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Anti-proliferative activity of L-Asparaginase enzyme from fungi on breast cancer

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Abstract
The present investigation deals with the anti-proliferative activity of an extracellular enzyme known as L-Asparaginase from *Aspergillus fumigatus* screened from infected leaves of the medicinal plant *Annona muricata*. The anti-proliferative activity of L-Asparaginase from *Aspergillus fumigatus* was checked on human breast cancer cell line MDA-MB-231.The influence of pH, temperature, substrate concentration, incubation period and concentration of metal ions were analyzed in the production of L-Asparaginase. The highest production of enzyme noticed at a pH of 8, 20mM substrate concentration, 20 minutes of incubation period, Presence of Fe^{2+} ions and 40 ^{0}C of temperature. These were the optimum conditions for the effective yield of enzyme. The enzyme isolated from *Aspergillus fumigatus* is a promising anti-proliferative agent and can be used in the expansion of further compositions for the therapy of tumors.

Keywords: L-Asparaginase, *Annona muricata*, *Aspergillus fumigatus*, characterization

Introduction
Annona muricata is a rich source of L-Asparaginase enzyme producing fungus [1]. L-Asparaginase (L-Asparagine amidohydrolase; EC 3.5.1.1) is used in the therapy of cancerous growths because of its biodegradable and oncological importance. L-Asparaginase is responsible for the hydrolysis of L-Asparagine into aspartic acid and ammonia [2]. L-Asparaginase has obtained considerable importance in the treatment of acute lymphoblastic leukemia [3]. Tumor cells require high amount of asparagine for their survival whereas the normal cells are self-sufficient for their own growth. For the reason that the hydrolysis of L-Asparagine into aspartic acid and ammonia leads to the death of tumor cells [4]. L-Asparaginase has been isolated from various sources such as plants, animals and microorganisms [5]. The property of L-Asparaginase is varied in accordance with their source. Studies showed that L-Asparaginase from bacterial sources leads to allergic conditions whereas fungal L-Asparaginase does not possess any unfavorable conditions [6]. An L-Asparaginase injection causes various side effects include inflammation of pancreas, liver disease, kidney disorders [7] and its use in gestation is dangerous to baby [8].
As an anticancer agent, L-Asparaginase enzyme is also employed in the food industry. According to the recent results, the performance of L-Asparaginase in various food products and assessed that it diminish the quantity of acrylamide in the final products [9].

Materials and Methods
Isolation of fungus and screening of L-Asparaginase
Infected leaves of the plants *Annona muricata* was used as the source of the fungus which was collected in paper bag from southern Western Ghats regions of Kollam District of Kerala and ground to a very fine paste with the help of mortar and pestle. The obtained fungi were subjected for qualitative analysis to produce L-Asparaginase enzyme by modified Czapek Dox agar plate method [10] supplemented with pH 6.2 and indicator phenol red.

Identification of the fungal organism
According to Gulati *et al*., (1997) [11], after the selection of positive isolates, allowed to regrown to attain pure culture. Lacto phenol cotton blue staining technique [12] was used for the identification based on the morphological and microscopical characteristics. The precise order of the nucleotides and the gene sequence was checked by using DNA sequencing [13] and similarity of the genes were determined with BLAST [14].

Preparation of inoculum
The inoculum was prepared by growing the fungal isolate on the modified Czapek Dox medium for 72 hours at 37 ^{O}C.

By using a sterile inoculation loop, the discs were taken from the culture plate and then it was suspended in 100 ml of the modified Czapek Dox medium.

Preparation of crude extract
After the incubation centrifugation took place to recover the culture filtrates. The supernatant was considered as the crude enzyme extracts.

Assessment of L-Asparaginase activity
The Factors affecting the L-Asparaginase activity was checked by following the method of Imada *et al*., (1973) [15].

Estimation of protein
Estimation of protein was done according to Lowry *et al.*, (1951) [16]

Characterization of Enzyme
Various factors such as pH, temperature, incubation period, substrate concentration and metal ion concentration were characterized.

Effect of pH
The effect of pH was evaluated by using different range (6 to10) with incubation at 37 ^{O}C for 30 minutes in water bath and after that L- Asparaginase was assayed.

Effect of Temperature
Activity analysis of the L-Asparaginase activity was carried out by different temperatures ranges from 25 ^{o}C to 45 ^{O}C.

Effect of Substrate concentration
In order to optimizing the substrate concentration different asparagine concentrations were used (1mM to 60mM)

Effect of Metal ion concentration
Enzyme was assayed with the presence of various metal ions such as Mg^{2+},Fe^{2+},Na^{+} and K^{+} and the activity of the enzyme was optimized.

Effect of Incubation period
The optimum incubation period for the enzyme activity was determined by using different reaction time ranges from 10 to 60 minutes at 37 ^{O}C.

Anti-proliferative Assay
An epithelial human breast cancer cell line MDA-MB-231 was used for the assay and Trypan blue exclusion method [17] was followed to determine the cell viability.

Result and Discussion
Isolation and screening of fungal isolate
It is first time report of isolation of fungal strain from infected leaves of *Annona muricata* for the determination of anti-proliferative activity. Fungal isolates were processed from infected leaves of *Annona muricata* growing in southern Western Ghats regions of Kollam District of Kerala, cultured on modified Czapek Dox agar media. As per the pink color developed around the colonies, it is considered that the fungal organism has the capability to produce L-Asparaginase enzyme [18] (Figure1).

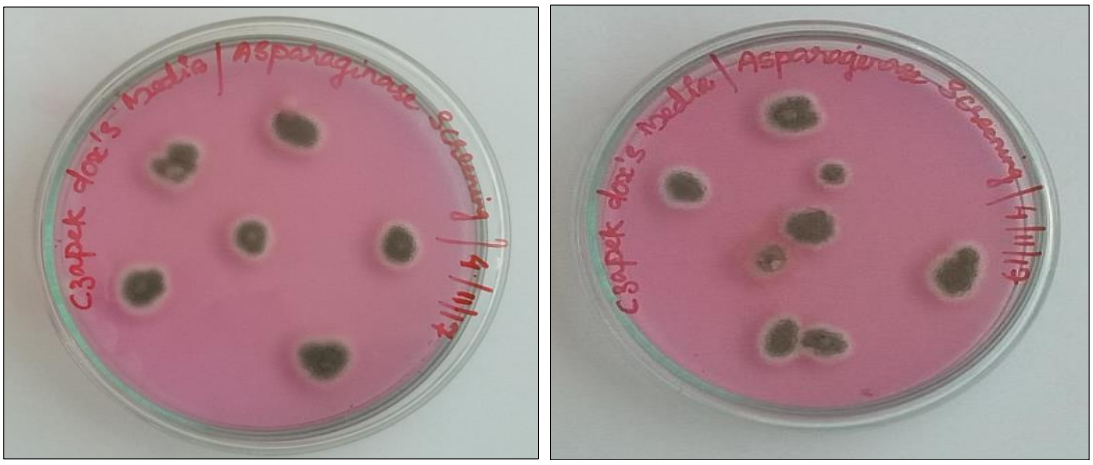

Fig 1: Development of pink color around the colonies on Modified Czapek Dox agar pH 6.2 at room temperature.

Identification of fungus
Lacto phenol cotton blue staining was employed for the evaluation of morphological characteristics of the fungal isolate. The fungal strain was identified as Aspergillus species on the basis of external attributes. The process of identification extended to molecular level with DNA Sequencing and BLAST, it was identified as *Aspergillus fumigatus.*

Characterization of enzyme
Effect of pH
The change in the pH influences the rate of enzyme activity. To determine the optimum pH of the enzyme activity, the medium adjusted to different range of pH from 6 to 10 and it revealed that highest enzyme production took place at pH 8 which was 23.83 U/ml. (Figure 2)

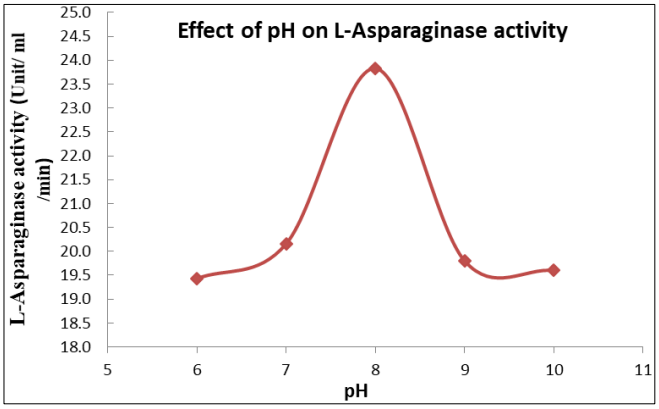

Fig 2: Effect of pH on L-Asparaginase activity

Effect of Temperature

The impact of temperature on enzyme production was checked and maximum was observed at 40 ^{0}C which was 21.26 U/ml. (Figure 3)

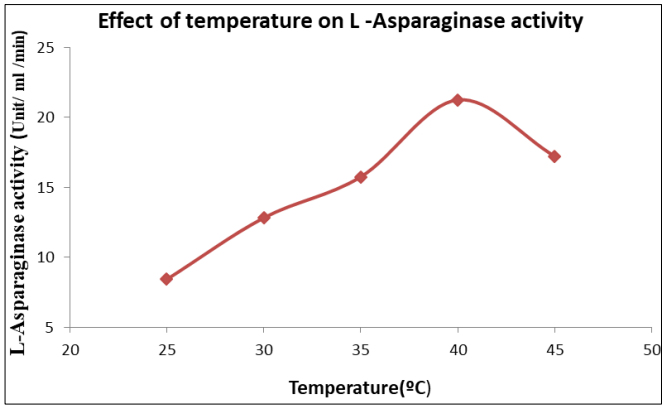

Fig 3: Effect of temperature on L -Asparaginase activity

Effect of Substrate concentration

It was showed that at 20 mM substrate concentration, the enzyme possess maximum production which was 17.6 U/ml. (Figure 4)

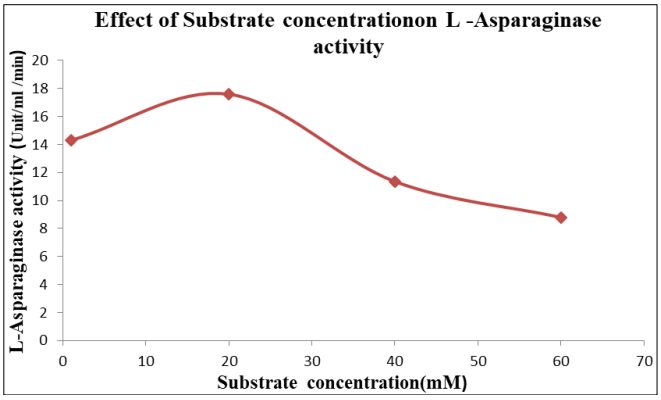

Fig 4: Effect of Substrate concentration on L -Asparaginase activity

Effect of Metal ion concentration

To investigate the effect of various metal ions in enzyme production, Fe^{2+},Mg^{2+},K^+ and Na^+ were employed and reported that maximum activity was took place in the presence of Fe^{2+} which was 32.26 U/ml and the lowest activity was noticed in presence of K^+ which was 2.93 U/ml. (Figure 5)

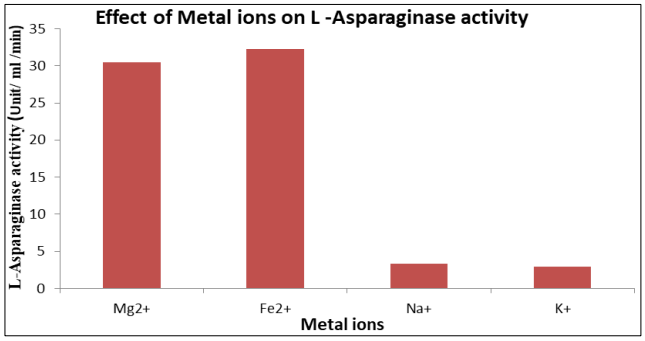

Fig 5: Effect of Metal ions concentration on L –Asparaginase activity

Effect of Incubation period

Considerable variation was observed on enzyme production when analyzed under different reaction periods and detects maximum production at 20 minutes which was 19.9 U/ml. (Figure 6)

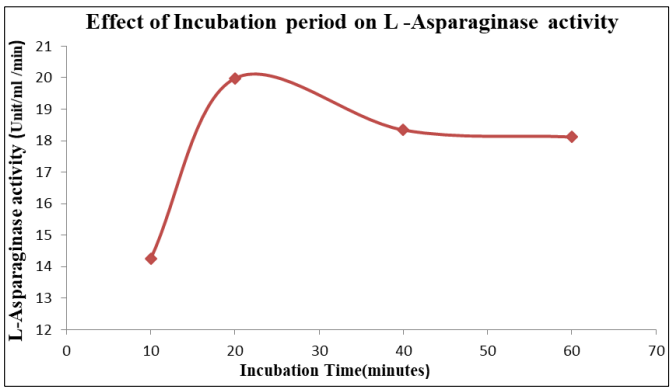

Fig 6: Effect of Incubation period on L -Asparaginase activity

Anti-proliferative assay

Anti-proliferative assay by L-Asparaginase enzyme from the fungus Aspergillus fumigatus was examined on human breast cancer cell line MDA-MB-231 and observed that 71 %, 87.7 % and 96.5 % of cell death occurred when 5U, 10U and 20U of drug used respectively.

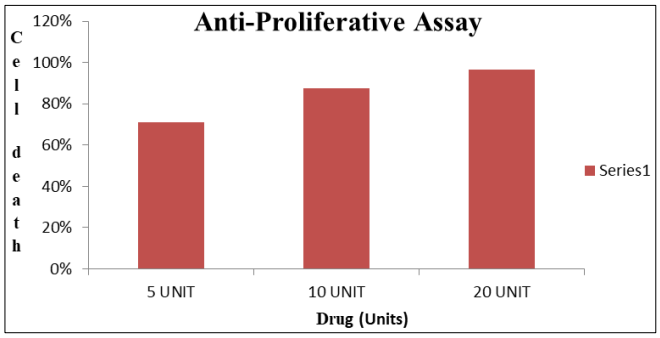

Fig 7: Effect of L -Asparaginase on MDA-MB-231

Conclusion

The fungal species *Aspergillus fumigatus* have shown potential to produce L-Asparaginase on modified Czapek Dox medium. The present study showed that optimum conditions for the maximum production of L-Asparaginase were pH 8, Temperature 40 ºC, Incubation period 20 minutes, Substrate concentration 20Mm and presence of Fe^{2+} concentration showed maximum activity, moreover L-Asparaginase

produced *Aspergillus fumigatus* performed as an anti-proliferative agent on MDA-MB-231. Considering all these characteristics, the production of L-Asparaginase from *Aspergillus fumigatus* suggested for the treatment of Human Breast cancer.

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