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PRODUCTION OF ASPARAGINASE ENZYME BY FILAMENTOUS FUNGI

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***Abstract*: About 31 different moist humus rich soil samples were collected from different locations in and around Egmore, Chennai and preliminarily screened by plate assay using Czapex dox medium without carbon source, instead asparagine is used as carbon source and Phenol red is used as indicator which shows pink holo zone from pale yellow colour indicating production of the asparaginase enzyme. From the screening, eight different isolates were showed positive results indicating pink holo zone around the colony. Among the eight isolate N1 showed maximum zone of 5.3 cm whereas enzyme assay by nesselerisation method also revealed isolate N7 showed higher asparaginase activity which was found to be 5.0622 U/ml . From the slide culture the isolate N1 & N7 is identified as *Fusarium sp*.**

***Keywords*: Asparaginase, *Fusarium sp* and Phenol red indicator.**

1. INTRODUCTION

Amino acid is needed for the proliferation and metabolic processes (Luhana et al., 2013). It is important in the role of biosynthesis of protein, DNA and RNA. The non- acidic hydrophilic amino acid are obtained through blood serum or they are self synthesized (Narta et al., 2007). Cells also need asparagine to proceed cell cycle and cell division (Rytting., 2012). Hence, L- asparagines is a nutrient supply for the tumor cells to proliferate.

L-Asparaginase is an enzyme which belongs to homologous amidohydrolases family, which catalysis the breakdown of L-asparagine into aspartic acid and ammonia. Normal cells have the sufficient enzyme (asparagines synthetase) for their catalyzing activity whereas cancerous cells have low level of asparaginase enzyme production. So there is need of L-asparaginase enzyme to break the tumor cells. L-asparaginase has increased attention in the recent year for its anti carcinogenic potential and is used as a chemotherapeutic agent for acute lymphocytic leukemia and less frequently for acute myeloblastic leukemia, chronic lymphocytic leukemia, hodgking's lymphoma. The reason it is preferred for this purpose is that, it is biodegradable, non-toxic and can be administered at the local site quite easily (Lalitha Devi A.S & Ramanjaneyulu R., 2016).

Moreover, the demand for L-Asparaginase will increase several folds in coming years due to its potential application in food processing addition and its clinical application (Pedreschi et al., 2008). To overcome this constraint, productions of L-Asparaginase by other microbes should be explored. Therefore there is a search for other sources of L-Asparaginase production (Soliman et al., 2005). Hence, the sources of L-Asparaginase is documented from organisms such as animal, bacteria (Howard and Carpenter, 1972a), reported in bacteria such as *E. coli* and *Erwinia caratovora* (Narta et al., 2007), fungi (Sarquis et al., 2004), actinomycetes (Sudhir et al., 2012), algae (Paul, 1982) and plants (Oza et al.,2011b)

L-Asparaginase produced by bacteria leads to adverse side effects in human trails. Actinomycetes are the least studied organisms among all the L-asparaginase producing sources (Sudhir et al., 2012). In algae, the enzyme shows less antitumor activity in an anti lymphoma assay in vivo (Paul, 1982). L-asparaginase is also efficiently produced by fungi and the enzyme from fungal source has fewer side effects in comparison to the bacteria (Sarquis et al., 2004). Hence, to overcome these few side effects the research has to carry by altering the toxic gene into non toxic though genetic engineering technology must to be a non- pathogenic drug. Hence, the present study is to find asparaginase producing fungi.

2. MATERIALS AND METHODS

2.1. Collection of Soil Samples:

Different moist humus rich soil samples were collected from different locations of Egmore and used for screening Asparaginase producing fungi. The soil samples were air dried and used for the study.

2.2. Screening for Asparaginase Enzyme-Plate Assay:

Czapek dox medium KH_2PO_4-1g; $NaNO_3$-2g; $MgSO_4$-0.5g; KCL-0.5g; $FeSO_4$-0.01g; Asparagine-30g; Phenol Red-0.05g; Agar-30g; p^H-7 was prepared for 1000ml. Asparagine acts as a sole Nitrogen source instead of carbon source along with Phenol red as indicator was used. About 10mg of soil samples were spread on to sterile petriplate. Czapek dox agar was poured and swirled gently. After solidification it was incubated at 30± 2^0C and observed for the appearance of the fungal colonies in the medium. The fungal colonies which showed a pink holo around them were streaked on PDA medium (3.9g of commercially available PDA medium was suspended in 1000ml of distilled water and boiled. The pH was adjusted to 5.5 and transferred into 250ml conical flask and autoclaved at 121^0 C, 15 lb pressure for 15 min. They were distributed equally in the petriplates. After the fungal growth on PDA, a disc of 5mm diameter of the fungi was made using a sterile cork borer inoculated again on Czapek dox with phenol red. Colonies producing pink halo were positive for asparaginase activity. The diameter of the pink holo was measured and recorded.

2.3. Estimation of Biomass of Fungi:

Czepek dox broth with asparagine (as a sole nitrogen source instead of carbon) without agar and phenol red was inoculated with different isolates of fungi. After incubation for 7 days the growth of fungi was estimated. The fungal mat was harvested by filtering using pre weighed filter paper. The fresh weight of fungal isolates was estimated and the supernatant was used to estimate the extracellular protein.

2.4. Characterisation of Filamentous Fungi:

2.4.1. Biochemical Characterization (Estimation of Protein):

To 1ml of supernatant, 2ml of Coomassie Brilliant Blue (10mg of CBB G-250 was dissolved in 5ml of ethanol and 10ml of ortho-phosphoric acid and made up to 100ml with distilled water) was added. The test tubes were shaking thoroughly and the absorbance was read at 595nm. A corresponding BSA standard was used to estimate the protein and it was expressed in ug/ml.

2.4.2. Morphological characterization (Slide culture method):

A PDA disc of 5mm diameter was placed on the sterile glass slide. The fungal isolates were inoculated; a sterile cover slip was placed above the disc and tapped gently. The slides were incubated in moist chamber for 5days. After incubation the slides were stained in Methyl Blue, mounted in glycerol, sealed and were observed in 40X magnification in the compound microscope and identified. The potato Dextrose Agar was inoculated with the isolated fungi, incubated for 5 days at 27± 2 ^{0} C for colony morphology.

2.5. Assay for Asparaginase:

Czapek dox broth with Asparagine was prepared and the isolated fungi were inoculated and incubated at 27± 2 ^{0} C for 7 days. After the incubation period the culture was filtered and the filtrate served as the source of enzyme. The assay was performed according to Venil et al., 2009. Briefly, to 1ml of filtrate, 0.5ml 0f asparagine (0.2M) was added and incubated for 10 minutes at 37 ^{0} C. after 10 minutes 0.5ml of 15% TCA was added to all the samples and centrifuged. The pellet was discarded and the supernatant which contains ammonia was collected. To 1ml of supernatant 2ml of Nesserler's reagent was added and read at 500nm. A standard was prepared using ammonia solution and read at 500nm.It is measured as; release of 1umole ammonia was equivalent to 1 unit of enzyme activity.

3. RESULTS AND DISCUSSION

3.1. Screening for asparaginase producers:

Screening for asparaginase producing fungi was carried out in Czapek dox agar with asparagine along with phenol red indicator. The fungal colonies of eight soil samples showed pink holo around fungal colonies were designated as N1,N2,N6,N7,N9,8,8A,8A1(Table-1; Plate-1)

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Table 1: Screening for asparaginase producing fungi (Plate assay)

S.No	SAMPLE	DIAMETER OF PINK HOLO(CM)
1	N1	5.3
2	N2	4.5
3	N6	4.3
4	N7	1.7
5	N9	4.3
6	8	1.6
7	8A	4
8	8A1	5

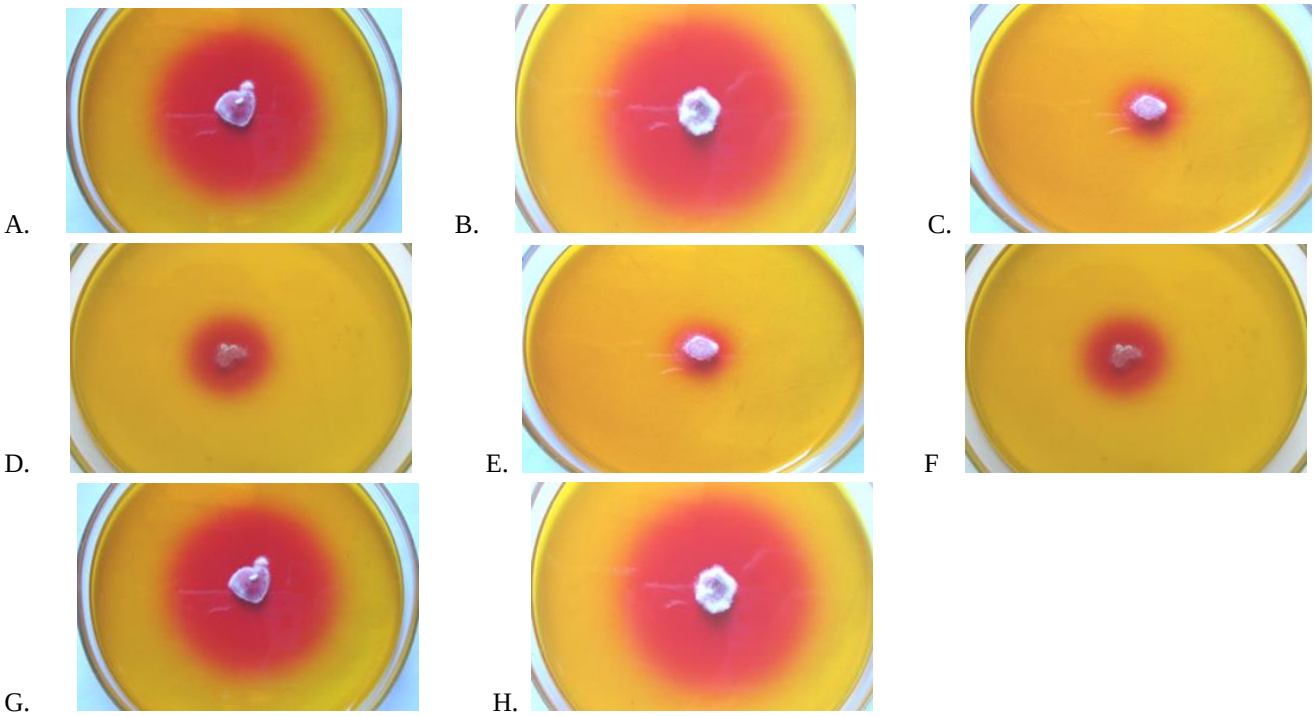

PLATE -1 SCREENING FOR ASPARAGINASE PRODUCING FUNGI - PLATE ASSAY

A - *Fusarium* sp. Isolate 8A1 ; B - *Fusarium* sp. Isolate N 1 ; C- *Fusarium* sp. Isolate 8

D - *Fusarium* sp. Isolate N7 ; E - *Fusarium* sp. Isolate 8A1 ; F - *Fusarium* sp. Isolate N 1

G- *Fusarium* sp. Isolate 8 ; H - *Fusarium* sp. Isolate N7

Among the 8 isolates N1, 8A1 showed the maximum diameter of pink zone while isolate 8 showed the minimum diameter. While the rest of the isolates N9, N6, 8A, N2 exhibited moderate halo, while the least zone was produced by N7. The eight fungal colonies demonstrated a varied pattern of pink halo in a solid medium.

The production of L-Asparaginase leads to ammonia formation which increases in the pH of the medium. The phenol red indicator changes from yellow (acidic condition) to pink (alkaline condition). The pink zone around the fungal colony indicates the pH alteration which originated from ammonia accumulation in the medium.

Since this method is very simple and rapid for the detection of L-asparaginase activity, it has been used for primary screening of L-asparaginase production from fungi such as *Aspergillus, Penicillium* , *Fusarium sp* and endophytic fungi isolated from Thai medicinal plants. Yones Ghasemi et al., 2008 investigated the various nitrogen sources and found modified M9 medium to be the best for the identification of L-asparaginase in *E.Coli*.

3.2. Fresh weight of fungal isolates:

A minimal medium supplemented with asparagine supported good growth of the fungal isolates. A higher biomass was recorded by N2 and N7 isolate with 141mg and 135mg respectively. Moderate growth of 128mg was recorded by 8A1

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and the least growth was exhibited by N1 isolate which had a biomass of 40mg (Fig-1). The highest amount of cell dry weight was obtained in the medium with 0.4% glucose. Maximum cell dry weight (1.560.06mg/ml) of fungi was observed with 1.0% L-asparagine as nitrogen source (Lapmak et al., 2010). It was suggested that L-asparaginase production was regulated by glucose and nitrogen level which were congruent with those investigated by Sarquis et al., 2004

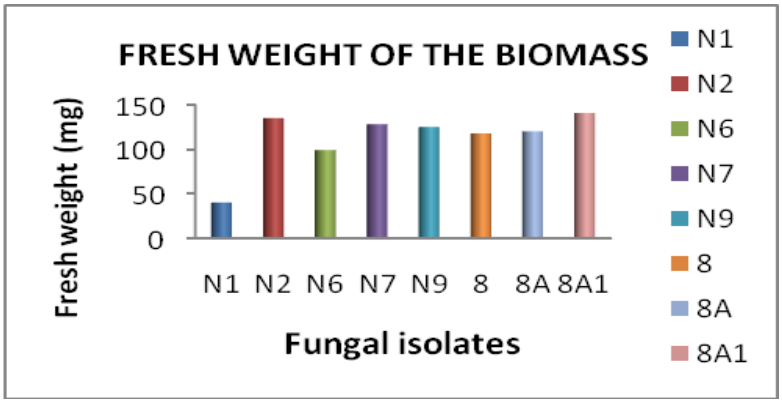

FIG 1: Fresh weight of fungal isolates

3.3. Estimation of Protein:

The extracellular protein was estimated from eight isolates that were positive asparaginase producers. Isolate N1, 8, 8A had maximum quantity of protein while the least protein was present in 8A1 isolate (Fig-2)

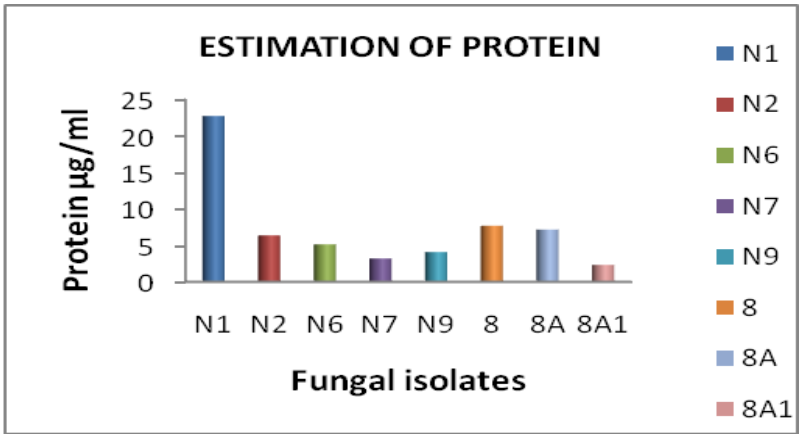

FIG 2: Extracellular protein of the fungal isolates

3.4. Identification of the Fungi:

The fungal colonies N1, N2, N6, N7, N9, 8, 8A, 8A1 were identified to be *Fusarium sp*, based on colony morphology, pigmentation and spores morphology. Among *Fusarium sp*. N1 isolate produced more asparaginase when compare to other fungi in agar plate assay. Yones Ghasemi et al., 2008 reported that the microorganisms such as *Acetobacter, Bacillus, Erwinia, Pseudomonas, Serratia, Xanthomonas, Photobacterium, Streptomycetes,Proteus, Vibrio* and *Aspergillus* produce asparaginase enzyme.

L-Asparaginase has been found in a variety of yeast and algae. It has been observed that the eukaryotic microorganisms like yeast and filamentous fungi such as *Aspergillus, Penicillium* and *Fusarium* are commonly reported to produce Asparaginase (Sarquis et al., 2004)

3.5. Assay for Asparaginase:

The fungal isolates that exhibited pink zone around colonies were tested for asparaginase production in liquid medium by nesserlerisation. The fungal isolates exhibited asparaginase activity between 0.1333-5.0622 U / ml (table-2). Isolates N7, N9, N2 exhibited high asparaginase activity of 5.0622, 4.0631 &2.7309 U/ml, respectively. Similar concordance of asparaginase activity in agar plate assay and spectrophotometer was not evident. Isolates N7 had lesser pink halo around

the colony in agar plate assay. However it showed enhanced activity in the liquid medium. This result was similar to earlier work of Holker et al., 2004; Lee et al., 2005 & Theantana et al., 2007 who suggested that the ability of fungi to produce enzyme was different in solid and liquid state condition. It is also reported that *Fusarium sp* was the most active group that can give zone diameter of 1.5-2.5cm in agar plate assay. *Xylaria sp* and *Phomopsis sp* were the second group (Zone diameter of 0.5cm). Imada et al., 1973 also reported that L-asparaginase was produced in all *Fusarium sp*. where the activity was 9.42 U /ml. However in the present study *Fusarium sp* isolated produce extensive zonation bit relatively lesser activity when compare to the reported *Fusarium sp*. However, *Bipolaris sp* isolate BR438 showed the highest L-asparaginase activity of 5.54u/ml in 0.4% of glucose (Lapmak et al., 2010). In the present study, *Fusarium sp* showed enhanced activity with no carbon source being provided in the medium. *Colletotrichum sp* demonstrated an enhanced asparaginase activity of 1.530 U /ml (Theantana et al.,2009). *Fusarium*, *Penicillium*, *Talaromyces* and *Eupenicillium* also demonstated high asparaginase activity. However, in the present study a minimal medium such as Czapek dox without carbon source proved to be efficient in identification of L-Asparaginase producing fungi.

Table 2: Asparaginase enzyme activity in Fusarium sp

S.No	FUSARIUM ISOLATE	U/ml
1	N1	0.8659
2	N2	2.7309
3	N6	0.9991
4	N7	5.0622
5	N9	4.0631
6	8	1.3987
7	8A	0.1332
8	8A1	2.0642

4. CONCLUSION

Asparaginase is a anti-neoplastic agent; anti-leukemia agent, which has a high potential against acute lymphoblastic leukeamia in children. Since, asparaginase enzyme which is produced by bacteria is toxic and causes adverse effects and allergic reactions to humans. so, other source of asparaginase from microorganisms like filamentous Fungi which is less toxic to humans is inevitable. Eight isolates of *Fusarium sp* was promising asparaginase producers. Among them isolate N7 produced maximum asparaginase. Further, investing the effect of solid waste substrate on asparaginase production can result in extensive yield of this potent enzyme.

Conflict of Interest:

The authors declare that they have no conflicts of interest.

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