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Isolation, Screening and Optimization of L-Asparaginase Producers from Soil Samples

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Abstract: ***L-Asparaginase, a group member of homologous amidohydrolases family, expressed and produced naturally by animal tissues, plants and large group of microorganisms. This enzyme typically, catalyses the hydrolysis of amino acid L-asparagine to L-aspartate and ammonia. Recently, L-Asparaginase has received increased attention for its therapeutic potential and therefore present study aimed to isolate and screen L-Asparaginase producers, and determines optimized conditions for its production. We have screened 55 isolates and from them 23 isolates were identified as L-Asparaginase producers. DU1 isolate was recognized as higher producers and used for optimization procedures.***
Keywords: L-Asparaginase, Therapeutic potential, Optimization.

I. INTRODUCTION

Enzymes are proteins that catalyze (i.e., increase the rates of) chemical reactions. In enzymatic reactions, the molecules at the beginning of the process are called substrates, and they are converted into different molecules, called the products. Almost all processes in a biological cell need enzymes to occur at significant rates and enzymes are selective for their substrates [2]. L-Asparaginase (E.C. 3.5.1.1) belongs to a group of homologous amidohydrolases family, which catalyses the hydrolysis of amino acid L-Asparagine to L-Aspartate and Ammonia [3].

L-Asparaginase usually exists in a tetrameric form, but hexamers, dimmers and monomers are also found to occur in some species. Most bacterial, mainly *Escherichia coli* and *Erwinia* species, enzymes are present in similar three-dimensional quaternary and tertiary structures [4]. Hydrolysis of L-Asparaginase is a two-step process. In step one, a beta-acyl enzyme intermediate is formed by nucleophilic attack and in step two, the intermediate formed is attacked by a water molecule that generates aspartic acid and ammonia [5].

L-Asparaginase is found in diverse groups of plants and animals but owing to its difficult extraction procedures, only the microbial sources are explored. The bacterial population in the soil is predominant as compared to all other groups of microorganisms in both number and variety. Soil bacteria play crucial role in decomposition of complex organic matter, ammonification, nitrification, and denitrification, biological fixation of atmospheric nitrogen, oxidation and reduction of sulphur and iron compounds and have been used in industries on large scale. L-Asparaginase is produced by Gram positive as well as Gram negative bacteria found both in terrestrial and marine environments, but Gram negative species has got more importance. *Escherichia coli, Erwinia chrysanthemi, Erwinia carotovora* and many other bacterial species are in clinical use, over years, as antitumor agents in the treatments. Fungi are potential source of L-Asparaginase along with bacteria. Fungi, being eukaryotic closely resemble humans, therefore; the chances of immunological reactions are lesser. The main reason of their importance is that they produce enzyme extracellularly, so it is easy to extract and purify it [6].

II. MATERIALS AND METHODS

A. Collection of Samples

The present study was conducted over a period of three months, i.e., from December 2018- March 2019. Soil samples were collected from the various places with view to isolate potent L- Asparagine producing microorganism. All the samples were collected in polythene bag from the depth of 10 cm from different location and characteristics.

B. Isolation of Bacteria

Different soil samples treated for bacterial isolation by serial dilution technique using nutrient agar medium and antifungal agents (Fluconazole 75ug/ml, Ketocanazole75ug/ml) were added to control the fungal contamination. The inoculated agar plates were incubated at 37°C for 24 hrs. (A.S. Lalitha Devi et al., 2016).

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C. Screening of L-Asparaginase Producing Microorganisms

About 1 gram of each of the above samples was taken into separate conical flasks. The suspension was kept on rotary shaker for 30 min and kept aside to settle the suspending matter. 1 ml of the supernatant was serially diluted with sterile water. 0.1ml from each, of $10^{-2,}$ 10^{-4} and 10^{-6} dilutions were added to sterile Nutrient agar medium containing 1% L-Asparagine and phenol red indicator (2.5%) as pH indicator and incubated at 37ºC for 24 hours. Antifungal agents were incorporated to control the fungal contamination. After 24 hours of incubation, the selected bacterial colonies picked up and transferred onto modified nutrient agar medium and incubated at 37ºC for 24 hours. Control plates were of modified nutrient agar medium without dye and without L-asparagine. Colonies with pink zones around the colony were considered as L-Asparaginase producing strains.

D. Phenotypic Identification

The morphology and colony characteristics were studied using nutrient agar plate and isolates were identified by their Biochemical profiling.

E. Secondary Screening of Potential Isolates

Agar cup method was carried out to quantify the enzyme. Sterile nutrient agar was modified by adding 1% L- Asparagine and (2.5%) phenol red indicator. The isolates were grown in 10 ml sterile nutrient broth for 24 hours and add in respective wells. Plates were incubated for 24 hours at 37^{0}C. Diameter of pink zones around the well were measured to quantify the amount of enzyme produced. Maximum zone given organism take for further study.

1) *Quantity Assay for Enzyme Production:* Broth based assays were performed as an effort to screen the isolate that would produce highest yield of extracellular L-Asparaginase was examined for enzyme activity by submerged fermentation in Tryptone glucose yeast extract broth (TGY-Broth with 1% Asparagine at pH7.0). Inoculum were prepared in Erlenmeyer flask containing 50 ml (TGY) broth After 24 hours incubation on rotary shaker at (180 rpm /min) at 37^{0}C, then transferred for enzyme production, a 250 ml volume of TGY broth in 500-ml Erlenmeyer flask was inoculated 5 ml of inoculums (2.304 OD at 600nm) of the bacterium to be tested. The broth is incubated in a shaker incubator at (180 rpm/min) at 37^{0}C. For different time interval 24 hours, 48 hours, 72 hours, 96 hours, 120 hours and 144 hours. After Incubation the culture broth was centrifuged at 10,000 ×g for 10 min, supernatants were collected, and enzyme assay done was by Nesslerization.
2) *Assay for L-Asparaginase Activity:* The enzyme L-Asparaginase was assayed calorimetrically and for that purpose standard curve was prepared using ammonium sulphate concentration gradient. Assay of enzyme was carried by taking 1.0 ml of 50 mM Tris Buffer, pH 8.6 in a test tube, to which 0.1 ml of 189 mM Asparagine 0.5ml of enzyme and 0.9 ml of deionized water was added to make up the volume up to 2.0 ml and incubate the reaction mixture for 10 minutes. After the incubation period 0.1 ml of enzyme is added in test tubes and incubate it at 37^{0} C for 30 minutes and then reaction was stopped by adding 0.1 ml of 1.5M TCA (Trichloroacetic acid). After that take 0.2 ml supernatant then add 4.3 ml deionized water and then add 0.5 ml Nessler's reagent. Then incubate at room temperature for 15 to 20 min mixed well and centrifuged at 4000 rpm at room temperature. Then after take OD at 436nm. The blank was run by adding enzyme preparation after the addition of TCA. The enzyme activity was expressed in unit.
3) *International Unit (IU):* One IU of L-Asparaginase is the amount of enzyme which liberates 1µmol of ammonia per minute per ml [µmole/ml/min].
4) *Calculation:*

$$\text{Units/ml enzyme} = \frac{(\mu\text{mol of ammonia released})\ (2.20)}{(0.2)\ (30)\ (0.1)}$$

Where, 2.20 = Initial volume of enzyme mixture (mL), 0.2 = Volume of enzyme mixture used in final reaction (mL), 30 = Incubation time (minutes) and 0.1 = Volume of enzyme used (mL)

F. Estimation of Protein

Protein content of the supernatant was determined by using Lowry et al Method and the absorbance was measured at 750nm using UV-Vis Spectrophotometer.

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G. Optimization Studies for L-Asparaginase Producers:

The selected isolate for production that was carry forward for optimization process using Tryptone glucose yeast extract broth (TGY). Optimization of Carbon source (0.1%W/V) like Glucose, Sucrose, Trehalose, Galactose. Different Nitrogen sources (0.1%W/V) like Yeast extract, Beef extract, Peptone, Tryptone, Asparagine, and Glutamine and Time interval on assay of enzyme activity is 15 minutes, 30 minutes, 45 minutes, 60 minutes, is 75 minutes and 90 minutes.

H. Partial Purification of L-Asparaginase

The purification was carried out at 4^0C on the crude extract, according to the modified method of Distasio et al. (1976). For purification Ammonium sulphate fractionation and Dialysis method were used.

I. 16s-rRNA Sequencing of Screened Isolate

Molecular identification and classification on the basis of 16s-rRNA sequence analysis is the highest pink color zone giving isolate selected and identified as *Pseudomonas aeruginosa*. The results of sequences were submitted to National Centre for Biotechnology Information (NCBI) Gene Bank.

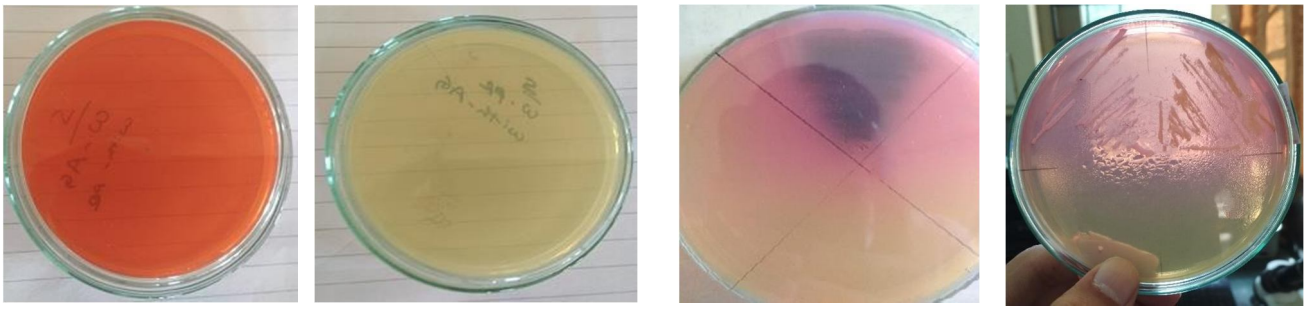
J. Identification of Molecular Components and Structure by FTIR Analysis

Fourier-transform infrared spectroscopy, also simply called as FTIR Spectroscopy, is a technique that provide information about the chemical bonding or molecular structure of material, whether organic or inorganic. The technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. A molecule that is exposed to infrared rays absorbs infrared energy at frequencies, which are characteristic to that molecule. During FT-IR analysis, a spot on the specimen is subjected to a modulated IR beam.

III. RESULTS AND DISCUSSION

A. Primary Screening OF L-Asparaginase Producing Bacteria

Based on the primary screening by serial dilution technique on Nutrient agar plate 55 isolates were found. Among them 23 isolates gave pink color around the colony on modified nutrient agar plate.



[A] [B] [C] [D]

Figure 1: Modified nutrient agar plate [A] showing un-inoculated control PR+, LASP- plate; [B]showing un-inoculated control PR-, LASP+ and [C] and [D] showing production of L-Asparaginase by microorganism on plate in pink color and negative as in yellow color.

Out of total 55 screened isolates only 23 isolates were found to be positive for L-Asparaginase production by rapid plate assay method. Pink zone around the colonies indicated production of L-Asparaginase**.** The isolates were further tested for LA production. Selected 23 isolates through primary screening were positive in that they showed intense pink zone around the colonies on modified Nutrient agar medium.

B. Phenotypic Identification

Microbial identification was carried out by standard microbiological procedures.

C. Secondary Screening of L-Asparaginase Producing Bacteria

In this study, Secondary screening, by Agar cup method L-Asparaginase producing 23 isolates gave different zone of diameter after 24 hours of incubation. Out of this Dumas beach soil sample isolate DU1 was found good L-Asparaginase producer as it exhibited 52 mm pink zone. So the DU1 Isolate was selected for further study. By zone assay DU1 isolate give highest zone 52 mm found

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maximum zone giving organisms from forest soil. However, there are many reports that justify the selection of potential isolate depending on the diameter of pink zone around the L-Asparaginase producing colonies. The zone obtaining isolates showing in below Figure 2:

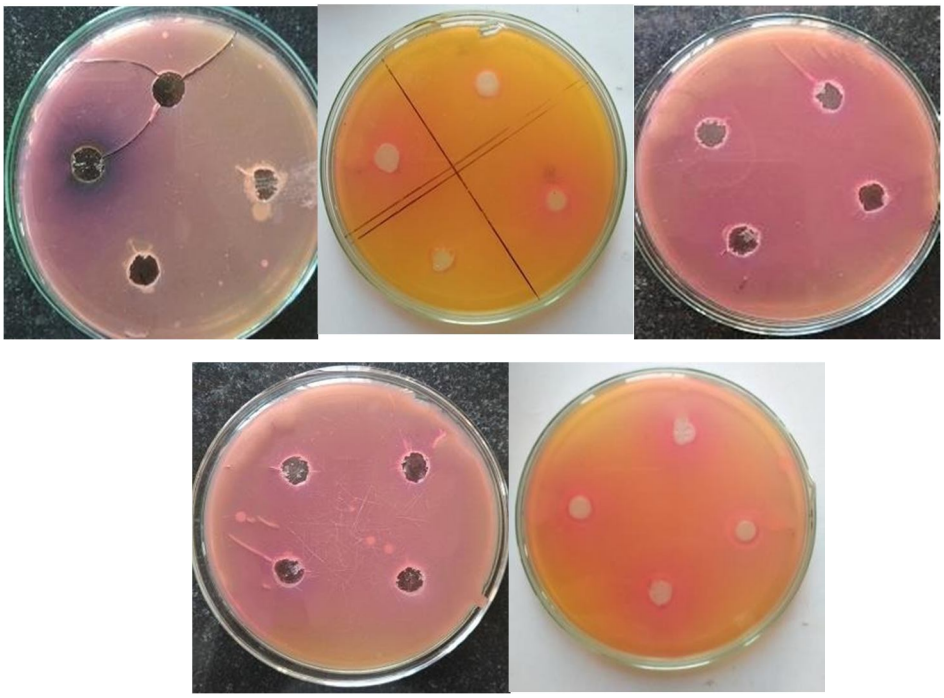


Figure 2: Showing the diameter of Pink zone obtained by various primary screened L- Asparaginase producing colonies on Modified Nutrient Agar medium by Agar cup method. Pink coloration around wells indicates L-Asparaginase production due to breakdown of present L-Asparagine.

D. Determine of Enzyme Activity

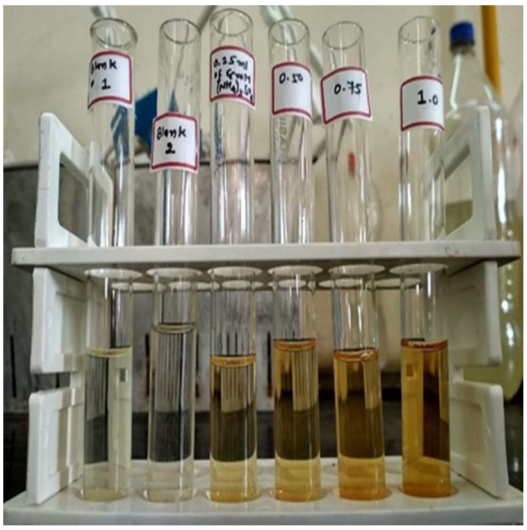


Figure 3 : Result of Standard of Ammonium sulphate

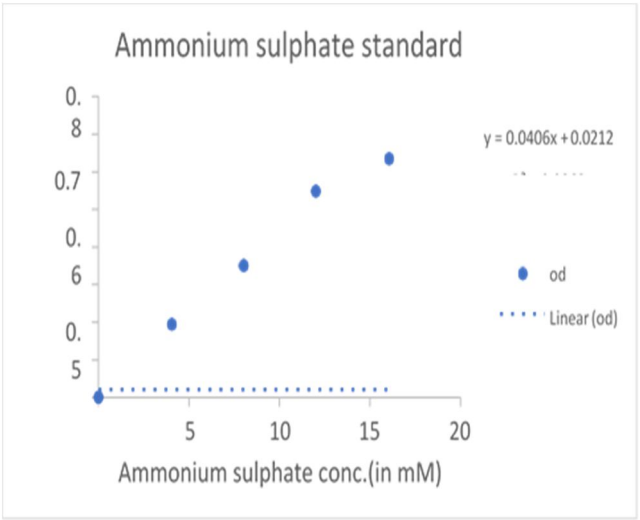


Figure 4 : Standard graph of enzyme assay

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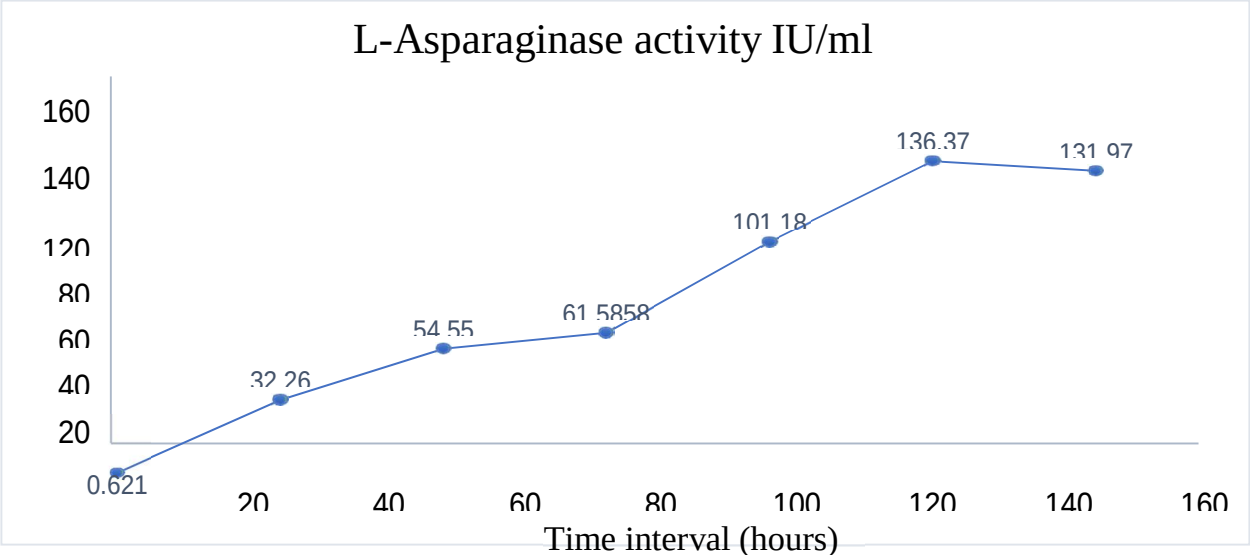


Figure 5: Growth curve of Enzyme assay

In the enzyme production at 24 hours, 48 hours,72 hours,96 hours, 120 hours and 144 hours at the log phase gave increasing amount of activity after 120 hours the production of enzyme and enzyme activity is 136.37 IU/ml found 13.53 IU/ml enzyme activity.

E. Protein Estimation

Protein estimation was done by Folin-Lowery method using bovine serum albumin as standard.

Protein estimation was performing by taking undiluted 0.1 ml aliquot of supernatant of production broth.

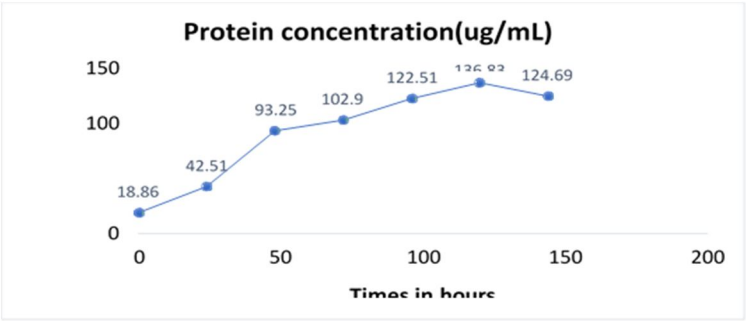


Figure 6: Estimation of protein

F. Optimization of Different Parameter

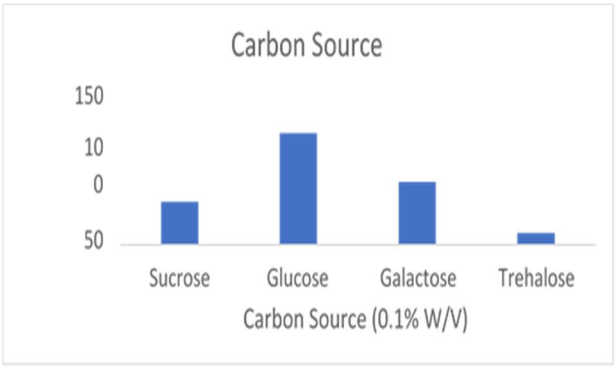


Figure 7: Effect of carbon source on L-Asparaginase production

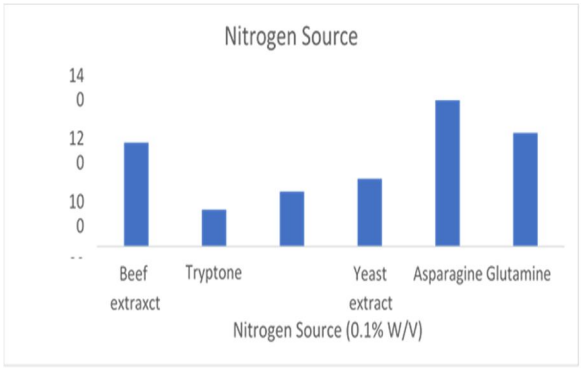


Figure 8: Effect of Nitrogen source on L-Asparaginase production

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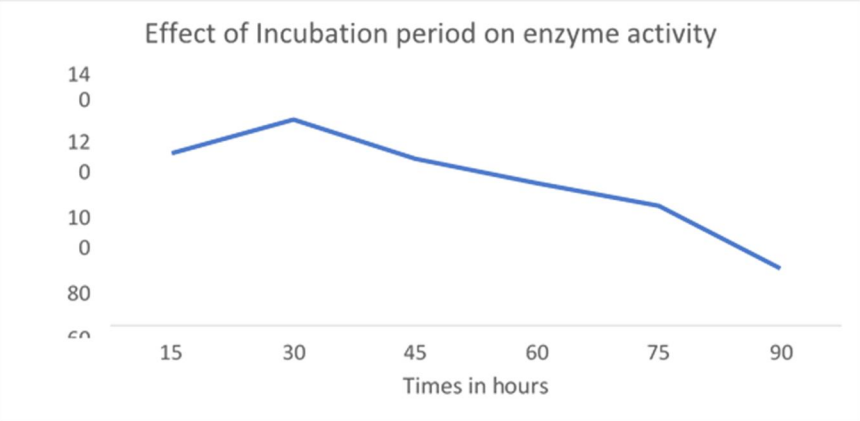


Figure 9: Effect of Incubation period on enzyme activity

G. Purification of L-Asparaginase Enzyme

The partial purification was done by ammonium sulphate precipitation technique. The partially purified L-Asparaginase enzyme showed a specific activity of enzyme was 4.256 IU/ml, 11.92-fold purity with a yield of 56.52%. Lingappa, K.; Vivek Babu, C.S. (2005) found 1.8-fold purity with a yield of 50.6%.



Figure 10: Purified L-Asparaginase Dialysis

Table 1: Result of purified L-Asparaginase enzyme

	Total activity (IU/ml)	Total protein(µg/ml)	Specific activity (IU/µg)	Fold purification	% yield
Crude enzyme (20ml)	1.25	3.5	0.357	1	100
Precipitate enzyme (5ml)	70.66	16.6	4.256	11.92	56.52
Dialysis	18.3	12.6	1.452	4.06	14.64

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H. Partial Molecular Identification (16s-rRNA sequencing) of Screened Isolate:

Isolate DU1 (*Pseudomonas aeruginosa*) was identified as highly L- Asparaginase producers amongst and therefore selected for partial molecular identification. Comparison of partial 16s-rRNA sequence with known sequence database using BLAST tool of NCBI. Partial sequence of isolate showed 98.47% identified with 16s-rRNA partial sequence *Pseudomonas aeruginosa.*

I. Identify Molecular Components and Structure by FTIR:

The FTIR analysis of our purified L-Asparaginase is shown in Figure 11**.** The X-axis represented the Infrared spectrum that plots the intensity of Infrared spectra. The Wave number on Infrared spectrum is plotted between 4000 to 400 cm^{-1}. The Y-axis represented the amount of Infrared light transmitted by an enzyme. The relative intense peak at 1057 cm^{-1} corresponds to bending of amine group. Absorption peak between 1548 to 1653 cm^{-1} are associated with N-H bending of amide group. The relatively broad peak at 3304 cm^{-1} represents the N-H stretch of amine group. The results thus confirm the presence of amide and amine group present in L-Asparaginase.

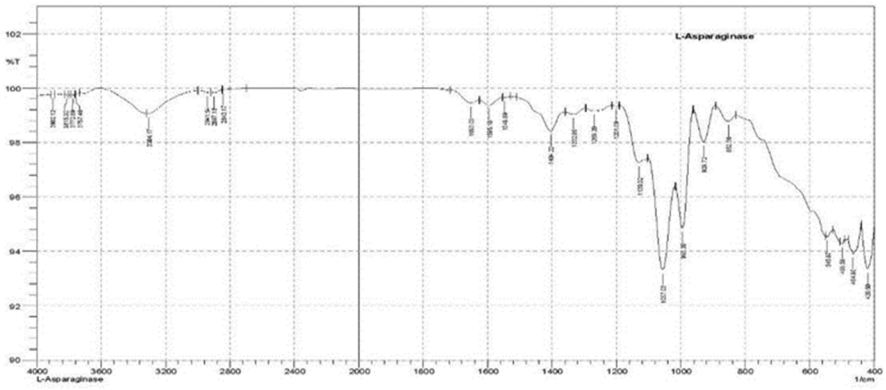


Figure 11: FTIR Analysis

IV. CONCLUSION

The preliminary work of Screening L-Asparaginase producing isolates from chosen sampling site show variety of microorganisms. By performing agar well diffusion method maximum zone given DU1 isolate was carried out for enzyme production. It was may be *Pseudomonas* spp. After 120 hours it shows enzyme activity 136.37 IU/ml. Among 55 isolates, 23 isolates were found as L-Asparaginase producers while DU1 isolate screen as highest production during optimization: Glucose and Asparagine observed as best Carbon & Nitrogen sources respectively, 30 minutes time interval as optimizes incubation period found enzyme activity. After performing partial purification and Dialysis 14.64% and 56.52% yield was obtained. Molecular identification of Isolate DU1 and this isolate is *Pseudomonas aeruginosa*. Identification of amide group and amine group from isolated L-Asparaginase enzyme by analysis of FTIR.

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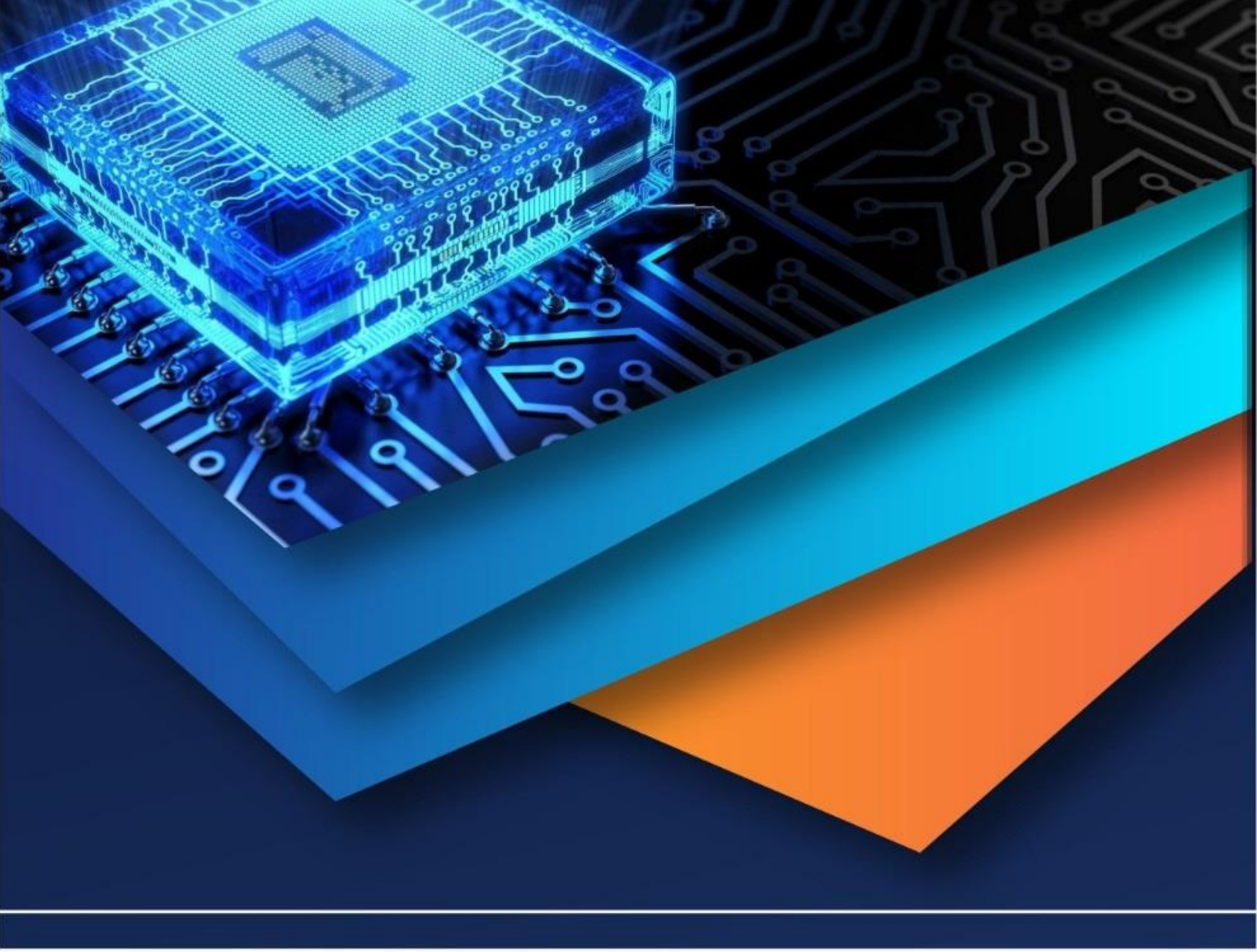
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