Acta Biomed 2010; 81: 104-108

Original article

Biotechnological advancement in isolation of anti-neoplastic compounds from natural origin: a novel source of L-asparaginase

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Abstract. L-asparaginase catalyzes the hydrolysis of L-asparagine into aspartate and ammonia, which is used as an anti-neoplastic agent. Isolation of asparaginase from microorganisms may be cardinal for producing this anticancer agent at industrial level. A total of three hundred fungal isolates were screened for L-asparaginase production. These fungal isolates were growing on various fruits and vegetables. Among these, the *Fusarium sp.* isolate that was growing on green chilly showed highest enzyme production. This study may give an outstanding contribution for finding organisms with high yielding L-asparaginase. Fungal L-asparaginase is superior in terms of its eukaryotic origin that may be responsible for its lesser toxicity. (www.actabiomedica.it)

Key words: L-asparginase, anti-neoplastic agent, *Fusarium sp.*

Introduction

L-asparaginase (EC 3.5.1.1) is an anti neoplastic agent, generally used for treatment of a type of cancer that is Acute Lymphoblastic Leukemia (ALL) and non Hodgkin's Lymphoma (NHL), which are prevalent in children aged up to 10 years and some adults. Anti neoplastic activity of this enzyme was initially suggested by Broom (1) and proved by Mashburn and Wristun (2). Cells in ALL patients are unable of asparagines synthesis due to deficiency or absence of asparagines synthetase enzyme (3). Due to lack of asparagine, these cells depend on uptake of asparagine from surrounding cells and tissues. In this condition, when we inject the L-asparaginase intravenously then it decreases the blood concentration of L-asparagines by breakdown of L-asparagine into L-aspartate and ammonia (4,5). Inability of ALL cells to uptake L-asparagine resulted in inhibition of protein synthesis and their ultimate death. L-asparaginase have been isolated, purified and experimentally used as an anti cancer agent in human patients (6, 7)

This enzyme is routinely screened in laboratory using Nessler's reagent (8). Although this enzyme is produced by various microorganisms including prokaryotes and eukaryotes, bacterial L-asparaginase can cause hypersensitivity in the long term use due to allergic reactions and anaphylaxis (9). Several scientific groups have studied L-asparaginase production and purification in attempt to minimize impurities that produce allergic reactions (10-12)

It has been observed that eukaryotic microorganisms like yeast and filamentous fungi have a potential for L-asparaginase production (13-15). Some researchers also purified L-asparaginase from plants (16).

In the view of toxic effect of bacterial L-asparaginase, the present study was designed for screening of L-asparaginase producing fungal strains isolated from various sources. This source may provide better L-asparaginase producing fungal strains with lesser toxicity.

Materials and Methods

Organisms

Fungal strains were isolated from various plant sources like spoiled fruits and vegetables and various plant parts and maintained on potato dextrose agar. Fungal strains were identified by cultural and morphological characteristics (17) in Department of Microbiology, Cancer Hospital & Research Institute, Gwalior (India).

Media components

All chemicals used in the study were of analytical and equivalent grade (Sigma). The preparation of media for screening was based on Gulati et al (18). Modified Czapek Dox medium (pH 6.2) was used for fungal screening. The media components included Glucose, L asparagine, KH_2PO_4, $MgSO_4.7H_2O$, $CuNO_3.3H_2O$, $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$ and Agar. The media was also supplemented with 0.003% of phenol red dye as pH indicator and pH was adjusted with 1M NaOH.

Cultivation and Enzyme assay

Solidified Czapek Dox media was prepared in petri plates and then ninety six hour old fungal culture was inoculated and kept for incubation at 37°C for 48 hours. After incubation, the fungal strains showing positive results were studied for measurement of zone index. The positive result was observed by formation of pink colored zone around the inoculated fungal mass. The ratio of diameter of the fungal colony to total zone diameter produced by L-asparaginase was calculated as zone index.

Zone index = Diameter of zone produced by L-asparaginase (mm) / Diameter of fungal colony (mm)

For quantitative estimation of the enzyme activity, the liquid modified Czapek Dox medium was inoculated with the fungal strains and incubated in a shaker incubator at 37°C for 96 hours with 250rpm. After incubation, these inoculated media was filtered and the filtrate was used as enzyme extract. The activity of enzyme was measured with Nessler's reagent at 450 nm absorbance. Total protein concentration was measured by the method of Lowry (19). The specific activity of enzyme was also calculated.

Results

A total of 29 fungal strains out of the 300 isolated in the present study recorded the L-asparaginase activity (Table 1). The zone index varied from 1.42 to 3.85 with majority (24) of the strains recording the zone index below 3.0. The strains isolated from the soil samples have generally recorded the minimum enzyme activity, whereas those from the fruits recorded relatively higher activity (Table 1). The five fungal strains, namely F1, F2, F3, F4 and F5, showing the maximum enzyme activity in excess of 3.0 on the basis of zone index were isolated from fruits of different plants and were selected for further experimentation. These strains were identified as *Absidia* sp. (F2) from the fruit of *Cicer arietinum*, *Aspergillus flavus* (F1) isolated from the fruit of *Lycopersicon esculentum*, *Fusarium* sp. (F4) from the fruit of *Capsicum anum*, *Mucor* sp. from the fruit of *Cucurbita maxima*, and *Penicillium* sp. (F3) from the legume of *Tamarindus indica* (Table 2).

The enzyme activity (IU/ml) was the highest and the protein content (mg) was the lowest from *Fusarium* sp. (F4) isolated from the fruit of *Capsicum anum*, thereby resulting in the maximum specific activity of L-asparginase of 273 IU/mg among the five isolates (Table 3). Aspergillus flavus (F1) and Absidia sp. (F2) have recorded relatively lesser specific activity of the enzyme than that by Fusarium sp., but the differences were not statistically significant among the three isolates ($Fp > 0.05$). The remaining two isolates, namely, Mucor sp. and Penicillium sp. have shown much lesser specific activity of the enzyme (Table 3) relative to that in the other isolates ($Fp < 0.05$).

Table 1. Qualitative L-asparaginase activity by isolated organisms

S. No.	Isolate	Source of Isolation	Enzyme diameter (mm)	Colony diameter (mm)	Zone Index
1	*F1*	*Lycopersicon esculentum (Fruit)*	77	20	3.85
2	*F2*	*Cicer arietinum (Fruit)*	65	21	3.09
3.	*F3*	*Tamarindus indica (Legume)*	35	10	3.5
4	*F4*	*Capsicum anum (Fruit)*	65	20	3.25
5	*F5*	*Cucurbita maxima (Fruit)*	73	21	3.47
6.	*F6*	*Solanum melongena (fruit)*	60	27	2.22
7.	*F7*	*Raphanus sativus (*root)	32	18	1.77
8	*F8*	*Lycopersicon Esculentum* (fruit)	80	32	2.5
9	*F9*	*Solanum tuberosum* (bulb)	70	27	2.59
10	*F10*	*Mangifera indica* (fruit)	50	26	1.92
11	*F11*	*Musa paradisiaca*(fruit)	55	27	2.03
12	*F12*	*Psidium guajava* (fruit)	20	13	1.53
13	*F13*	*Carica papaya (*fruit)	20	13	1.53
14	*F14*	*Brassica oleracea* (fruit)	55	21	2.61
15.	*F15*	*Cajanus cajan* (fruit)	30	15	2.0
16	*F16*	*Pisum sativum* (fruit)	65	27	2.40
17	*F17*	*Malus domestica* (fruit)	40	15	2.66
18.	*F18*	*Abelmoschus esculentus* (lome)	35	20	1.75
19	*F19*	*Citrullus lanatus* (fruit)	43	15	2.86
20	*F20*	*Citrus sinensis* (fruit)	25	11	2.27
21	*F21*	Spoiled Sweet	18	10	1.8
22	*F22*	*Arachis hypogea (*fruit)	19	08	2.375
23	*F23*	Soil 1	15	09	1.66
24	*F24*	*colocasia esculenta (root)*	25	12	2.08
25	*F25*	Soil 2	35	23	1.52
26	*F26*	Soil 3	30	21	1.42
27	*F27*	*Manilkara zapota (fruit)*	55	27	2.03
28	*F28*	*Ananas comosus* (fruit)	30	13	2.30
29	*F29*	Soil 4	35	20	1.75

Table 2. Identification of fungi with highest activity of L- asparaginase.

S No	Fungal strains	Name of species	Source of Isolation
1	F1	*Aspergillus flavus*	*Lycopersicon esculentum (Fruit)*
2	F2	*Absidia sp.*	*Cicer arietinum (Fruit)*
3.	F3	*Penicillium sp.*	*Tamarindus indica (Legume)*
4	F4	*Fusarium sp.*	*Capsicum anum (Fruit)*
5	F5	*Mucor sp.*	*Cucurbita maxima (Fruit)*

Table 3. Quantitative enzyme activity of selected fungi (the values are the mean of three replicates)

S No	Fungal strains	Enzyme Activity (IU/ml)	Protein Content (mg)	Specific activity (IU/mg)
1	*Aspergillus flavus*	*29.65*	*0.113*	*261*
2	*Absidia sp.*	*28.60*	*0.109*	*261.7*
3.	*Penicillium sp.*	26.56	0.108	245
4	*Fusarium sp.*	*29.76*	*0.100*	*273.33*
5	*Mucor sp.*	*23.94*	*0.106*	*225.84*

Discussion

The use of L-asparaginase in translational medicine may be an important establishment in the treatment of ALL. Fungi with good L-asparaginase activity like *Absidia* sp. *Penicillium* sp., *A. flavus*, *Mucor* sp. and *Fusarium* sp. *Fusarium* sp. isolated from Green Chilly plant was showing highest L-asparaginase activity as indicated by primary screening. Green chilly (*Capsicum anum*) itself shows L-asparaginse enzyme with Km value 3.3 mM. The enzyme from green chillies has thus a lesser affinity for the substrate L-asparagine and such enzyme has also been isolated from other organisms like *Pseudomonas* (16, 20). In the view of evolutionary perspective, it may be concluded from these observations that such type of enzyme can only exist in organisms with high content of L-asparagine. Perhaps, this may be the reason for the isolation of highly efficient producer of L-asparaginase. Isolation of high level of L-asparaginase producing fungi from *Capsicum anum* itself paved the way for isolation of many other organisms from sources rich with L-asparagine. Moreover the work carried out over fungal L-asparaginase is meager, so this study may be significant in developing isolation and cultivation system for such organisms. As far as L-asparaginase activity of other fungal isolate is concerned, it can be correlated with the widespread occurrence of isolated fungal genera.

Moreover, *Fusarium sp*. was also found to have highest L-asparaginase activity as indicated by primary screening. So the further analysis of these selected fungal strains was performed for their enzyme activity and specific activity. While analyzing the above mentioned parameters, the results were found to be consistent with primary screening results. Highest activity of L-asparaginase was found with *Fusarium sp* followed by *Aspergillus flavus, Absidia sp, Penicillium sp*. and *Mucor sp.,* respectively. The results of specific activity were also consistent and the highest specific activity was found in *Fusarium sp*. followed by *Absidia sp, Aspergillus flavus, Penicillium sp*. and *Mucor sp.*

The results suggest that *Fusarium sp*. may be a good source for isolation and purification of L-asparaginase that act as anti-neoplastic agent. The strain improvement method can further increase its production manifold and detailed research is warranted in relation to this. These results can be correlated with the results of many other studies carried throughout the globe (21). However, isolation of high L-asparaginase activity containing *Fusarium sp*. from Green chilly is a new approach. This approach may pave the way for isolation of many other high L-asparaginase activity containing fungi from such sources.

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Accepted: April 21th 2010
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