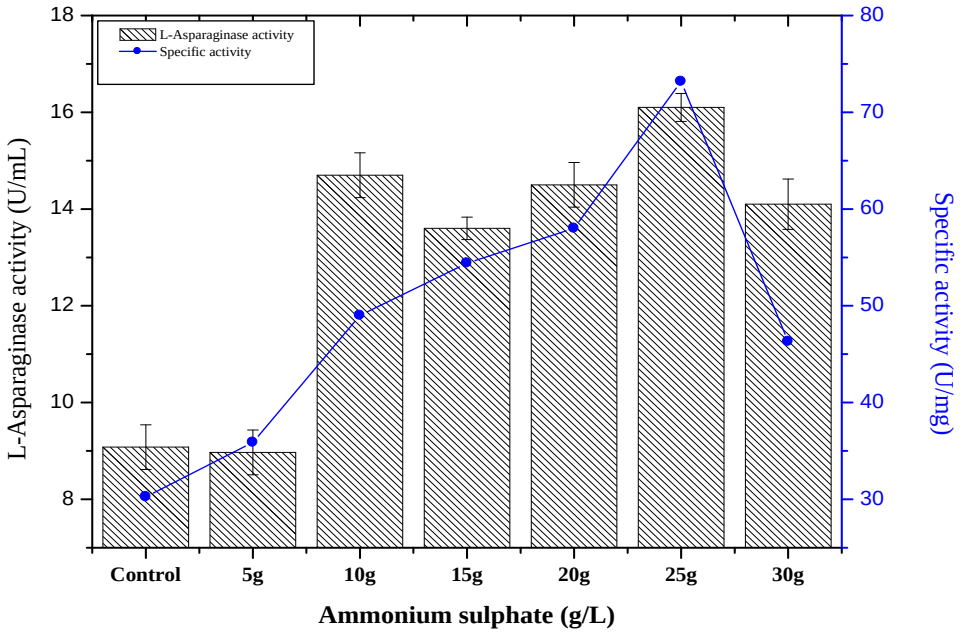

Figure 4 Influence of ammonium sulphate concentrations on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

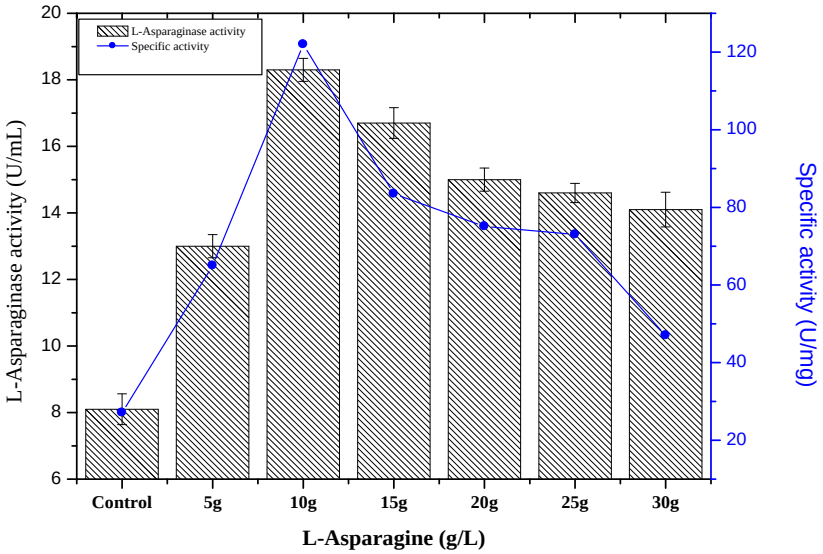

Figure 5 Influence of L-Asparagine concentrations on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

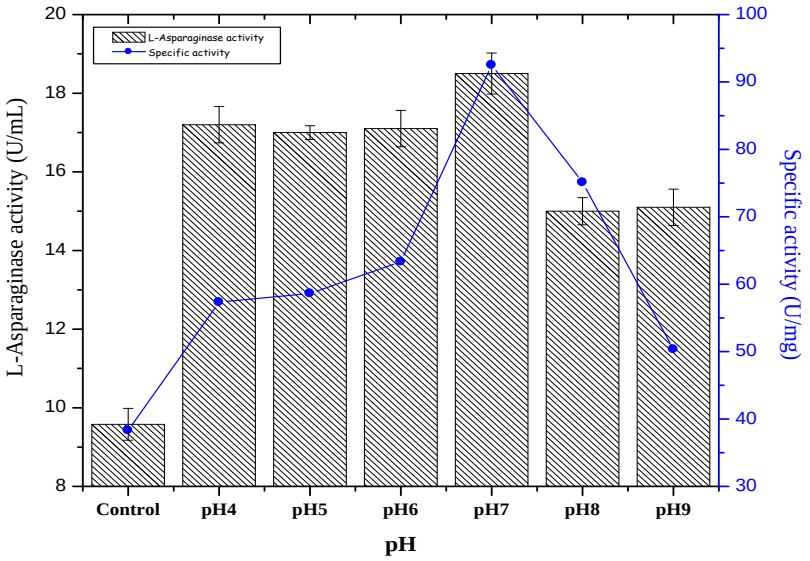

Figure 6 Influence of pH on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

L-Arginine, L-Tryptophan, L-Glutamic acid, and L-Tyrosine recorded L-ASNase production of about 14.5 ± 0.462 U/mL, 15 ± 0.115 U/mL, 14.7 ± 0.173 U/mL, and 14.9 ± 0.462 U/mL respectively (Table 2). L-ASNase production was also studied at various L-Asparagine concentrations (5-30 g/L). 10 g/L showed optimum L-ASNase production of 18.3 ± 0.346 U/mL for *Curvularia* sp. LCJ413 (Figure 5). L-Asparagine has been reported to be used as an inducer for increasing L-ASNase production (Elshafei and El-Ghonemy 2015). Abbas Ahmed et al. (2015) reported high L-ASNase production of 35.16 U/mL from marine endophytic fungus *Aspergillus* sp. ALLA2000 was achieved using the amino acid arginine as an inducer.

3.6 Influence of pH

The effect of different pH (4 - 9) on L-ASNase production was evaluated in MCDB. At pH 7, the highest L-ASNase activity of 18.5 ± 0.52 U/mL was recorded. L-ASNase production reduced as the pH increased (Figure 6). Kalyanasundaram et al. (2015) reported endophytic *Aspergillus terreus* produced high L-ASNase activity at pH 7 (32.25 U/mL). The pH of the growth medium is critical for nutrient transfer across the cellular membranes and the increase in L-ASNase production (Farag et al. 2015). For optimal L-ASNase production, a pH range of 6.3 to 9.0 culture medium was used (da Cunha et al. 2019).

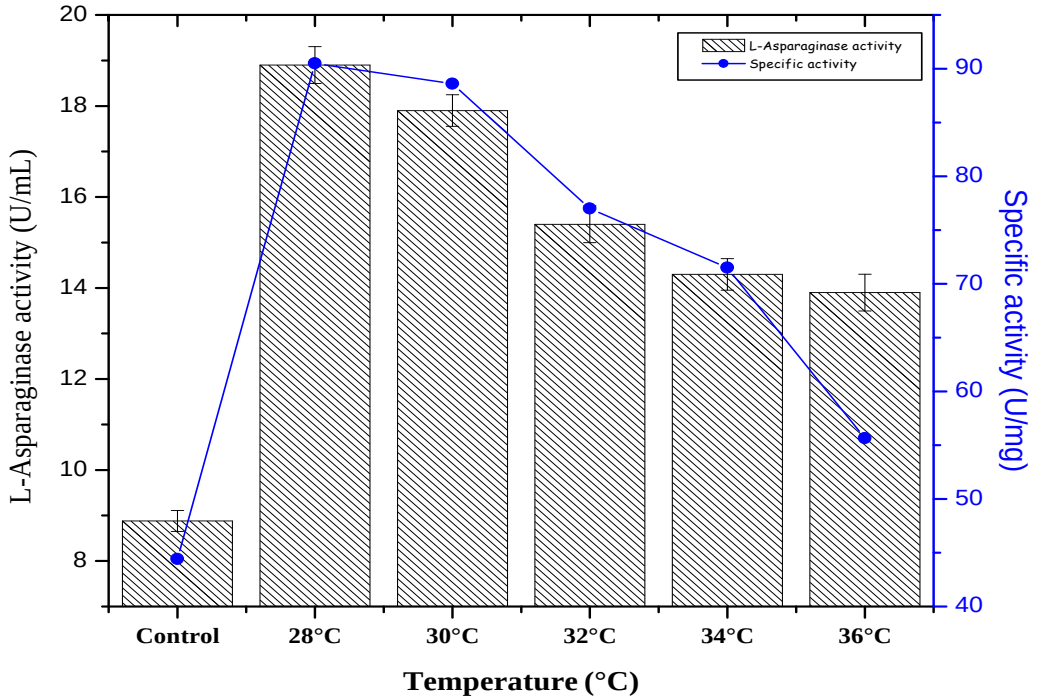

Figure 7 Influence of temperature on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

3.7 Influence of temperature

Curvularia sp. LCJ413 could thrive and produce L-ASNase at various temperatures (28°C - 36°C). At 28°C, L-ASNase production reached a maximum of 18.9 ± 0.404 U/mL. Higher temperatures were found to reduce L-ASNase activity. The lowest level of L-ASNase production was 13.9 ± 0.404 U/mL at 36°C (Figure 7). The reduction in L-ASNase production might be due to heat generation inside the medium, which causes enzyme denaturation. Temperature influences the stability and rate of catalysis of the enzyme. Temperature tolerance and stability of L-ASNase vary between the isolated fungal species (Chand et al. 2020), and it was reported that 27°C was the ideal temperature for L-ASNase production from *Fusarium* sp. (Thirunavukarasu et al. 2011), 30.5°C for *Fusarium proliferatum* (Yap et al. 2021), and 35°C for *Aspergillus terreus* (Farag et al. 2015). The L-ASNase enzyme was most active between 25°C and 45°C (Castro et al. 2021).

3.8 Optimizing the L-ASNase production using RSM

The effect of four variables such as maltose, ammonium sulphate, pH, and L-Asparagine were studied by FCCCD using RSM. Additional trials were investigated in triplicate at the shake flask level with the optimized medium to validate the model's prediction. The FCCCD results examine the influence of four independent variables, namely maltose, ammonium sulphate, pH, and L-Asparagine, on L-Asparagine production, together with the experimental and predicted response (Table 3).

The p-value in the model was 0.0001 based on the ANOVA test, which is <0.005, representing that the model terms are substantial. The R^2 value of this study was 0.99, indicating that the model describes 99% of the variance in the response. The predicted sum of squares was 11.86, the adequate precision ratio was found to be 39.11, and the coefficient of variance was 2.70% (Table 4). All these responses validate that this model has the potential to initiate the design space for L-ASNase production. The FCCCD experimental findings were modelled with the following polynomial equation that predicted L-ASNase activity;

$$Y= 19.18 + 1.58A + 1.64B – 0.5989C + 0.7267D – 2.04A2 – 0.7090B2 – 1.68C2 – 0.4490D2 + 0.5456AB + 0.2744AC – 0.4494AD – 0.2069BC – 0.5356BD – 0.0794CD$$

Where Y is the activity of L-ASNase (U/mL); A, B, C, and D are maltose, ammonium sulphate, pH, and L-Asparagine, respectively

Three-dimensional graphs were created using RSM to interpret the association of selected components and the optimal concentration necessary for maximal L-ASNase synthesis (Figure 8-10). The highest L-ASNase production of 20.11 U/mL was attained using the combination of 2 g/L maltose, 10 g/L L-Asparagine, pH 7, and 30 g/L ammonium sulphate. The highest enzyme yield in this experiment was 2.28 times higher than the unoptimized medium. At the highest levels of all four parameters, L-ASNase production decreased. The model was evaluated in a designed space, the predicted responses were similar to the experimental responses,

and the model was successfully validated. The optimization of L-ASNase using RSM was helpful to this research because it aided in quickly identifying essential factors and their interactions. It also suggested the importance of the range of factors at altered levels. In the study conducted by Baskar and Renganathan (2011), optimization of L-ASNase using RSM yielded 35.73 U/mL of the enzyme from *A. terreus* MTCC under ideal conditions. The highest L-ASNase production (15.7808 U/mL) was attained using RSM resulting in a 108.62% increase in the L-ASNase production from *A. niger* (Vala et al. 2018).

Table 3 Experimental and predicted responses for L-ASNase production using FCCCD

Run	Maltose	Ammonium sulphate	pH	L-Asparagine	L-ASNase activity (U/mL)	
					Experimental value	Predicted value
1	-1	-1	-1	-1	10.38	10.50
2	-1	1	1	-1	12.34	12.17
3	-1	-1	1	-1	9.49	9.32
4	1	-1	1	-1	12.94	12.85
5	-1	1	-1	-1	13.93	14.17
6	1	1	1	-1	17.42	17.88
7	1	1	-1	-1	19.12	18.78
8	0	0	0	-1	18.24	18.01
9	1	-1	-1	-1	12.74	12.92
10	0	0	0	0	19.21	19.18
11	0	0	0	0	19.46	19.18
12	0	0	0	0	18.86	19.18
13	0	0	-1	0	18.11	18.10
14	0	0	0	0	19.57	19.18
15	1	0	0	0	18.74	18.72
16	0	-1	0	0	16.5	16.83
17	0	0	0	0	19.36	19.18
18	0	1	0	0	20.11	20.12
19	0	0	1	0	16.56	16.90
20	-1	0	0	0	15.2	15.55
21	0	0	0	0	19.64	19.18
22	-1	1	1	1	13.24	13.29
23	-1	-1	1	1	12.57	12.59
24	1	1	-1	1	18.02	18.43
25	1	1	1	1	17.64	17.20
26	-1	1	-1	1	15.84	15.61
27	1	-1	1	1	14.32	14.31
28	1	-1	-1	1	14.86	14.71
29	0	0	0	1	18.89	19.46
30	-1	-1	-1	1	14.3	14.08

Table 4 ANOVA analysis to validate the model's adequacy

Statistics	Model's response (L-ASNase production)
Standard deviation	0.3902
Mean	16.25
R^2	0.9914
F-value (lack of fit)	2.32
Adjusted R^2	0.9834
Coefficient of Variance	2.40
Predicted R^2	0.9554
PRESS	11.86
Adequate precision	39.11

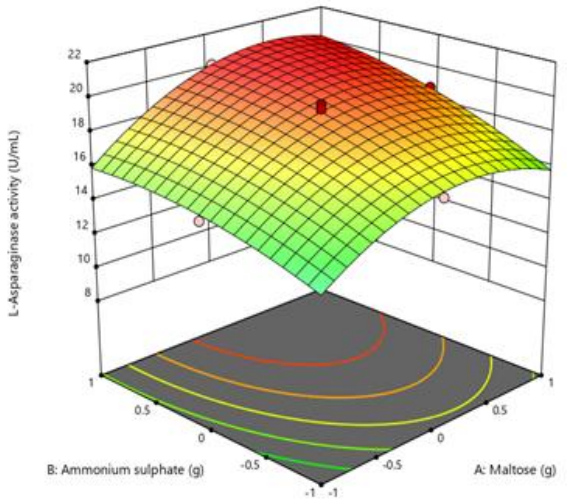

Figure 8 RSM plot representing the effect of interaction between maltose and ammonium sulphate on L-ASNase production

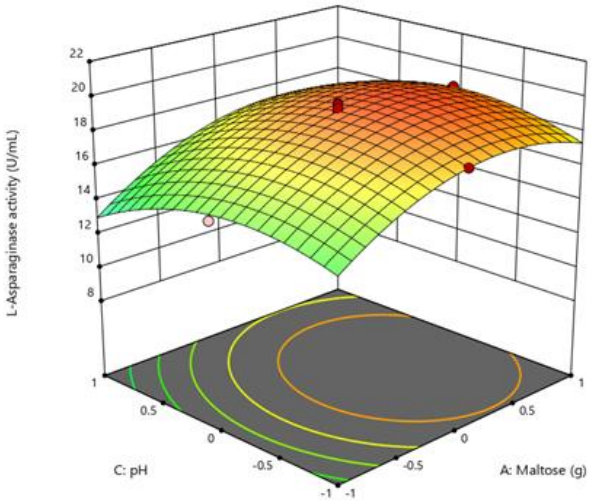

Figure 9 RSM plot representing the effect of interaction between maltose and pH on L-ASNase production

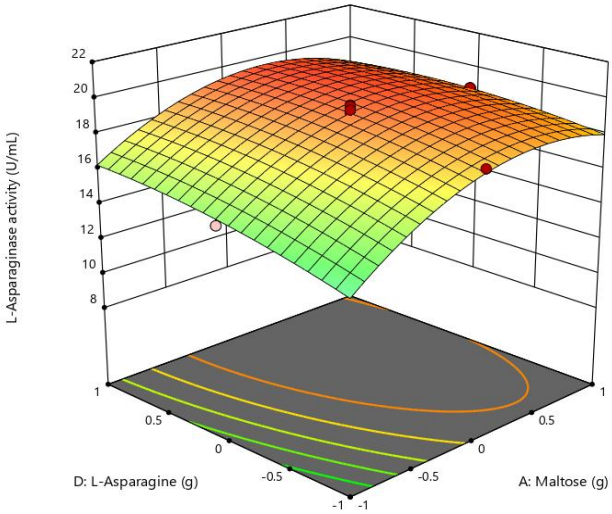

Figure 10 RSM plot representing the effect of interaction between maltose and L-Asparagine on L-ASNase production

Table 5 Comparative study of original and optimized medium for L-ASNase production

Medium	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Original medium	2.91 ± 0.231^{e}	3.86 ± 0.173^{de}	5.98 ± 0.346^{bc}	8.79 ± 0.346^{a}	6.54 ± 0.173^{b}	5.08 ± 0.289^{cd}
Optimized medium	12.7 ± 0.346^{e}	14.8 ± 0.404^{cd}	17.6 ± 0.346^{ab}	18.98 ± 0.52^{a}	16.2 ± 0.462^{bc}	14.1 ± 0.462^{de}

ANOVA (one-way) and TUKEY tests were performed. Values are in means ± SEM

3.9 Comparative study of original and optimized medium for L-ASNase production

The maximum L-ASNase production from *Curvularia* sp. LCJ413 in the original (standard MCDB) medium was 8.79 ± 0.346 U/mL. The observed L-ASNase activity in the MCDB after conventional optimization of parameters was found to be 18.98 ± 0.52 U/mL (Table 5). Thus, traditional one-factor optimization resulted in a 2.15 fold increase. Numerous studies on optimizing the process parameters for L-ASNase production were reported by Krishnapura and Belur (2016), Yap et al. (2021), Jenila and Gnanadoss (2018), and Abhini et al. (2022).

Conclusion

Endophytic fungus *Curvularia* sp. LCJ413 was an effective producer of the L-ASNase enzyme under submerged fermentation. On the 6th day, L-ASNase production in submerged fermentation was enhanced to 18.9 ± 0.404 U/mL with pH 7, an incubation temperature of 28°C, 2 g/L (maltose), 25 g/L (ammonium sulphate), and 10 g/L (L-Asparagine). Furthermore, statistical optimization with RSM raises L-ASNase production to 20.11 U/mL. A good level of resemblance was noticed with the experimental and predicted data indicating the reliability and applicability of RSM for enhancing L-ASNase production. Response surface plots also revealed the substantial interaction between four variables and their impact on L-ASNase production. The optimization approach used in this study can be implemented to upscale the yield of other enzyme production. Additional studies on the purification and characterization of L-ASNase will help in establishing its application in the food (for acrylamide mitigation) and pharmaceutical (as a chemotherapeutic agent) industries. The application of cutting-edge recombinant technology may increase the overall yield of this therapeutic enzyme L-ASNase.

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Conflicts of interest

The authors have no conflicts of interest.

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