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ISOLATION, CHARACTERISATION AND OPTIMIZATION OF IMMOBILIZED L-ASPARGINASE- ANTICANCER ENZYM FROM ASPERGILLUS NIGER

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ABSTRACT
L-Aspariginase, an anticancer enzyme fits to a cluster of homologous amino hydrolases which catalyses the hydrolysis of aminoacid L-asparigine to L-aspartate and ammonia to regulate can cereous disorders. The present study deals with screening, isolation and optimization of L-aspariginase Producing fungal strain of soil sample from different areas of AP , India. L-Aspariginase activity was detected on the basis of pink colored surrounding the growth colony. A total of 132 colonies were screened and isolated from all the samples. Based on the zone diameter L-aspariginaseacitivity is concluded, L- aspariginaseacitivity is optimized at 28^{o}c and Immobilized Aspariginase showed more activity than the free enzymes.
KEY WORDS:L-Aspariginase, Immobilization, Asparigine, Temperature.

INTRODUCTION

MICROBIAL ENZYMES:

Enzymes have amazing catalytic power and their high level of specificity for their substrate makes them suitable for biological reactions. They are crucial for cellular metabolism. Each and every reaction that plants, micro-organisms and animals proceeds at a quantifiable rate as a direct result of enzymatic catalysis. In 1837, Berzelius documented the catalytic nature of fermentation.In the 1850 s Louis Pasteur reported that fermentation was a process initiated by living organisms. During this study it was reported that the fermentation of sugar into alcohol by yeast was catalyzed by ferments. He also hypothesized that these ferments are close to the structure of yeast. These ferments were later called enzymes (in yeast) .The key breakthrough in the history of the enzymes came in 189 when Edward Buchner isolated from yeast cells, the soluble active form of the set of enzymes that catalyses that fermentation of sugar to alcohol.Emul Fischer reported the first systematic studies on enzyme specificity in the early twentieth century [1]. Later, in 1926, James Sumner extracted urease in pure crystalline form from jack beans [2]. He also recognized the protein nature of urease. In 1930, John Northrop and his co-workers crystalized pepsin and trypsin and established them as proteins [3]. In subsequent year's enzymology developed rapidly.

At present, 2000 different enzymes have been recognized, each of which catalyzes a different chemical reaction. Currently, more focus is being directed towards the application of enzymes. The high efficiency of enzymes makes them commercially valuable and their specificity of action is offering diverse advantages in clinical medicine[4]. Asparaginase is an enzyme that is used as a medication and in food manufacturing [5] [6]. As a Medication, L-asparaginase is used to treat acute lymphoblastic leukemia [ALL] [7]. It is given by injection into a vein, muscle, or under the skin. [8] A pegylated version is also available [9] In manufacturing it is used to decrease acrylamide [10].

ENZYMATIC ACTIVITY OF L -ASPARIGINASE:

L-Asparaginase (EC 3.5.1.1) catalyses the hydrolysis of asparagine to aspartate and ammonia in mammals, birds, plants, and microbes. L-Asparaginases have become popular because of their capacity to remove asparagine from complicated systems. There are numerous uses in medicine and the food industry (11). L- Asparaginases, for example, are formed from treatment protocols for acute infection caused by E coli and Erwiniachrysanthemi are required. Lymphoblastic leukemia is a type leukemia that affect children (12). They work by diminishing the extracellular L- asparagine pool, which is necessary for growth and development. The growth of cancer cells Normal cells, in contrast to malignant cells extracellular L- asparagine is not required. In heat treated foods, L-asparaginases are also utilized to prevent the development of acrylamide (1314). Asparagine and reducing sugars may react via the malliard process to produce acrylamide during high temperature cooking (16-18). Although the relevance of such low acrylamide levels in not fully understood, the food industry benefits. Because of this, the industry has taken voluntary initiative to limit acrylamide levels in final products. Concerns about its carcinogenic potential (19-22). Pre-treating raw meat is one of the most important strategies for achieving the goal. L-asparaginase and a few commercial enzyme preparations are used to lower asparagine levels because of this are available for any reason (23, 24). However, industrial scale enzymatic activities are frequently carried out in hostile environments, such as high temperatures or salts, which can deactivate enzymes derived from common bacteria.

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Enzyme produced by halophilic or halotolevant bacteria, on the other hand, have a tendency to be more halophilic or halotolevant. Increased tolerance for severe circumstances such as high salt concentrations, high temperatures, non-physiological PH levels, and organic solvent (25). This research was carried out in a try to find halophilic or halotolevant. Microorganisms that can produce L-asparaginases using industrial applications are possible. L-asparaginase (EC 3.5.1.1), a medium-sized enzyme. L-asparagine (essential amino acid) is hydrolyzed to treptomycesid and ammonia. Because certain types of tumor cells require L-asparagine for protein synthesis, when L-asparaginase is present, they are deprived of an important growth factor. As a result, leukemic cells become cytotoxic. L- Asparaginase is widely distributed enzyme that can be found in a variety of microbes, including bacteria (Peterson and Ciegler, 1969) Aerobatic, Bacillus, pseudomonas, serratia, xanthomonas, and Photo bacterium. Streptomyces (Dejong, 1972), Proteus (Tosaetal, 1971), Vibrio(kafkewitz and Goodman 1974), and Aspergillus Niger.(sarquis et al 2004).The fact that not all L- asparaginase has anti-tumor capabilities appears to be linked to the enzyme is substrate affinity and other parameters. The systems clearance rate (cornea et al 2002) L-asparaginases have recently been discovered to be beneficial anti-tumor activity derived from E coli and Erwiniacarotovara. Lymphoblastic leukemia is a type of blood cancer that effect children and adolescents (Mash burn and wriston, 1964).

However, the use of an enzyme protein for this purpose is not recommended. In general, a long period of time causes the tissues to manufacture the appropriate anti-body, which results in an anaphylactic shock may occur, as well as the drugs impact being neutralized. As a result, finding a novel serologically distinct L-asparaginase with a similar therapeutic effect is a top priority. The goal of the research is to find new possible species that can produce L-asparaginase (26).

MATERIAL AND METHODs:

COLLECTION OF STRAIN: Strain: Aspergillus Niger The fungus Aspergillus Niger is mainly present in moist and shady places, where there is continuous Presence of the water. They have collected from onions, apples, tomatoes, canteen backyard, city garden and shore of a pond at mangalagiri, and from surrounding on our college area.

ISOLATION OF FUNGUS:

The collected samples were serially diluted up to 10-14 dilution in sterile water. After dilution 0.1 mL from 2nd, 3rd and 4th dilutions were spreader on PDA & CDA plates. The pH of the PDA & CDA medium was maintained at 5.6 & 6.8 respectively. [27], [28-29].The cultural characteristic was determined by its appearance on culture plates while the Morphological features was determined microscopically using lacto phenol cotton blue staining Technique. The pure culture was maintained on Potato Dextrose Agar (PDA) slants (HIMEDIA, India), regularly sub cultured and maintained at 4 °C (30).

PREPARATION OF PO TATO PEEL STARCH HYDROLYSATE (SPPSH):

The procedure described by Betiku and Ajala [31-33] was adopted in this work with some Modification to produce the potato peel starch hydro lysate (SPPSH). Freshly harvested mature potato Tubers were purchased from a local market in Vijayawada, AP. The potato tubers were washed with clean potable water to remove dirt, soil and adhering latex. The tubers were manually peeled with a clean stainless-steel knife and the peels were sun dried for four days. The peels were further dried to Constant weight using a plate dryer and were subsequently milled into powder form. Five hundred-Gram (500 g) powder form was dissolved in 5 liters of distilled water, sieved with Teflon cloth and Allowed to settle for 24 h (34-35). Water was decanted from the settled starch and dried in an oven at 60°C, overnight.

A measure of twenty-five grams (25 g) of the starch was dissolved in 100 mL of sterile distilled water to make 25% (w/v) slurry. A solution of 5 mm CaCl2 was added for the stability of the enzyme. The PH of slurry was adjusted to 6.5 with citrate – phosphate buffer. Gelatinization was done by heating the mixture to 97°C and held at this temperature for 10 min. The gelatinized starch was then cooled to 55°C. Liquefaction was carried out by adding 2% (w/v) of amylase at PH 6.5 and heated 60°C for a period of 1hour. For the saccharification, 2ml of glucoamylase was added at PH 5.0 after which the temperature is maintained at 55°C for a period of 1hour (36-38). The enzyme activity was stopped by heating the mixture to 100°C for 15min. The final mixture was then centrifuged at 10,000rpm for 10min.

SCREENING OF FUNGAL ISOLATE:

The fungal strain was screened qualitatively in Petri dish for the production of citric acid following the methods described by Rao and Reddy [39]. Sterile Czapek-Dox Agar medium (20 mL) Incorporated with 5% Bromo-cresol green dye and streptomycin to inhibit the growth of bacteria was Poured into separate sterile Petri plates and maintained at room temperature. The Czapek-dox agar Contains (g/L): Sucrose 120 g, $NaNO_3$ 5 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 1 g, $CuSO_4 \cdot 7H_2O$ 0.02 g, $FeSO_4 \cdot 7H_2O$ 1 g, $ZnSO_4 \cdot 7H_2O$ 1 g, Agar 15 g and Distilled water 1000

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Ml (40). The pH of the Medium was adjusted to 6.5. A sterile cork borer of 5 mm diameter was used to bore holes in the petri Dishes. Each of these plates was then inoculated with 0.5 mL of the spore suspension of the culture. The plates were incubated at 28±2 °C for 5 days and observed for the appearance of yellow zone Which indicates citric acid production by the test organism(41).

MEDIUM COMPOSITION FOR ASPERGILLUS NIGER PRODUCTION: Fermentation medium used for this study is composed of ammonium nitrate (NH4NO3), magnesium Sulphateheptahydrate (MgSO4•7H2O), potassium dihydrogen phosphate (KH2PO4) and (1–5%v) of methanol concentration [42]. The carbon source used in this study was sweet potato peel starch Hydrolysate. All media and flasks were sterilized in an autoclave at 121 °C for 15 min. Sabaurds Agar medium is used for cultivation of fungi.

IDENTIFICATION OF ASPERGILLUS NIGER:

From the plates inoculated with the samples, a loop full of the culture was taken and placed on the slide. Then a drop of lacto phenol blue was poured on it for staining. It was observed under the microscope at 40X magnification power to check its morphology to confirm that the isolated fungus was Aspergillus Niger (43). It was present in two of the samples.

Collection of Enzyme: The enzyme can be collected from strain by following processes. They are: 1.Filtration 2. Ammonium sulphate precipitation 3.Dialysis 4. Ion Exchange Chromatography.

CHARACTERISATION OF THE ENZYME:

About 1 gm 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.1ml each, of 10-5 dilutions were added to 20 ml of sterile modified M9 medium (as described below) maintained at 45ºC, Mixed thoroughly and plated in 10 cm diameter sterile petridishes and incubated at 37ºC(44). Anticancer agents were incorporated to control the cancer contamination. After 24 h of incubation, the selected bacterial colonies with pink zones around them were picked up and transferred onto M-9 medium slants (Gulati et al., 1997). Control plates were of modified M9 medium without dye and without L-asparagine (45). The zone and colony diameter were measured after 24 hours. Colonies with pink zones around the colony were considered as L-asparaginase producing strains.

PURIFICATION OF L-ASPARAGINASE:

Purification of intracellular L-AsparIginase was done using gel permeation column Chromatography using Sephardim G-100 and its purity was checked using SDS PAGE (46). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out in using slab gel of 7% acrylamide in a Tris-HCl buffer pH 8.3 containing 0.1% SDS. The gels were stained with 0.025 Coomassie brilliant blue R-250 and destined (Stegemann, 1979) (47).

OPTIMISATION OF ENZYME:

The most potent fungal isolate was Aspergillusterreus which yielded the highest l-Asparaginase specific activity (4.81 U/mg protein). The highest enzyme productivity was observed on the 5th day and the optimized fermentation parameters were pH 6.0, temperature 35 °C (48). The yield was also high up on using dextrose and asparagine (8.26 U/mg protein) as carbon and nitrogen sources (49).

Table 1: OPTIMISATION OF CULTURE MEDIA:

CULTURE MEDIA	MEDIA COMPONENTS	WEIGHT	% OF ENZYME
1.	Czepak s dox agar medium	35 gms	3.5%
2.	Modified czepaksdox agar medium	49.01 gms	0.12%
3.	Potato dextrose agar medium	20 gms	4.5%
4.	Brain heat infusion method	19.5gms	0.3%
5.	Soya bean casein digest medium	40 gms	2.0%

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The more percentage enzyme activity was seen in PDA media.

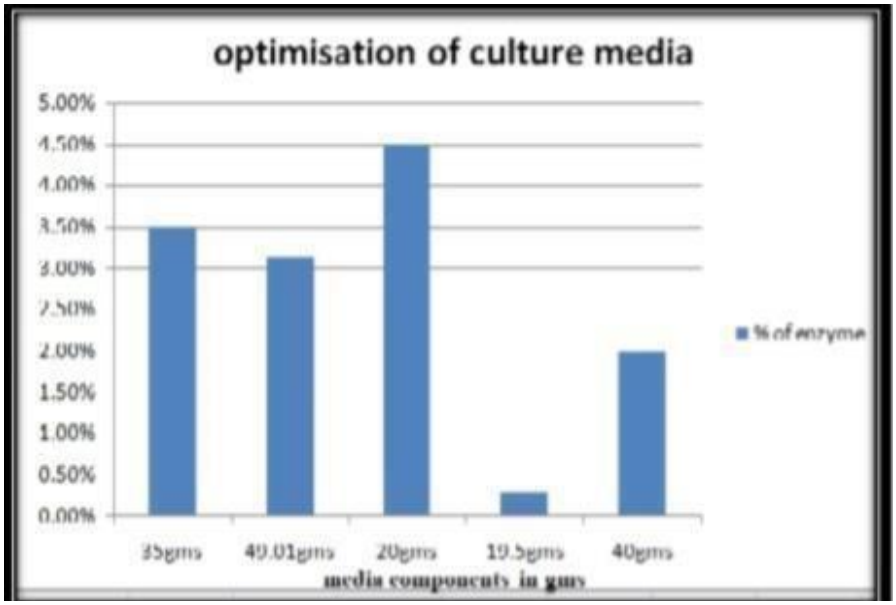

Fig 3.1EFFECT OF CULTURE MEDIA ON ASPARIGINASE.

Table 2: OPTIMISATION OF TEMPERATURE:

S No	TEMPERATURE	CONCETRATION OF ENZYMES
1.	4.5	20
2.	5.5	29
3.	6.5	41
4.	7.5	42
5.	8.5	49
6.	9.5	43
7.	10.5	33

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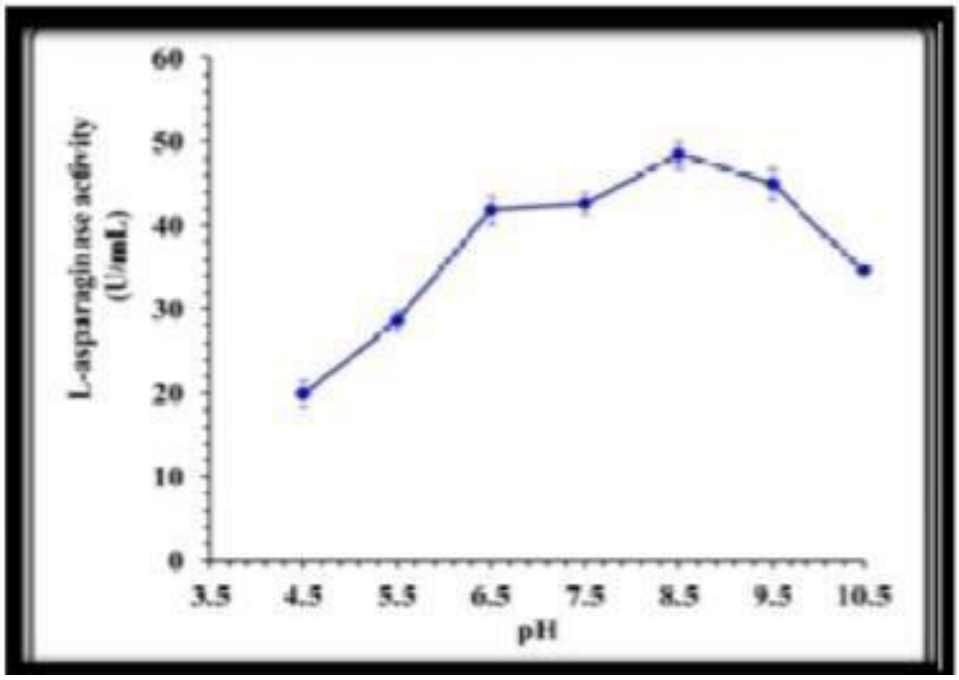

Fig 3.2.EFEECT OF PH ON ASPARIGINASE

Table3: OPTIMIZATION OF INCUBATION TIME:

S No	Incubation period	Concentration of enzyme
1.	10	33
2.	20	41
3.	30	48
4.	40	52
5.	50	71
6.	60	64
7.	70	66
8.	80	60

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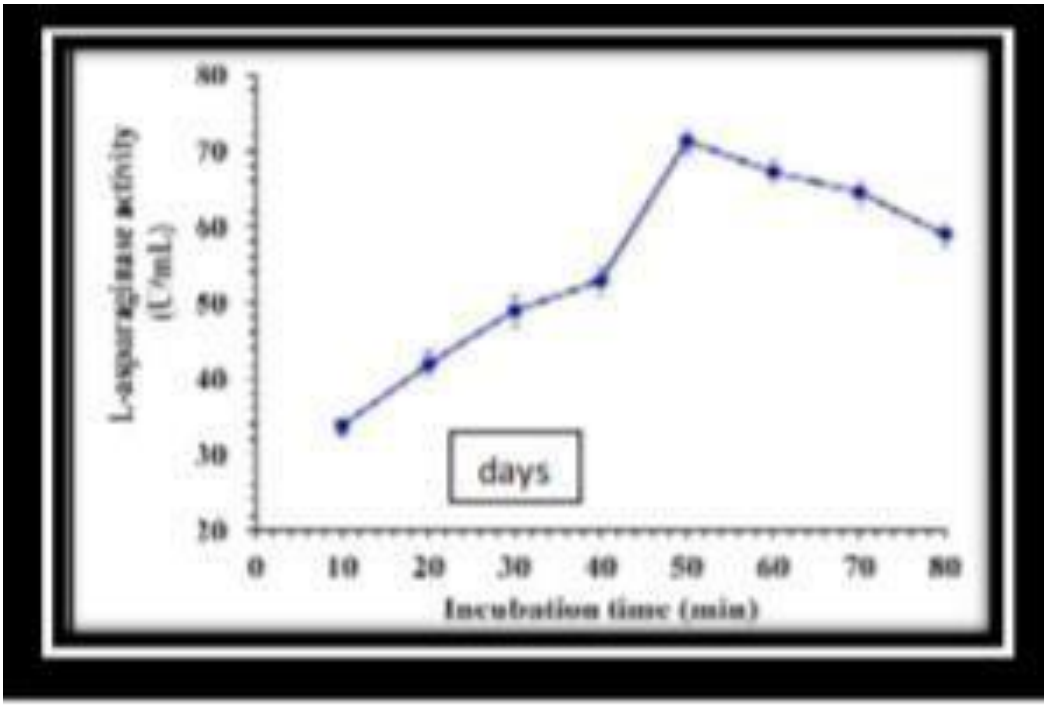

Fig no 3.3.EFEECT OF INCUBATION ON ASPARIGINASE.

IMMOBILISATION OF L-ASPARGINASE:

- Enzyme immobilization is an attractive field to improve l-asparginase activity and stability (50).
- The covalently immobilized l-asperginase showed higher enzyme activity than free enzyme at pH 8 with the maximum recovered activity of 100%, 90.5% and 40.6% after 24 hrs. of incubation at 20° , 40° and 60°c respectively(51).

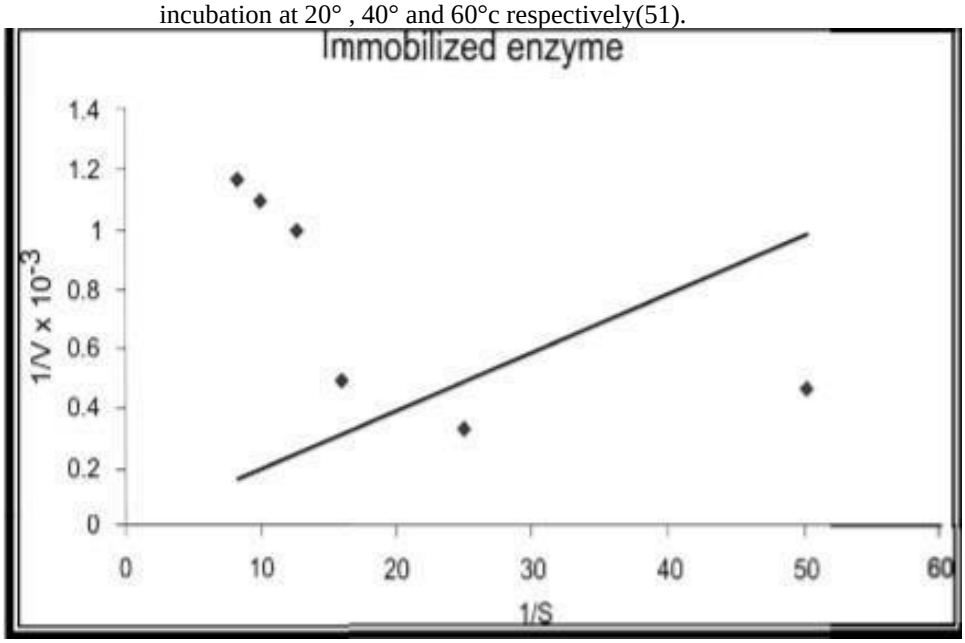

Fig 3.4ACTIVITY OF IMMOBILIZED ENZYME

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IMMOBILIZATION OF L-ASPARGINASE CARRIER MATERIALS

Carrier for l-asparginase immobilization:

Carriers such as organic inorganic and hybrid materials have been used for -ASNase are immobilized in the literature. Almost all the carrier matrixes for L-ASNase are catalogued. The aim of this review is to give an overview because there are no papers in this topic. Therefore for better understanding, we classified the carrier type and the advantages and disadvantages of L-ASNase (52) were compared to that of the free one. At the same time we thought it was necessary to summarize the available immobilization information. For this reason we compiled the most interesting result, optimum pH temperature Michael's constant (Km) and stability properties of the immobilized L-ASNase (53) And these results are listed.

These tables will provide important information to interested researchers for the selection of the appropriate carrier method for L-Asparinase immobilization (54).

METHODOLOGY OF IMMOBILIZATION OF L-ASNase

GELATIN METHOD:

- Dissolve 1.0gms of gelatin in 10 ml of water to prepare 10%of aqueous solution.
- Heat the solution gently to facilitate the diffusion process.
- Approximately 20microlitre enzyme (0.50) and 2ml of hardening solution (20% of formaldehyde, 50% ethanol and 30% water) were added.
- Add 10microlitre of phenol red indicator to the solution.
- Then pour it into mould and allow to freeze it at -20°c for 4 hrs. to facilitate Gel formation.
- When the gel is set warm the gel to room temperature.

DETERMINATION OF ASPARAGINASE ACTIVITY:

(a) Qualitative assay for production of L- asparaginase by isolated Aspergillus Niger Cultures:

Agar diffusion technique was used for qualitative assay of L-asparginase by isolated fungal cultures. Modified CzapekDox's Medium (pH 6.8) supplemented with sodium nitrate 2.0gm, potassium chloride 0.5gm, magnesium glycerophosphate 0.5gm, ferrous sulphate 0.01gm, potassium sulphate 0.35gm, sucrose 30.0gm, agar agar 12.0gm, L-asparagine (1%w/v), distilled water 1000 ml and phenol red (0.009 % w/v) was used as an assay medium(55). Sterilized medium (10 ml) was distributed in the presterilized culture tubes to prepare stabs. After that a loopful culture of each isolate was inoculated on the surface of solidified stabs and incubated at 37°C for 24 to 48 hours. UN inoculated was regarded as a negative control (56). Stabs were examined for change in color of medium from yellowish to pink due to change of pH indicating the positive asparaginase activity. Gradation of asparaginase activity was done On the basis of the extent of color change of medium. Every experiment was conducted in triplicate [57]

ASSAY OF Aspariginase:

Davis et.al 1947 reported a colorimetric method for the measurement of aspariginase Content it was modified by Bram and Solomon as such to develop color by incubating 28oC [58]. A standard curve was prepared with pure asparagine and which is final Hydrolysis product. The method of preparing from asparagine was that of suitable Blanks and controls were made.

Reagents:

1. Diethylene glycol – 90% of analytical grade (from merk) as a solvent for asparagine Davis 1947[59].

2. NaOH (4N) – Dissolving 16 gm. of NaOH pellets in 100ml of distilled water. Standard solution 25gm of pure asparagine sample was dissolved in about 40ml of distilled water and was heated up to 70oC ,precipitated for several hours Davis-1947[60].

Procedure:

10ml of diethylene glycol, 0.2 ml of different concentration of standard asparagine solution were taken 0.2ml of NaOH was added and kept aside for 5 min. The curve is plotted as asparagine concentration vs. Absorbance. Five different types of media with varied concentrations were studied for the production and results are tabulated which Indicates medium 3 was found to be the best with a titer of this production medium is Used for the submerged studies [61].

AGAR WELL DIFFUSION TECHNIQUE:

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The 100µl of cell free culture broth was poured into the agar well of diameter 8mm prepared in plates containing modified CzapekDox's Medium. The filtrate was allowed to diffuse into the medium for 12 hours at 4°C [62]. The diameter of zone (mm) of L asparaginase activity, as Indicated by the formation of pink colored zone around the well against the yellow background, was measured. For further studies, cultures showing greater enzyme production were selected [63].

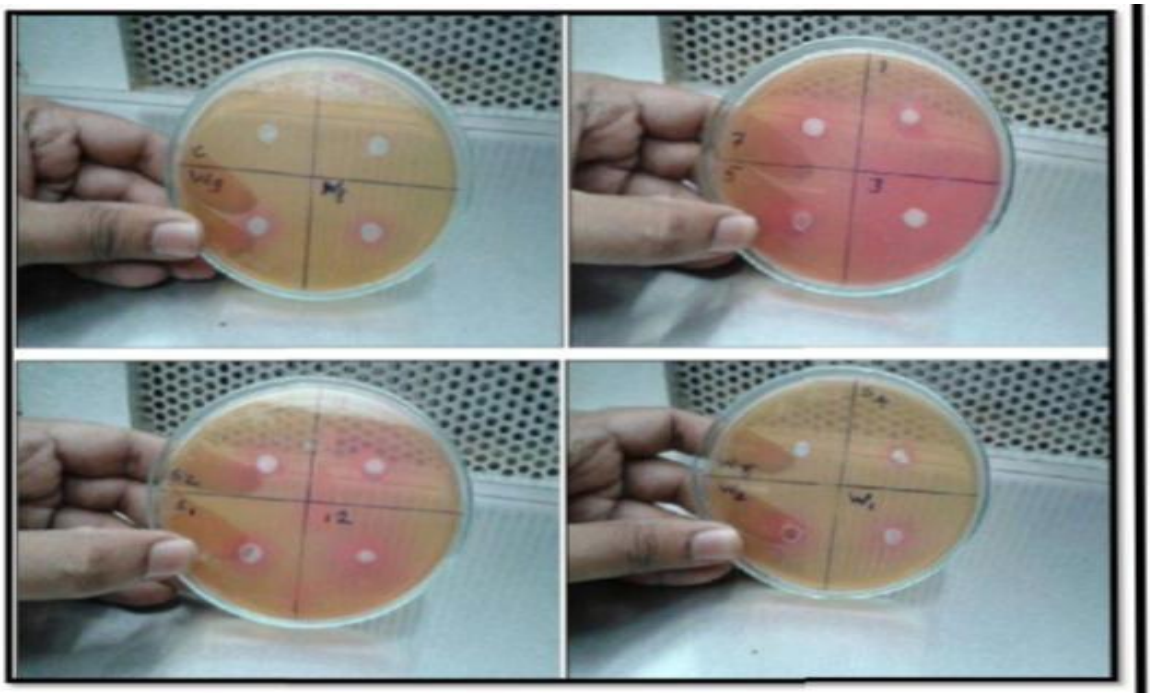

FIG 4.1 ASPARIGINASE ACTIVIVTY IN PINK COLONIES

QUANTITATIVE ASSAY FOR PRODUCTION OF L-ASPARGINASE BY ISOLATED FUNGAL CULTURE

The quantitative estimation of enzyme activity was done with selected culture i.e., A.niger isolates VRY-8, VRY-14 and VRY-15. Asparginase activity was measured by method of mash burn and wristen (1963)[64].The rate of hydrolysis of L-ASNase was determined by measuring the release of ammonia using nessler's reaction. The reaction mixture contained 0.5 ml of enzyme sample ,0.5 ml of 0.05 M tri HCL buffer pH 8.6 and 0.5 ml of 0.04 M L asparagine. The reaction mixture was incubated for 30 mints at 37°c (65]. The enzyme activity was stopped by the addition of tri chloroacetic acid TCA10% w/v [66]. The mixture was then centrifuged at 10000 rpm s for 5 mints and 0.5 ml of supernatant was taken and to it 3.7 ml of distilled water was added, 0.2 ml of Nessler's reagent [67] was added to the reaction tube and kept at 20°c for 20mins. The absorbance was measured at 450 nm using spectrophotometer [68]. The amount of ammonia liberated was calculated by using ammonia standard curve. One unit of L-asparginase activity is defined as the release of one micromole of ammonia per hour at 37°c and pH 8.6 [69].

Results and Discussion:

Determination of Asparginase activity has been carried out as described. In the present study a total of 4 media components were subjected or studied by using different optimizing parameters.

S.No	Types of culture media	Temperature	PH	Incubation period (days)	Concentration of enzyme activity
1.	Czapek s dox media	40^{0c}	8.5	50	3.8%
2.	PDA media	40^{0c}	8.5	50	4.5%

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3.	Modified czapek s dox media	40^{0c}	8.5	50	2.0%
4.	Brain heart infusion media	40^{0c}	8.5	50	0.3%

Table4: OPTIMISED PARAMETERS OF ENZYMETIC ACTIVITY

EFFECT OF CULTURE MEDIA : By comparing 4 different media components it is found more concentration of enzyme is shown by potato dextrose agar medium which is showing about 4-5%of enzymatic activity.

EFFECT OF TEMPERATURE: The highest peak concentration of enzymatic activity is observed at 40°C, it is suitable for the growth of culture that is Aspergillus Niger.

EFFECT OF PH: The concentration of enzymatic activity got highest percentage at 8.5 PH range. This is because the Aspergillus Niger grows comfortable at this specific pH conditions.

EFFECT OF INCUBATION PERIOD: The more concentration of enzymatic activity is observed at 50days of incubation period.

CONCLUSION:

We have isolated and optimize parameters for the Aspariginase production from Asparigillu. Niger Immobilized aspariganase showed more potency than the free enzyme.

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