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Production, Purification, Characterization And Anti-Leukemic Activity Of L-Asparaginase From Streptomyces Spp Aia 1 Section: Microbial And Enzyme Technology

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Abstract
Future therapeutic applications of L asparaginase in the treatment of leukemia and lymphoid system cancers are expected to cause the demand for the enzyme to increase several times over. It is therefore imperative to find appropriate sources of production. The novel L asparaginase producer (Streptomyces sp AIA 1) produced in this study offers a proof of concept for its production. It was isolated and then optimized by a solid state fermentation process employing a low-cost, straightforward medium made of soybean meal and rice bran. Examined were the effects of seven distinct independent parameters on the synthesis of L-asparaginase in solid state fermentation. Using the Plackett–Burman design, the effects of seven distinct independent parameters were investigated with regard to L-asparaginase synthesis under solid state fermentation conditions. Ammonium sulfate, FeSO4.7H2O, and soybean meal were the main variables influencing the synthesis of L-asparaginase. Thus, the ideal values of these variables were ascertained by the use of central composite design. Ammonium sulfate was used to purify L-asparaginase, and the final purification fold was 39.87,this was achieved by Sephadex G25 column chromatography and DEAE cellulose 52 ion exchange chromatography. The SDS-PAGE approach yielded a monomeric molecular weight of 30 kDa for the purified L-asparaginase. When L-asparaginase was tested in vitro on the leukemic cancer cell line K562 and the normal cell line PBMC, it was discovered to exhibit potent anti-proliferative properties. The outcomes demonstrated that the K562 cell lines (IC50 = 17.63) was subjected to the greatest cytotoxic effect of L-asparaginase.

Keyword: Anticancer Activity; Characterization; Plackett–Burman Design and Central Composite Design;Purification; Solid State Fermentation; Streptomyces Spp AIA 1

I. INTRODUCTION

L-Asparaginases remain an appealing study topic 120 years after their discovery, and were primarily introduced in the 1960s as an effective anti-leukemic agent (Loch *et al*., 2021). Asparaginase ((L-asparagine amidohydrolase EC 3.5.1.1) is a significant catalyst utilized in the drug and food enterprises and has hostile to cancer-causing potential for the therapy of acute lymphoblastic leukemia, lymphomas and other malignancies (El-Gendy*et al*., 2021).

By attacking the metabolic pathways of cancer cells and causing cellular death from malnutrition, L-asparaginase transforms L-asparagine into L-aspartic acid and ammonia (Martinez-Outschoorn, 2017). Asparagine synthetase, which is necessary for asparagine synthesis and is regulated by the cytosine methylation process, is a component of normal cells (Asthana and azmi, 2003). Asparagine synthetase is encoded by a gene located on chromosome 7q21.3 (Lomelino*et al*., 2017). A crucial amino acid needed for the growth and multiplication of cancer cells is asparagine. The synthesis of asparagine amino acid is inhibited by the absence of asparagine synthetase. When asparagine levels are low, RNA and protein synthesis is inhibited, which causes leukemia cell types to enter cell cycle arrest and undergo apoptosis(Prasad *et al*., 2014)
L-asparaginase has a broad distribution in microorganisms (bacteria, fungi, yeast, actinomycetes), plants, animals (fish, mammals, and birds), and tissues (liver, pancreas, brain, kidneys, and lungs). However, L-asparaginase is not found naturally in humans (Castro *et al*., 2021).Commercial L asparaginase is derived from *Escherichia coli* and *Erwiniacarotovora*(Ei-naggar*et al*., 2020). Therapeutically, some of the most successful preparations of asparaginase are the native *E.coli* L-asparaginase (Elspar), its peg-tag version (Oncaspar), and *E.chrysenthemi* L-asparaginase (Erwinaze) (Brumano*et al*., 2019). However, this bacterial asparaginase has a low substrate binding affinity and significant glutaminase activity. Glutaminase activity causes liver dysfunction, pancreatitis, leucopenia, neurological convulsions, and coagulation problems. As a result, a novel source of L-asparaginase with improved stability, decreased glutaminase activity, strong substrate affinity, and low Km values is required for therapeutic usage.

The effect of this catalyst is proven by the high worldwide interest, which came to $380 million in 2017 and is supposed to build up to $420 million by 2025 (Alam*et al*., 2019). The goal of the current study is to identify actinomycete that can successfully generate L-asparaginase as a substitute source of bacteria that are more productive, stable, and efficient.

Actinomycetes are believed to be a little-known source of L-asparaginase. The advantage of microorganisms, mostly actinomycetes in enzyme production is increased by their high yields, cost-effectiveness and vulnerability to genetic manipulation. But the research studies on actinomycetes are very limited, especially in respect of microbial habitats of rhizospheric soil. So, we have to explore the rhizosphere soil of medicinal plants to isolate actinomycetes for the production of important metabolites.

II. MATERIAL AND METHODS

L-asparagine used for the study was purchased from SiSCO Research Laboratories PVT Ltd (SRL). Other chemicals (analytical grade) used were obtained from different commercial sources.Blood cancer cell line K562 and normal cell line PBMC were procured from National Center for Cell Science (NCCS Pune, India).

Microorganism used in this study and cultural conditions

The *Streptomyces sp*. strain AIA 1 utilized in this investigation was isolated from a *C.urens* plant rhizosphere soil sample that was acquired from Maroli, Gujarat, India. The colony was isolated and maintained on actinomycete isolation agar medium that contained the following components (g/l): glycerol (5g), sodium caseinate (2 g), sodium propionate (4 g), asparagine (0.1 g), K_2HPO_4 (4.5g), $MgSO_4$ (4.1g), $FeSO_4$ (4.001 g), and asparagine (0.1 g). The organisms were sequenced using 16S rRNA at Saffron Life Science in Surat, India. After being placed in the gene bank, the data was given the accession number OP001983 (Gayatri*et al*., 2022).

Qualitative and quantitative screening for L-asparaginase production

Potential actinomycetes strains that produced L-asparaginase were identified through screening using M9 medium (6.0 g Na_2HPO_4; 3.0 g KH_2PO_4;NaCl 0.5 g; $MgSO_4$ 2.0 g; KCl 0.5 gm; glucose 3.0 g; L-asparagine 10.0 g; agar 20.0 g and 1000 ml distilled water). The pH was adjusted to 6.8 and supplemented with phenol red (prepared in ethanol) as a pH indicator (2.5%) and L-asparagine as a source of carbon and nitrogen. Incubation of the plates lasted seven days at 35°C. The pH indicator changed from yellow to pink as a result of the synthesis of L-asparaginase raising the culture medium's pH. The isolate with the pink zone around it was identified as a L-asparaginase-producing strain and was selected for more research. Control plates were made using a dye-free media. Rather than using L-asparagine as a substrate for L-glutaminase synthesis, L-glutamine was employed [Gulati *et al*., 1997].

Further, quantification of enzyme performed with positive isolates. Strong actinomycete strains were inoculated into a 250 ml Erlenmeyer flask that held 50 ml of ADS (Asparagine dextrose broth) medium, which contained 1% dextrose, 0.05% asparagine, 0.05% K_2HPO_4, 0.2% meat extract, and pH 7.0. After adding 5% inoculum to the media, the flasks were incubated for five days at 150 rpm and 35ºC. The medium was centrifuged for 20 min at 10000g after being filtered with Whatman filter paper No. 1. To measure the enzyme activity and perform a protein test, the supernatant was utilized as a crude enzyme (Imada*et al*., 1973)

Inoculum preparation

The spore inoculum was prepared using a seven-day-old culture that was grown on an actinomycete isolation agar slope. The spores were suspended in 10 ml of sterile distilled water containing 0.1% (v/v) of Tween 80 using a sterile loop (lingappa*et al*., 2005).The spore suspension containing 10^6–10^7 spores/ ml was used as the inoculum.

Comparative evaluation of substrates for L-asparaginase production and solid state fermentation

The Maroli local market in Gujarat, India provided the wheat bran, rice bran, coconut oil cake, cotton oil cake, and soybean grains that were employed as the substrate for the synthesis of L-asparaginase. Particles of size between 1-3 millimeters were produced by grinding the grains. It was evaluated how well agro-industrial leftovers worked as a carbon source for L-asparaginase synthesis. 10 grams of substrate mixed with asparagine dextrose salts broth medium in 250 ml Erlenmeyer flask sets, which were used for solid state fermentation. The ingredients were completely combined and sterilized. To achieve the appropriate moisture content, 5% of the inoculum was added to the sterilized medium components. After completely mixing the contents of the flask using a sterile wooden spoon, it was incubated for five to seven days at 35 °C while in a stationary position.

Crude enzyme preparation

After incubation, the crude enzyme was extracted from the fermented substratum using a 50ml Tris/Hclbufer (pH 8.6, 0.05M). The fermented substratum and buffer were homogenized with a constant stirring by agitation in a rotary shaker at 150 rpm for 1 h. after that filter through whatman filter paper no.1 and centrifuged in a cooling centrifuge (at 4 °C) for 15min at 10000g. The resulting supernatant was used for L-asparaginase activity assay.

Selection of the significant variables using Plackett–Burman design

Using Design Expert (v.13.0.1) software (Stat-Ease Corporation, USA), a statistical procedure known as plackettburman design was carried out (Plackett and Burman, 1946). To identify and evaluate the key factors affecting the response, the Plackett–Burman design is frequently employed. Although the Plackett-Burman design is capable of quickly identifying the major physicochemical variables that have a significant impact on the response of several independent variables in a limited number of experiments, it is unable to explain the ways in which the

independent variables interact with one another. In this study, the effects of seven independent factors—A (xylose), B (ammonium sulfate), C (rice bran), D (soybean meal), E ($ZnSO_4.7H_2O$), F ($FeSO_4.7H_2O$), and G (asparagine)—on the production of L-asparaginase were screened using a Plackett–Burman experimental design consisting of 15 experiments. Each variable is represented at two levels, high (+) and low (−). Plackett-Burman experimental design is based on the first order model (Table 1):

$Y = \beta 0 + \Sigma \beta i Xi$(3)

Where, Y is production of L-asparaginase, β0 is the intercept of the model and $\beta i$is the linear coefficient, and Xi is the independent variable level.

Table 1 Actual values of the process variables

Serial no	Factors	Level 1	Level 2
1	Xylose	0.1	5
2	Ammonium sulfate	0.5	10
3	Rice bran	2.5	10
4	Soybean meal	5	20
5	$ZnSO_4.7H_2O$	0.01	0.5
6	$FeSO_4.7H_2O$	0.01	0.001
7	Asparagine	1	10

Central composite design (CCD)

Using RSM, more important variables influencing the production of enzymes that were chosen from PBD were optimized. Design Expert (v.13.0.1) created a 2^n factorial central composite design (CCD). Possible interactions between several important variables have been found using CCD. The three independent variables in the study—soybean meal, ammonium sulfate, and $FeSO_4.7H_2O$—were examined at five distinct levels (−1.68, −1, 0, 1, 1.68) across 20 experiments. Every experiment was run three times, and the average L-asparaginase activity was determined to represent the response (Y). Using the second order polynomial equation, the CCD findings were examined:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j$$

In which Y is the predicted response, X_i is the independent variable coded level, β_{ij} is the interaction coefficients, β_0 is the regression coefficients, β_{ii} is the quadratic coefficients and β_i is the linear coefficient.

Statistical analysis

The experimental designs and statistical analysis have been carried out using the softwareversion 7 of design expert for Windows. The 3D surface plots have been constructed with version 8.

Preparation of fermentation medium for AIA 1 strain: 12.5 g Soybean meal, 13.23g ammonium sulfate and 0.0055g $FeSO_4.7H_2O$ taken in 250ml Erlenmeyer flask. The pH of the medium was adjusted to 7.0. The media was sterilized at 121°C for 15 minutes. The flasks were inoculated with 3 ml spore suspension and incubated at 37°C for five days.

Extraction of L-asparaginase enzyme: Crude L-asparaginase was extracted from the fermentation medium by mixing thoroughly with 50 ml extraction buffer (0.05M sodium citrate buffer pH 8.6). The flask was kept on a rotary shaker for homogenization at 150 rpm and 35°C for 1 h. Then, filtered by using Whatman filter paper No.1 and the resulting clear filtrate was centrifuged at 10000g for 20 min. The supernatant was used as crude enzyme for the measurement of extracellular enzyme activity and protein concentration.

Purification of L-asparaginase from *Streptomyces sp AIA 1*

L-asparaginase purification has been carried out by ammonium sulfate precipitation, desalting by sephadex G25 chromatography and further purified by DEAE cellulose ion exchange chromatography.

Characterization of L-asparaginase

Several physicochemical parameters like reaction pH, reaction temperature, substrates specificity and incubation time were characterized. The kinetic parameters (*Km* and *Vmax*) of purified L-asparaginase were also determined.The reaction kinetics of the purified enzyme was determined from Lineweaver-Burk plots with L-asparagine as substrate under defined assay conditions. The Michaelis–Menten constant (*Km*) and maximal velocity (*Vmax*) were determined for the enzyme at each of the measured temperatures using the Michaelis–Menten equation:

$$V = \frac{Km+[S]}{Vmax[s]}$$

Where, V is the reaction velocity (a function of enzyme concentration), [S] is the substrate concentration, *Km* is the substrate concentration at half-maximal velocity, and *Vmax* is the maximal velocity. *Vmax* and *Km* values were determined using nonlinear regression [Lineweaver and Burk, 1934].

Enzyme molecular weight determination

In accordance with the Laemmlimethod, molecular weight of the purified L-asparaginase was defined by SDS-PAGE with protein marker ranges between 9 and 178 kDa.SDS-PAGE was performed by taking separating gel 12% (w/v) and stacking gel 4% (w/v) containing 10% (w/v) SDS.

The cell lines and cell cultures

K562 cell line (chronic myelogenous leukemia) and normal cell line PBMC (peripheral blood mononuclear cells) were obtained from NCCS, Pune. The cell line was maintained in RPMI-1640 medium with 10% Fetal Bovine Serum (FBS). The cells were cultured at 37°C in a humidified 5% CO2 incubator.

MTT colorimetric assay

The previously mentioned cell lines have been used to assess L-asparaginase'sinhibitory effects on cancer cell lines using the yellow tetrazolium bromide (MTT) test. MTT (3-[4, 5-dimethylthiazolyl-2-yl]-2, 5-diphenyl tetrazolium bromide) is a yellow water-soluble tetrazolium salt, measures metabolic activity of cells.At density of 1×10^3cells/well, 200µl of the cell suspension was added to each well and the plate was incubated at 37°C and 5% CO_2 atmosphere for 24hr. After 24hr, 200µl of various concentrations of L-asparaginase (5, 10, 25, 50, 100 µg/ml) were added to the respective wells and incubated for 24hr at 37°C. After L-asparaginase treatment, 100µl of medium with 10%, MTT reagent was added to obtain a final concentration of 0.5mg/mL and the plate was incubated at 37°C and 5% CO_2 atmosphere for 3hr. The culture medium was removed completely without disturbing the crystals formed. Then 100µl of DMSO was added and put on a shaker to solubilize the formazan. The absorbance was measured using a microplate reader at 570 nm and 630 nm [Alley *et al*., 1986, Mosmann*et al.*, 1983].

The effects of the L-asparaginase on the different cell lines were expressed as the % ofcytotoxicity (cell growth inhibition) using the following formula (cory*et al*., 1991):

% cytotoxicity = 100 − % cell viability

Where % cell viability = (A570 nm of treated cells)/(A570 nm of controlled) × 100

III. RESULTS AND DISCUSSION

Among all isolates in the qualitative assay for extracellular L-asparaginase, actinomycetesunder the isolation number AIA 1, AIA 2,AIA 4,AIA 5,AIA 6,AIA 7, KA 1,KA 2,KA 3, KA 4, KAP, SCAP, GLYAAP and SCANT showed pink zones around the colony which refer to their ability to produce L-asparaginase and then their productivities were quantitatively analyzed in SMF, they produced 436,419,250,118,374,299,122,132,222,330,175,123,133 and 230 U ml^{-1}, respectively. Then AIA 1 isolate was selected as the hyper L-asparaginase producer qualitatively (formed prominent pink zone) (Fig. 1) and quantitatively (436 U mL^{-1}) for the further studies. In order to check for the formation of L-asparaginase using both qualitative and quantitative techniques, a total of 14 actinomycete isolates were isolated and purified. Pink zones formed around the colonies, indicating the presence of L-asparaginase activity (Fig. 1). Moreover, isolate encoding AIA 1 was chosen as the most active isolate for additional research because it had a bigger zone compared to other strains and 436U/ml asparaginase activity. The strain colony and gram staining of the most potent asparaginase production are shown in Figure 2. Enzyme synthesis is known to be significantly impacted by the composition of the nutrition medium and the growth environment. Therefore, we sought to optimize the medium's composition in order to enhance L-asparaginase synthesis. SSF's most crucial step is selecting the right solid substrate for fermentation. The objectives of industrial scale SSF systems include increased L-asparaginase yield and decreased production costs by using less expensive substrate.

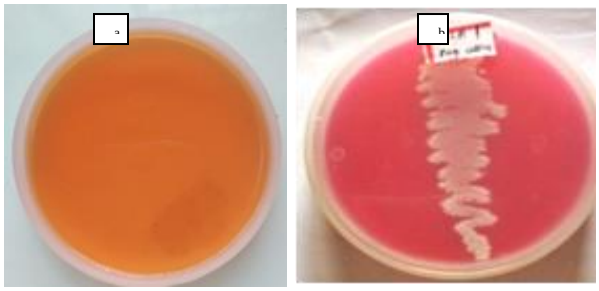

Fig 1 Production of L-asparaginase by *Streptomyces Sp. AIA 1* as detected by the plate assay, in which a color change in the medium from a standard yellow coloration to the presence of a pink zone around the colony after 5 days of incubation indicates the production of L-asparaginase

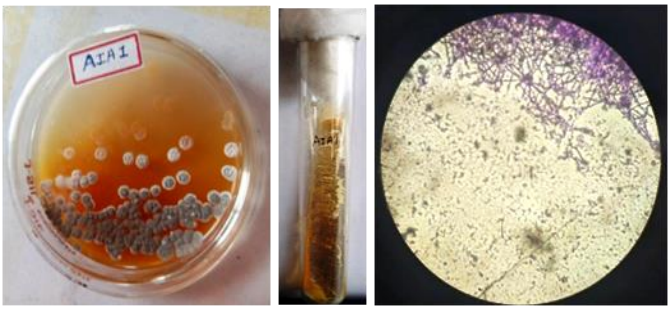

Fig 2 Cultural and morphological characteristics of AIA 1

Comparative evaluation of substrates as carbon source for L-asparaginase production under SSF conditions

The suitability of various agro-industrial leftovers, including rice bran, wheat bran, soybean meal, coconut oil cake, and cotton oil cake, as carbon sources for the manufacture of L-asparaginase was investigated through a comparative research. The *Streptomyces* strain AIA 1 exhibited the maximum activity of L-asparaginase (125U/gds) (Table 2). These findings led to the selection of the rice bran and soybean meal for further fermentation investigations on the synthesis of L-asparaginase under SSF. Strain AIA 1 was chosen from the examined strains and employed in the synthesis of L-asparaginase.

Table 2 Comparative assessment of natural substrates for L-asparaginase production

Substrates	Enzyme activity (U/gds)	Protein concentration (mg)	Specific activity (U/gds/mg)
Wheat bran	92	0.73	126
Rice bran	111	0.84	132
Coconut oil cake	83	0.70	118
Cotton oil cake	66	0.68	97
Soybean meal	125	0.95	131

Plackett–Burman design to identify significant factors affecting L-asparaginase production

In order to investigate the impact of each selected variable on the production of L-asparaginase, this experiment was carried out in 15 runs, with each run screening one of ten independent (assigned) variables (Xylose, Ammonium sulfate, Rice bran, Soybean meal, $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$, asparagines) and three unassigned variables (commonly referred to as dummy variables) in the Plackett–Burman experimental design (Table 3). In data analysis, dummy variables (Dummy1, Dummy2, and Dummy 3) are used to estimate experimental errors. Table 3 displays the design matrix that was chosen for the screening of important factors for the synthesis of L-asparaginase and the related response (Y) under SSF conditions. Every trial was conducted in two copies, and the response was defined as the average amount of L-asparaginase produced. Owing to the inclusion of ammonium sulfate, soybean meal, and $FeSO_4.7H_2O$ in the fermentation media, run number 02 showed the lowest level of L-asparaginase activity (720 U/gds), whereas run number 11 showed the highest level (3000 U/gds).

Table 3: Plackett-Burman experimental design for AIA 1

Run	Xylose (gm/L)	Ammonium sulfate (gm/L)	Rice bran (gm/L)	Soybean meal (gm/L)	$ZnSO_4$ (gm/L)	$FeSO_4$ (gm/L)	Asparagine (gm/L)	Enzyme Activity (U/gds)
1	2.55	5.25	6.25	12.5	0.255	0.0055	5.5	1820
2	5	0.5	2.5	5	0.5	0.001	10	720
3	5	0.5	10	20	0.5	0.001	1	789
4	5	0.5	10	20	0.01	0.01	10	1170
5	0.1	10	10	5	0.5	0.01	10	2875
6	0.1	10	10	20	0.01	0.001	1	2850
7	0.1	0.5	2.5	20	0.01	0.01	10	970
8	5	10	10	5	0.01	0.001	10	2445
9	5	10	2.5	5	0.01	0.01	1	2750
10	0.1	10	2.5	20	0.5	0.001	10	2900
11	5	10	2.5	20	0.5	0.01	1	3000
12	0.1	0.5	2.5	5	0.01	0.001	1	830

13	2.55	5.25	6.25	12.5	0.255	0.0055	5.5	1970
14	0.1	0.5	10	5	0.5	0.01	1	823
15	2.55	5.25	6.25	12.5	0.255	0.0055	5.5	1750

We fixed the significant variables that had a beneficial influence on the production of L-asparaginase at a high level for further optimization by the CCD, and we retained high levels of those variables that had a negative effect.

Table 4: ANOVA and fit statistic for AIA 1 asparaginase optimization

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	**1.137E+07**	**11**	**1.034E+06**	**122.65**	**0.0011**	**Significant**
A-Xylose	11656.33	1	11656.33	1.38	0.3245	
B-Ammonium sulfate	1.106E+07	1	1.106E+07	1311.39	< 0.0001	
C-Rice bran	3960.33	1	3960.33	0.4698	0.5423	
D-Soybean meal	1.273E+05	1	1.273E+05	15.10	0.0302	
E-$ZnSO_4.7H_2O$	705.33	1	705.33	0.0837	0.7912	
F-$FeSO_4.7H_2O$	92576.33	1	92576.33	10.98	0.0453	
G-Asparagine	120.33	1	120.33	0.0143	0.9125	
H-H	7701.33	1	7701.33	0.9135	0.4097	
J-J	49152.00	1	49152.00	5.83	0.0946	
K-K	21336.33	1	21336.33	2.53	0.2099	
L-L	3816.33	1	3816.33	0.4527	0.5492	
Residual	25290.73	3	8430.24			
Lack of Fit	24.07	1	24.07	0.0019	0.9692	**not significant**
Pure Error	25266.67	2	12633.33			
Core Total	1.140E+07	14				

Std. Dev.	**91.82**		R^2	**0.9978**
Mean	1844.13		**Adjusted R^2**	0.9896
C.V. %	4.98		**Predicted R^2**	0.9959
			Adeq Precision	27.7632

The determination coefficient was used to assess the model's goodness of fit (R^2). In this instance, the determination coefficient value (R^2 = 0.9978) shows that the provided independent variable accounted for 99.78% of the response's variability, leaving the independent variables to explain only 0.22% of the total variation. Furthermore, a high value of the adjusted determination coefficient (Adj. R^2 = 0.9896) suggests that the model is highly significant.

The P values, which are presented in Table 4, were used to assess the importance of each coefficient. Factors influencing L-asparaginase activity in the current experiment were deemed to have significant impacts at P values <0.05 (confidence levels >95%).

The most important factor was found to be ammonium sulfate, with a probability value (P) of 0.0001, followed by $FeSO_4$ (P value 0.04) and soybean meal (P value 0.03).

The model's F value of 122.65 (Table 4) suggests that it is significant, and the model terms are also significant when their F (P) values are less than 0.05 (0.001).

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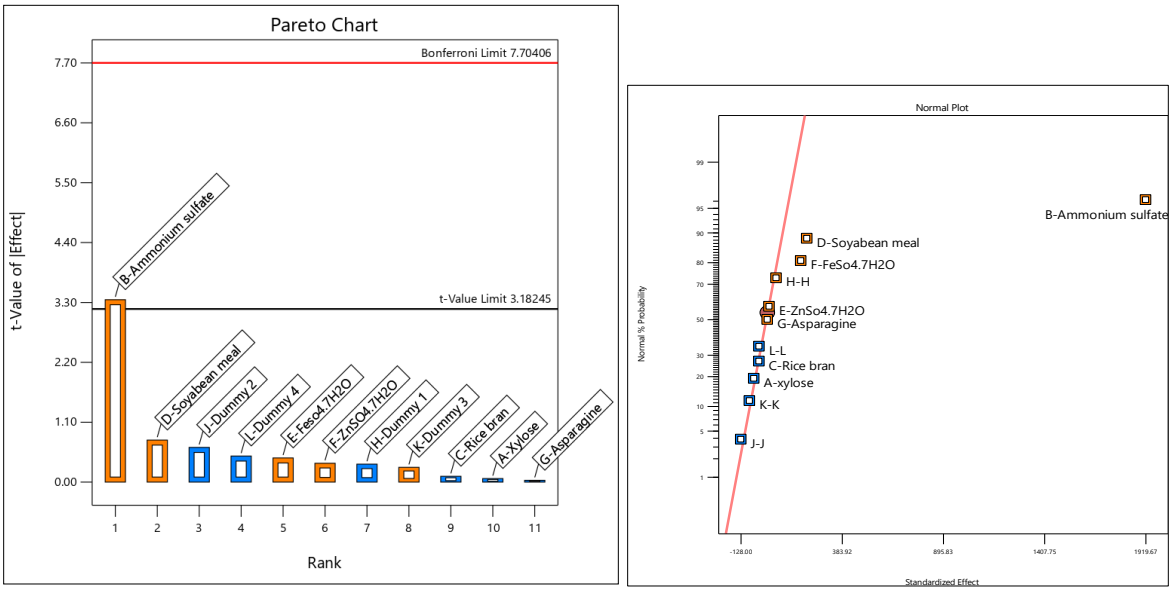

(a) (b)

Figure 3: (a) Pareto chart (b) Normal plot for AIA 1 asparaginase optimization

The relevance of the variables had an impact on the synthesis of L-asparaginase, indicating the Pareto chart in the Plackett-Burman experimental design. Figure 3 (a) & (b) shows the Pareto chart and normal plot for AIA 1 asparaginase. Based on the Pareto chart's computed t-value, the synthesis of L-asparaginase is positively impacted by four of the seven components (ammonium sulfate, soybean meal, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$), and negatively impacted by three others (xylose, asparagine, and rice bran).

Ammonium sulfate, soybean meal, and $FeSO_4.7H_2O$ were determined to be the most relevant components based on the Pareto chart and were selected for central composite design optimization. In the final experiment, the following medium composition (g/L) was employed to assess the correctness of the proposed Burman design: ammonium sulfate (20), soybean meal (10), and $FeSO_4.7H_2O$ (0.5). The inoculated flasks were cultured for five days at pH 7, 37ºC, and 3% of the inoculum was used for the medium preparation.

Optimization by CCD

The CCD was used to investigate the relationships between the important variables and to ascertain the ideal concentrations of each. The study's design yielded maximum yield in the Plackett-Burman experiments because the other variables were kept constant. The three most significant positive independent variables affecting L-asparaginase production (in the Plackett-Burman experiment) were chosen and further investigated using CCD. These variables included ammonium sulfate, soybean meal, and FeSO4.7H2O. We conducted 20 tests in all, varying the three independent variables. Table 5 shows the design matrix along with the expected and observed values for L-asparaginase production. ANOVA analysis of the data revealed significant variance in L-asparaginase activity. Asparaginase activity ranged from 312 U/gds at the lowest in run number 14 to 4400 U/gds at the highest in run number 15.

Table 5: CCD details for RSM optimization of AIA 1 asparaginase

Run	A:Soybean meal	B:Ammonium sulfate	C:$FeSO_4.7H_2O$	Enzyme activity
	gm/L	gm/L	gm/L	U/gds
1	12.5	5.25	0.013068067737283	1856
2	12.5	5.25	0.0055	1620
3	5	10	0.001	2723
4	-0.11344622880572	5.25	0.0055	1056
5	12.5	5.25	0.0055	1517
6	20	0.5	0.01	721
7	20	10	0.01	3000

8	12.5	5.25	0.0055	1369
9	12.5	5.25	0.0055	1478
10	5	0.5	0.01	643
11	12.5	5.25	0.0055	1484
12	12.5	5.25	0.0055	1292
13	25.113446228806	5.25	0.0055	1656
14	12.5	-2.7385159449103	0.0055	312
15	**12.5**	**13.23851594491**	**0.0055**	**4400**
16	20	0.5	0.001	660
17	5	0.5	0.001	506
18	20	10	0.001	2650
19	12.5	5.25	-0.0020680677372834	1400
20	5	10	0.01	2800

Table 6: ANOVA and fit statistics for AIA 1 asparaginase optimization

Source	**Sum of Squares**	**Df**	**Mean Square**	**F-value**	**p-value**	
Model	1.932E+07	9	2.147E+06	91.04	< 0.0001	**Significant**
A-Soybean meal	1.370E+05	1	1.370E+05	5.81	0.0366	
B-Ammonium sulfate	1.763E+07	1	1.763E+07	747.86	< 0.0001	
C-$FeSO_4.7H_2O$	1.419E+05	1	1.419E+05	6.02	0.0341	
AB	1378.12	1	1378.12	0.0584	0.8138	
AC	4851.12	1	4851.12	0.2057	0.6598	
BC	6555.12	1	6555.12	0.2780	0.6095	
A^2	43642.97	1	43642.97	1.85	0.2035	
B^2	1.284E+06	1	1.284E+06	54.47	< 0.0001	
C^2	24386.05	1	24386.05	1.03	0.3331	
Residual	2.358E+05	10	23578.10			
Lack of Fit	1.695E+05	5	33905.40	2.56	0.1628	**not significant**
Pure Error	66254.00	5	13250.80			
Core Total	1.955E+07	19				

Std. Dev.	153.55		**R^2**	0.9879
Mean	1657.15		**Adjusted R^2**	0.9771
C.V. %	9.27		**Predicted R^2**	0.9281
			Adeq Precision	35.2008

Table 6 displays the ANOVA findings, which are necessary to evaluate the model's and its coefficients' sufficiency and significance. ANOVA analysis showed that the model was significant at 99% confidence level with a p-value of less than 0.0001. Given that the model's F-value is 91.04, it is likely significant. There is a mere 8.96% probability of noise.

The measurement of the degree to which the observed response values can be described by the interactions and components of the experiment is indicated by the determination coefficient (R^2) values, which are consistently in the range of 0 to 1. According to Kaushik *et al*. (2006), a model is stronger and more accurate at predicting the response when its R^2 value is closer to 1. Relative to the overall variance, the model could account for 98.79 percent of the variability in the response, as indicated by the determination coefficient (R^2) of the model, which was 0.9879. As per Chen *et al*. (2009), a regression model that possesses a R^2 value more than 0.9 is deemed to exhibit a substantial correlation.

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As a result, the R^2 value discovered in our investigation indicates that the model is trustworthy for the synthesis of L-asparaginase and shows a very excellent fit between the observed and expected responses. Furthermore, our study's adjusted determination coefficient (Adj. R^2 = 0.9791) is likewise very high, indicating the model's high importance (Akhnazarova and Afarov 1982). As a result, we thought the model-based study of the response trend made sense.

The data displayed in Table 6 was interpreted by considering the signs, which indicate a positive or negative effect on the response, as well as the statistical significance of the coefficients (P<0.05). A negative coefficient indicates an antagonistic effect, whereas a positive coefficient indicates a synergistic effect resulting from interactions between two elements.

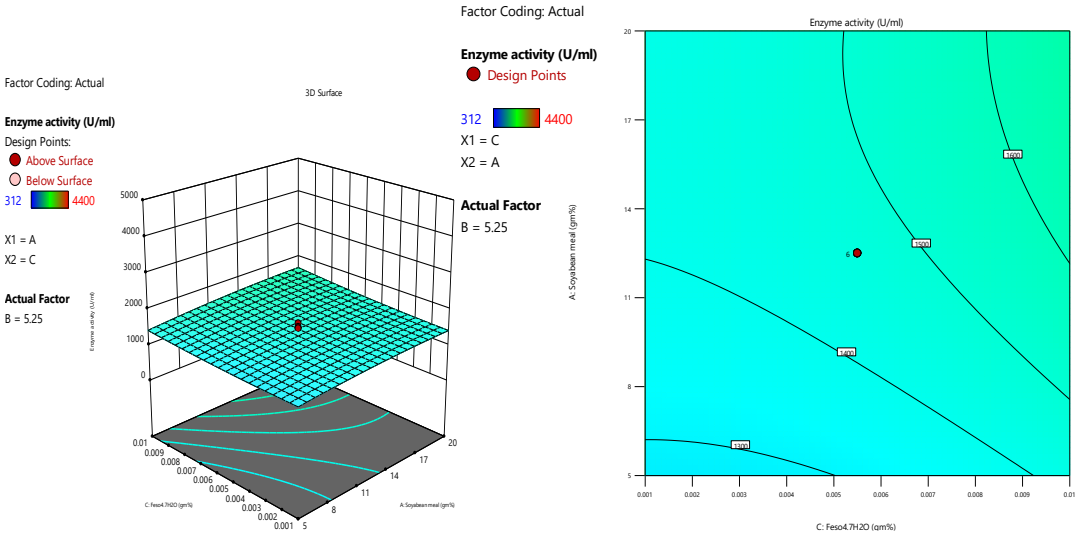

Figure 4a:Interaction between soybean meal and $FeSO_4.7H_2O$ in RSM optimization for AIA 1 asparaginase production (1) 3-D and (2) contour graphs

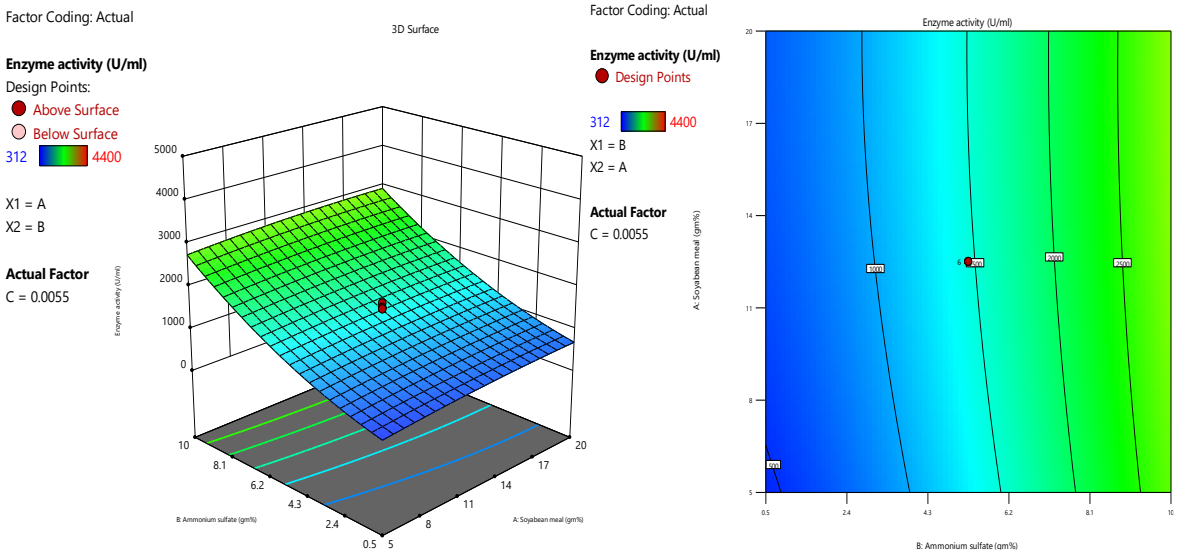

Figure 4b:Interaction between soybean meal and ammonium sulfate in RSM optimization for AIA 1 asparaginase production (1) 3-D and (2) contour graphs

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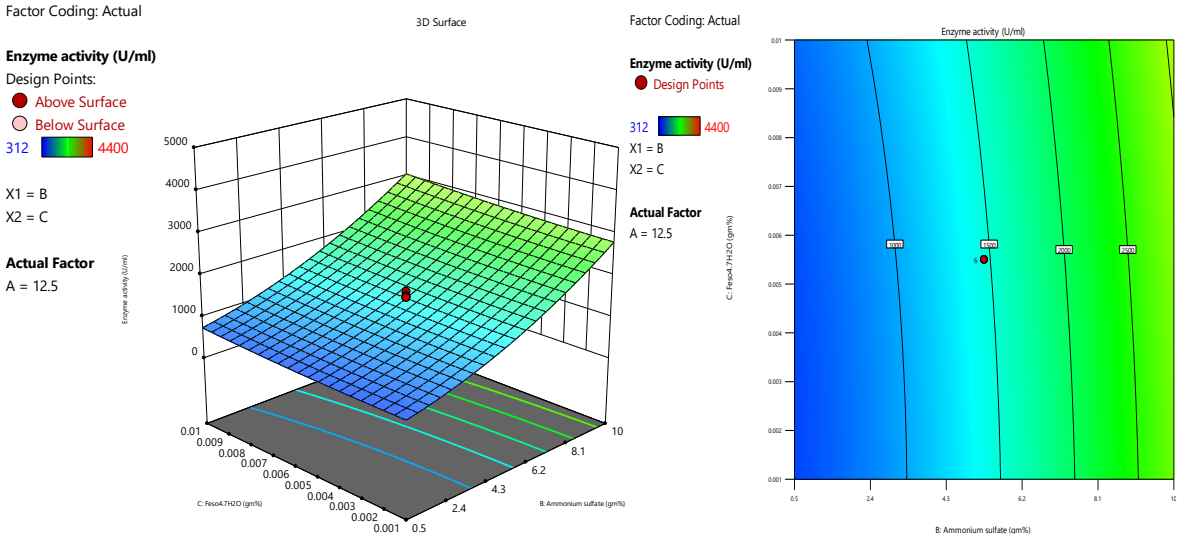

Figure 4c:Interaction between ammonium sulfate and $FeSO_4.7H_2O$ in RSM optimization for AIA 1 asparaginase production (1) 3-D and (2) contour graphs

By plotting the response surface curves (shown in Fig. 4a–c) when one variable was fixed at its optimum value and the other two were allowed to change, the interaction effects and optimal levels of the variables were revealed. Figure 4b and 4c shows the relationship between L-asparaginase activity and ammonium sulfate as well as soybean meal/$FeSO_4.7H_2O$, while maintaining a constant value for others. With the maximum value of L-asparaginase activity found at the middle range of ammonium sulfate levels. High concentrations of ammonium sulfate was required to reach the maximum L-asparaginase activity, and the activity of L-asparaginase increased accordingly.

Verification of the experimental model

In order to determine the accuracy of the model and to verify the result, an experiment was performed under the optimal conditions determined from the CCD–response surface methodology and the results compared with the predicted data. The measured L-asparaginase activity obtained from the experiment (2800U/gds) was very close to the response predicted by the regression model, revealing a high degree of accuracy (fig 5). This verification revealed a high degree of accuracy of the model indicating model validation under the tested conditions.

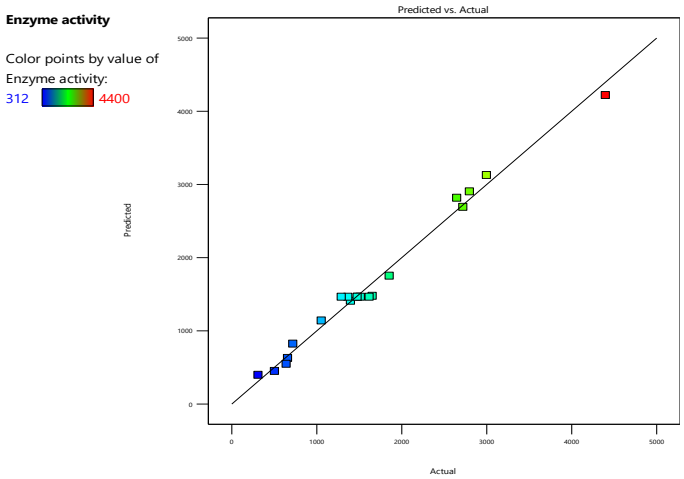

Fig 5: Predicted versus actual plot for RSM optimization of AIA 1 asparaginase production

L-asparaginase purification

Extracellular L-asparaginase from *AIA* 1 was purified from liquid culture by successive steps including $(NH_4)_2SO_4$ fractionation (80% saturation), Sephadex G25 column chromatography and DEAE cellulose 52 ion exchange chromatography.

For purification of L-asparaginase, crude filtrate of culture with overall activity 4,04,250U, protein content 780 mg and specific activity of 518 U/mg proteins was used. The ammonium sulfate precipitated enzyme had 179mg total protein; 1078U/mg specific activity and recovery of 47.76% with 2.08 fold purification. The desalted enzyme had 18.8 mg protein content, 4161U/mg protein specific activity and enzyme recovery of 22.6% with 9.38 purification fold (Table 7). The purified enzyme was obtained with the packed column of DEAE cellulose 52,

resulting in 15 fractions with a single peak (Fig. 6). The purified L-asparaginase collected from the packed column of DEAE cellulose 52 has 2.35 mg protein content with overall activity 48,540 U and the specific activity of 20655U/mg protein with 39.87 purification folds.

Recently L-asparaginase enzyme from strains *Streptomyces noursei* [Dharmaraj, 2011] and *Streptomyces gulbergenesis* [Amena*et al*., 2010] with an overall purification of 98.23 and 82.12 fold in final purification were carried out successfully. Sahu*et al*. extracted the L-asparaginase from the actinomycete strain LA-29 isolated from the gut contents of the fish and the enzyme was purified 18-fold and from which 1.9% of protein was recovered and showed the specific activity of about 13.57 IU/mg of protein.

Table 7: Summary of purification steps of L-asparaginase produced by AIA 1

Purification steps	Volume(ml)	Total activity (U)	Total protein (mg)	Specific activity(U/mg)	Purificationfold	Yield (%)
Crude	250	404250	780	518	1	100
Ammonium sulfate precipitation	100	193100	179	1078	2.08	47.76
Desalting	20	91400	18.8	4861	9.38	22.60
IEX	5	48540	2.35	20655	39.87	12.00

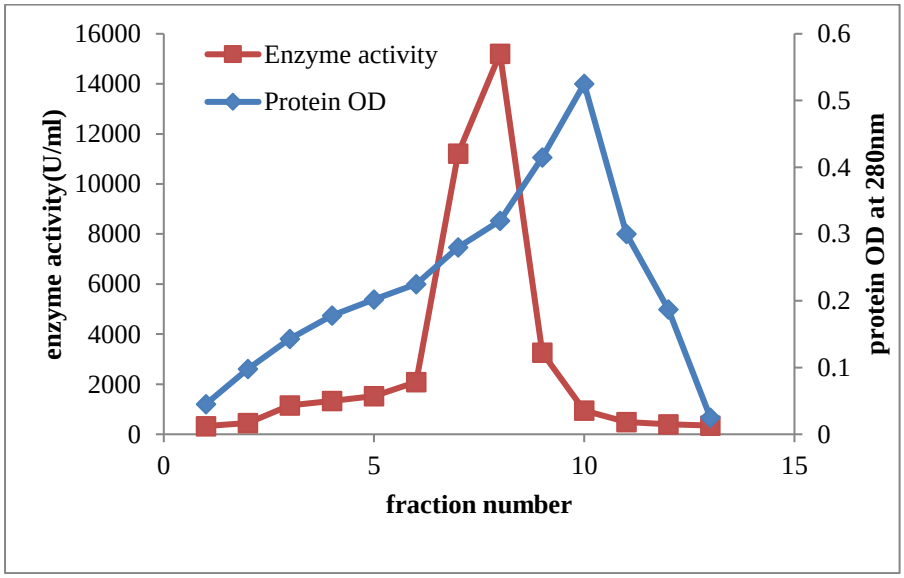

Figure 6: Chromatogram of AIA 1 asparaginase during ion exchange

SDS-PAGE and determination of monomeric molecular weight of the protein

By using Laemmli's method of SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) with a 12% separating acrylamide gel and a 4% stacking gel containing 10% SDS, the molecular weight of the purified enzyme was ascertained. The gel was stained with 0.025 Coomassie brilliant blue R-250 after electrophoresis and then destained with a 4:1:5 solution of methanol, acetic acid, and water. Using conventional molecular weight markers as a reference, the molecular weight of the purified L-asparaginase was calculated (molecular mass range: 9–178 kDa). For the pure production of L-asparaginase with an apparent molecular weight of 30 kDa, SDS–PAGE of the enzyme preparation revealed only one unique protein band (Fig. 7).

It was discovered that the molecular weight of L-asparaginase varied depending on the source of the enzyme, ranging from 53 kDa in *Streptomyces fradiae* NEAE 82 (Ei-naggar*et al*., 2016) to 140 kDa in *Streptomyces sp.* PDK22 (Dhevagi and Poorani, 2006) and 116 kDa in S. albidoflavus (Narayana *et al*., 2008). A unique protein band near 97.4 kDa was found by SDS-PAGE analysis of purified L-asparaginase from *S. tendae* (Kavitha and vijaylaxmi, 2010). According to this, L-asparaginases that were isolated from Thermusthermophilus, Escherichia coli, and Pseudomonas stutzeir MB-405 had reduced molecular weight values, ranging from 33 to 34 kDa (Manna *et al*., 1995; soares*et al*., 2002).

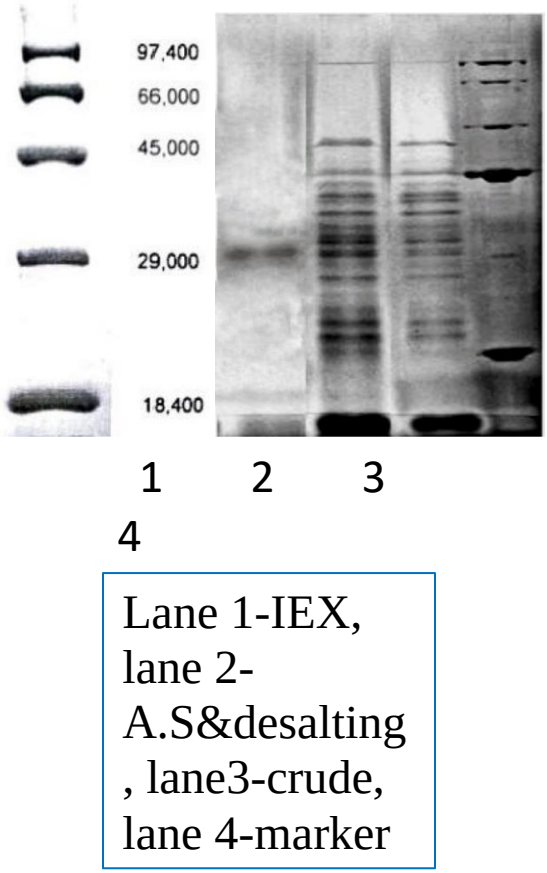

Fig 7: SDS-polyacrylamide gel electrophoresis of the purified L-asparaginase from *AIA 1*.

Kinetics properties of the purified L-asparaginase

The activity of L-asparginase of *AIA 1* was evaluated at different levels of pH, temperature, effect of substrate concentration and incubation time.

Effect of pH on L-asparaginase activity

The ionic form of the enzyme active site residues is affected by pH, which is a crucial determinant for the stability and activity of the purified enzyme. The relationship between L-asparaginase activity and pH in the range of 4.6–10.6 was investigated. The highest relative activity of an enzyme, with a pH of 7.6, was obtained. Enzyme activity was found to be lower at higher pHs. Figure 8 showed how pH affected pure asparaginase. The highest L-asparaginase activity of *Streptomyces sp*. PDK7 was reported by Dhevagi and Poorani to be between pH 8.0 and 8.5. These results are consistent with their findings. Peak activity was seen for L-asparaginase at pH 7.0 after it was extracted from *Streptomyces acrimycini NGP* (Selvam and Vishnupriya, 2013).

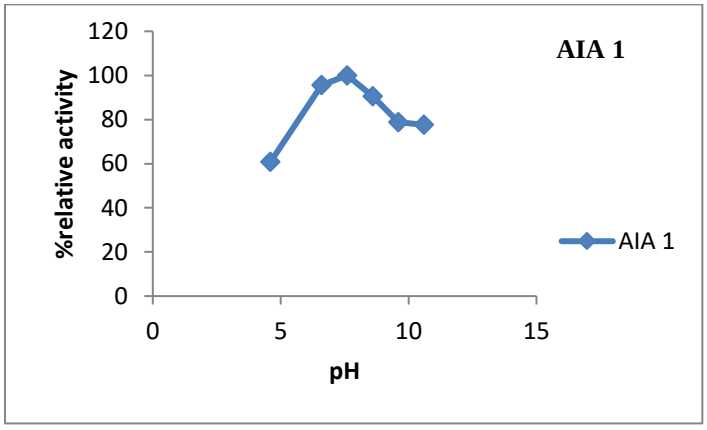

Fig 8: Effect of pH on asparaginase activity

Effect of temperature on L-asparaginase activity

One significant physical factor that affects the activity of enzymes is temperature. It functioned well in a broad temperature range of 25 to 60°C. The AIA 1 peak activity was measured at 40°C. At increasing temperatures, the activity of L-asparaginase decreased. Figure 9 shows the impact of temperature on pure L-asparaginase. Our findings concurred with those of a prior investigation that found that L-asparaginase isolated from *Streptomyces gulbargensis* exhibited its greatest activity at 40 °C (Amena*et al*., 2010). The ideal temperature for the enzyme activity extracted from *Pseudomonas stutzeri* MB-405 has been determined by Manna *et al*. to be 37 °C.

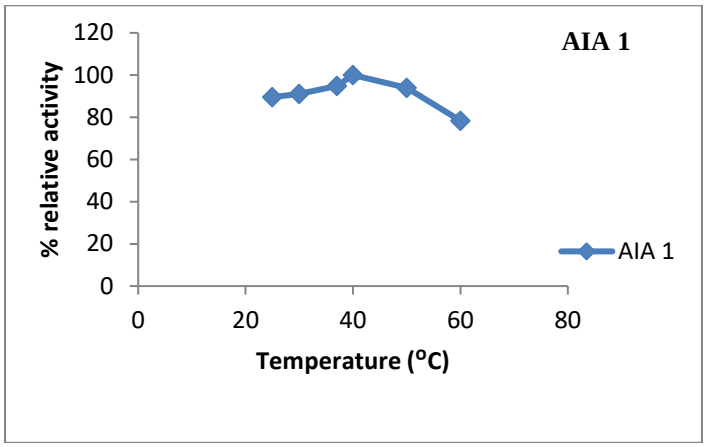

Fig 9: Effect of temperature on asparaginase activity

Effect of incubation time on enzyme activity

When the incubation time was extended to 30 minutes, the L-asparaginase activity (Fig. 10) rose. L-asparaginase activity was then only slightly reduced after that. According to El-Bessoumy*et al*., the isolated L-asparaginase from *Pseudomonas aeruginosa* 50071 reached its maximal activity within 30 minutes. Furthermore, the study examined the impact of incubation duration on the activity of purified L-asparaginase derived from *Streptomyces noursei*, revealing that the activity peaked at 35 minutes (Dharmaraj, 2011). Following a longer duration of substrate incubation, a decrease in L-asparaginase activity was visible. It's possible that product inhibition is to blame for the drop.

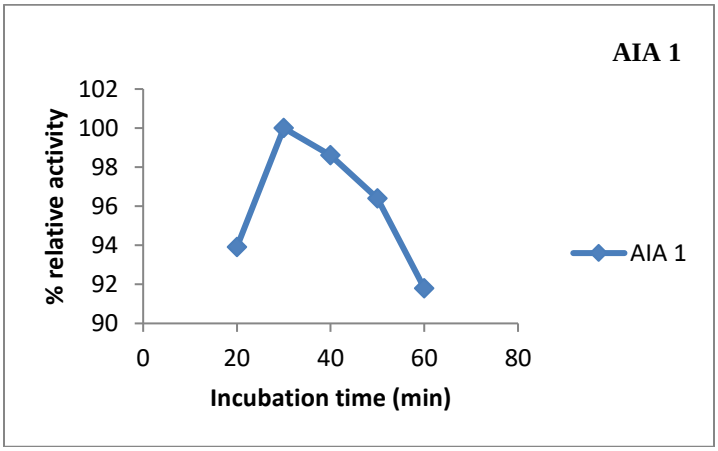

Fig 10: Effect of incubation time on asparaginase activity

Effect of substrate concentration on the activity of L-asparaginase

In order to ascertain the ideal substrate concentration needed to produce the maximum L-asparaginase activity, a range of substrate concentrations, from 0.02 to 0.1 molar, were used in this experiment to investigate the effect of substrate concentration on L-asparaginase activity. The findings in Figure 12 demonstrated a progressive rise in enzyme activity as substrate concentration increased from 0.02 to 0.1 molar. Using a steady-state kinetic study, the values Km and Vmax for the purified ASNase were found (Fig. 11). The enzyme employing L-asparagine as a substrate had Km and Vmax values of 6 mM and 1000 µmol/ml/min, respectively, according to the Lineweaver Burk plot.

As per El-naggar*et al*. (2016), Rahimzadeh*et al*. (2016), and Asha and Pallavi (2012), the line-weaver Burk analysis revealed that the Km and Vmax values for *Streptomyces fradiae*, *Bacillus PG 03*, and *Fusarium sp*. were 10, 23.08, 444 mM, and 30.6, NR, 2.9, respectively. A comparison of the Km value with that of L-asparaginase from *Escherichia coli* (3.5 mM) and *Erwiniacarotovora* (7.14 mM) was also made [Willis *et al*., 1974; Kamble*et al*., 2006].

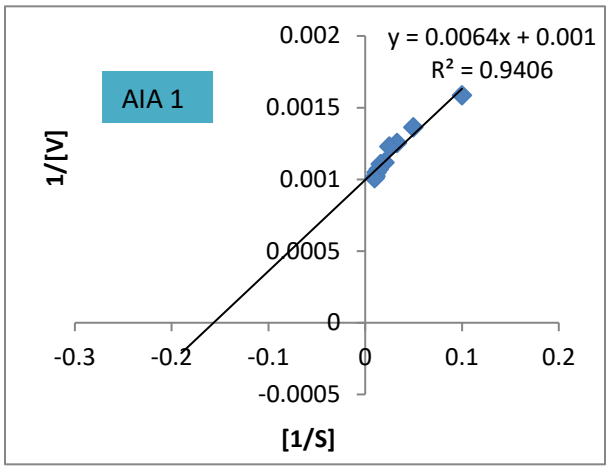

Fig 11:Effect of the substrate concentration of the reaction on L-asparaginase activity

Anticancer activity of L-asparaginase against human cancer cell lines

Purified L-asparaginase was tested for cytotoxicity using PBMC (peripheral blood mononuclear cells) and the K562 cancer cell line. The acquired results showed that L-asparaginase inhibited cell viability in a dose-dependent manner and that it had distinct cytotoxic activity against K562, with an IC_{50} of 17.63 µg/ml (Table 8). With IC_{50} values of 0.22, 0.78, and 0.153 IU, respectively, ASNases produced from marine *Bacillus sp*. and *B. licheniformis* strains showed the cytotoxic action against the cancer cell lines Jurkat clone E6-1, MCF-7, and K-562 [Arjun *et al*., 2016; Mahajan *et al*., 2014].

Table 8: Concentration Vs % Cytotoxicity toward K562 and PBMC cell line

		K562 cell line			**PBMC**		
Sample	Conc (µg/ml)	% cytotoxicity	% viability	IC 50	% cytotoxicity	% viability	IC50
AIA 1	5	15.03	84.97	17.63	1.78	98.22	N.A
	10	41.89	58.11		4.45	95.55	
	25	55.4	44.6		8	92	
	50	74.31	25.69		9.77	90.23	
	100	96.53	3.47		11.55	88.45	

IV. ACKNOWLEDGEMENT

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