L-asparaginase from marine derived fungal endophytes of seaweeds

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Symptomless endophytic fungi isolated from seven green algae, six brown algae and six red algae occurring along the southern coast of Tamilnadu, southern India were screened for the production of L-asparaginase enzyme. Of the 82 endophyte isolates, 64 were positive for the enzyme. A *Fusarium* sp. isolated from the thallus of *Sargassum wightii* and a sterile mycelial form isolated from the thallus of *Chaetomorpha* sp. showed maximum activity of the enzyme. The mycelial growth of *Fusarium* sp. was positively correlated with enzyme production. In a time course study, maximum enzyme activity was observed on the 5th day of growth of this fungus. The optimum pH for enzyme activity was pH 6.2. High concentration of glucose in the medium as C source inhibited enzyme production by the fungus. Endophytes of tropical seaweeds appear to be a good source of this therapeutic enzyme.

Key words – endophytes – tropical seaweeds – anti neoplastic – acute lymphoblastic leukaemia

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Introduction

The enzyme L-asparaginase (E.C.3.5.1.1) from bacterial source has been used as a therapeutic agent in the treatment of acute lymphoblastic leukaemia (Gallagher et al. 1999, Verma et al. 2007). This enzyme depletes tumor cells of L-asparagine and the cells die because of their inability to synthesize this amino acid. L-asparaginase is also used commercially to reduce the formation of acrylamide in fried foods (Pedreschi et al. 2008). Several bacterial species including *Erwinia* sp. (Borkotaky & Bezbaruah 2002), *Streptomyces albidoflavus* (Narayana et al. 2008) and actinomycetes from rhizosphere of medicinal plants (Khamna et al. 2009) have been studied for production of this enzyme. There are only a few studies on L-asparaginase production by fungi (Lapmak et al. 2010). These have established that filamentous fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium* and some yeast species produce this enzyme (Sarquis et al. 2004). In our study on pharmaceutical enzymes from endophytes, we found that several species of this ecological group of fungi isolated from tropical seaweeds are an excellent source of L-asparaginase.

Endophytic fungi live inside the living tissues of plants, either for a short or prolonged period, without initiating any visible symptoms on their host plants. Different ecological groups of fungi such as latent pathogens, entomopathogens and true endophytes can survive endophytically in aerial tissues of plants. The endophytic fungi produce an extraordinary diversity of extrolites some of which have

therapeutic value as novel antibiotics or anti-cancer chemicals (Gunatilaka 2006, Suryanarayanan et al. 2009, Weber 2009). They have not been studied to any extent for their enzyme potential (Govinda Rajulu et al. 2011). In the present study, we screened endophytic fungi isolated from seaweeds occurring along the coast of Tamilnadu for production of L-asparaginase.

Methods

Isolation of endophytes

The algae (thalli) were washed thoroughly in running tap water and cut into segments of approximately 0.5 cm^2. For each algal species, 100 segments were screened for the presence of endophytes. The segments were dipped in 70% ethanol for 5 sec followed by immersion in sterile distilled water 10 sec as this was found to be sufficient to surface sterilize the soft algal thalli (Suryanarayanan et al. 2010).

Incubation

The sterilized segments from each alga were plated on potato dextrose agar (PDA) medium amended with the antibiotic, Chloramphenicol (150 mgl^{-1}). Ten segments were placed on each of 20 mL PDA medium contained in a 9 cm dia. Petri dish. The Petri dishes were sealed using Parafilm^{TM} and incubated in a light chamber for 4 weeks at 26°C (Suryanarayanan 1992). The light regimen was 12 h of light followed by 12 h of darkness. The Petri dishes were observed periodically and the fungi that grew out from the tissues were identified. To prevent the rapidly growing fungi from inhibiting slow growing isolates, the former were removed following isolation and identification before they made contact with other isolates (Bills 1996). Sterile isolates were given codes using culture characteristic such as colony surface, texture and hyphal pigmentation and treated as morphospecies (Dobranic et al. 1995, Suryanarayanan et al. 1998).

Plate assay for L-asparaginase

A modified Czapek Dox medium (glucose 2 g, L-asparagine 10 g (Sigma-Aldrich), KH_2PO_4 1.52 g, KCl 0.52 g, $MgSO_4.7H_2O$ 0.52 g, $FeSO_4.7H_2O$ 0.01 g, agar 20 g, distilled water 1000 mL) was used for plate assay. A 2.5% stock solution of phenol red was prepared in ethanol (pH 6.2) and 3 mL of this was added to 1000 mL of Czapek Dox medium. A mycelial plug (5 mm dia.) cut from the growing margin of the colony of an endophyte was placed in a Petri dish containing 20 mL of this medium. After 72 h of incubation at 26±1°C, the appearance of a pink zone around the fungal colony in an otherwise yellow medium indicated L-asparaginase activity (Gulati et al. 1997).

Spectrophotometric assay of L-asparaginase enzyme

L-asparaginase activity was measured by the modified method of Imada et al. (1973). The fungus was grown for 5 days at 26±1°C in liquid modified Czapek Dox (CD) medium. A reaction mixture containing 0.5 mL of 0.5 M tris HCl buffer (pH 8.2), 0.1 mL of 40 mM L-asparagine, 1.0 mL of suitably diluted enzyme source (culture filtrate of an endophyte) and 0.4 mL of distilled water (total volume of 2.0 mL) was incubated at 37°C for 30 min. The reaction was terminated by adding 0.5 mL of 1.5 M tricholoroacetic acid (TCA). Blank tubes were prepared by adding the enzyme source after the addition of TCA. After termination of the reaction, 3.7 mL volume of distilled water and 0.2 mL of Nessler's reagent were added to 0.1 mL of the above reaction mixture and incubated for 20 min. The amount of ammonia released during the reaction was determined by measuring the absorbance at 450 nm. One international unit (IU) of L-asparaginase is the amount of enzyme needed to liberate 1 µmol of ammonia in 1 min at 27°C (Imada et al. 1973).

$$\text{Units/mL enzyme} = \frac{(\mu\text{mole of } NH_3 \text{ liberated})\ (2.5)}{(0.1)\ (30)\ (1)}$$

2.5 = Initial volume of enzyme mixture (mL)
0.1 = Volume of enzyme mixture used in final reaction (mL)
30 = Incubation time (minutes)
1 = Volume of enzyme used (mL)

Effect of incubation time and pH of the medium on enzyme activity

The fungus was inoculated in 20 mL CD broth with 1% L-asparagine and 0.2% glucose.

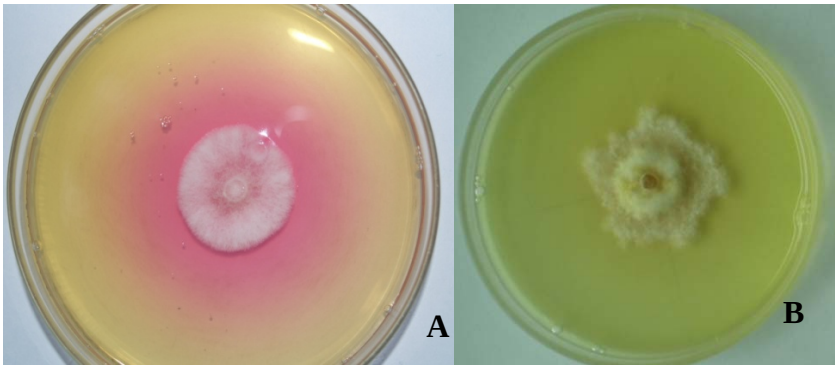

Fig. 1 – L-asparaginase activity detected by plate assay **A)** Color change in the medium (yellow to pink) around colony indicates production of enzyme. **B)** Non producer isolate.

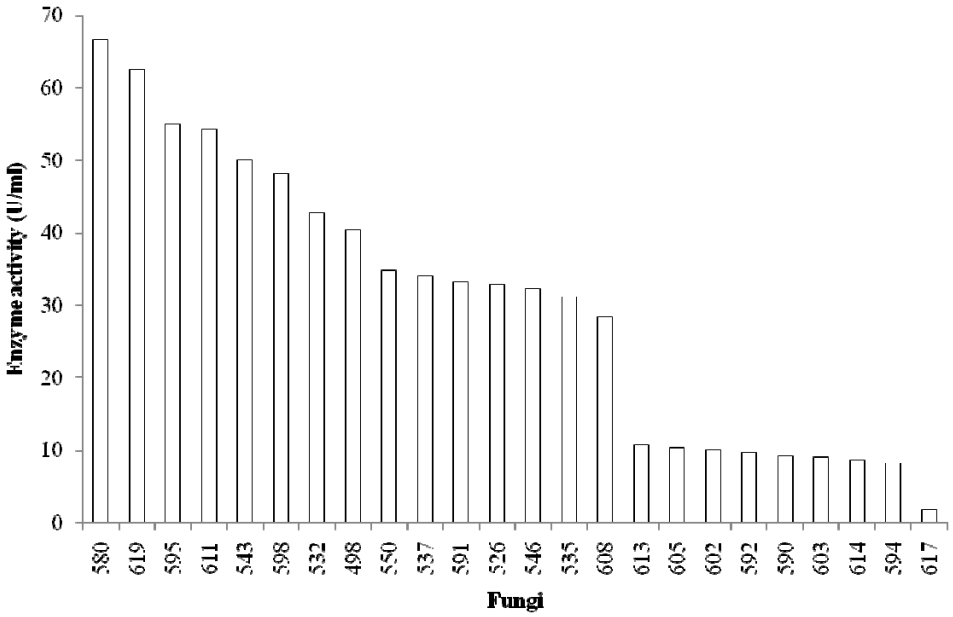

Fig. 2 – Spectrophotometric assay of L-asparaginase of some marine derived seaweeds endophytic fungi.

The culture was incubated 26°C for 5 days in an orbital shaker (120 rpm); the broth was sampled every 24 h for enzyme activity. To study the effect of pH on enzyme activity, the fungus was grown as mentioned above in CD medium, of different pH (4.2, 5.2, 6.2, 7.2 and 8.2) and assayed for enzyme activity.

Effect of carbon and nitrogen concentration on enzyme activity

The fungus was inoculated in 20 mL CD broth with different concentrations of glucose (0.2, 0.5, 1.0, 2.0 or 4.0 mg/mL) or L-asparagine (1.2, 2.5, 5.0, 10.0 or 20.0 mg/mL) and incubated as mentioned above. The initial pH of the medium was 6.2. The culture filtrate was assayed for enzyme activity on the fifth day of incubation.

Results and Discussion

Screening of microorganisms

Of the 82 endophyte isolates screened, 64 were positive for extracellular L-asparaginase. The remaining isolates, as discerned by plate assay, were non-producers (Fig. 1). More number of isolates belonging to genera such as *Aspergillus*, *Cladosporium*, *Fusarium*, and *Penicillium* produced the enzyme (Table 1). Twenty four endophytes isolates were tested quantitatively for L-asparaginase activity. *Fusarium* sp. 3 showed maximum enzyme production (Fig. 2).

Table 1 L-asparaginase activity of some marine derived endophytic fungi isolated from seaweeds (Plate Assay).

Fungus	Isolate code	Algal host	Organ	Location	Enzyme activity
Alternaria sp.	572	*Sargassum wightii*	Thallus	Sundramadam	+
Alternaria sp.	592	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Alternaria sp.	603	*Sargassum* sp.	Bladder	Kodiyakkarai	+
Alternaria sp.	626	*Stoechospermum marginatum*	Thallus	Mandapam	+
Aspergillus niger	523	*Caulerpa racemosa*	Thallus	Mandabam	-
Aspergillus niger	531	*Caulerpa scalpelliformis*	Thallus	Mandapam	-
Aspergillus niger	544	*Ulva lactuca*	Thallus	Mandapam	-
Aspergillus sp.	591	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Aspergillus sp.	597	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Aspergillus sp. 1	525	*Caulerpa sertularioides*	Thallus	Mandabam	-
Aspergillus sp. 2	545	*Ulva lactuca*	Thallus	Mandapam	+
Aspergillus sp. 2	581	*Sargassum wightii*	Thallus	Sundramadam	+
Aspergillus sp. 2	600	*Sargassum* sp.	Axis	Kodiyakkarai	+
Aspergillus sp. 2	533	*Caulerpa scalpelliformis*	Thallus	Mandapam	+
Aspergillus sp. 2	551	*Halimeda macroloba*	Thallus	Mandapam	+
Aspergillus sp. 3	526	*Caulerpa sertularioides*	Thallus	Mandabam	+
Aspergillus sp. 4	524	*Caulerpa racemosa*	Thallus	Mandabam	+
Aspergillus sp. 4	569	*Sargassum wightii*	Thallus	Sundramadam	+
Aspergillus sp. 10	570	*Sargassum wightii*	Thallus	Sundramadam	+
Aspergillus terreus	532	*Caulerpa scalpelliformis*	Thallus	Mandapam	+
Aspergillus terreus	537	*Sargassum ilicifolium*	Thallus	Mandapam	+
Aspergillus terreus	539	*Gracilaria edulis*	Thallus	Mandapam	+
Aspergillus terreus	543	*Ulva lactuca*	Thallus	Mandapam	+
Aspergillus terreus	550	*Halimeda macroloba*	Thallus	Mandapam	+
Aspergillus terreus	598	*Sargassum* sp.	Thallus	Kodiyakkarai	+
Aspergillus terreus	611	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Aspergillus terreus	615	*Ulva fasciata*	Thallus	Kovalam	+
Aspergillus terreus	632	*Halymenia* sp. 2	Thallus	Mandapam	+
Chaetomium sp.	628	*Grateloupia lithophila*	Thallus	Pondicherry	-
Chaetomium sp.	616	*Ulva fasciata*	Thallus	Kovalam	-
Chaetomium sp. 1	548	*Ulva lactuca*	Thallus	Mandapam	-
Chaetomium sp. 1	558	*Padaina tetrastromatica*	Thallus	Kizhakkari	+
Chaetomium sp. 1	559	*Halymenia* sp. 1	Thallus	Kizhakkari	-
Chaetomium sp. 1	576	*Stoechospermum marginatum*	Thallus	Mandapam	-
Cladosporium sp. 1	564	*Portieria hornemonii*	Thallus	Kizhakkari	+
Cladosporium sp. 1	575	*Gratelouipa lithophila*	Thallus	Mandapam	+
Cladosporium sp. 1	579	*Sargassum wightii*	Thallus	Sundramadam	+
Cladosporium sp. 1	601	*Sargassum* sp.	Bladder	Kodiyakkarai	+
Cladosporium sp. 1	547	*Ulva lactuca*	Thallus	Mandapam	+
Cladosporium sp. 1	520	*Caulerpa racemosa*	Thallus	Mandabam	+
Colletotrichum sp.	599	*Sargassum* sp.	Thallus	Kodiyakkarai	+
Colletotrichum sp.	613	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Curvularia lunata	602	*Sargassum* sp.	Bladder	Kodiyakkarai	+
Curvularia lunata	608	*Sargassum* sp.	Bladder	Kodiyakkarai	+
Curvularia sp. 1	593	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Curvularia sp. 1	535	*Caulerpa scalpelliformis*	Thallus	Mandapam	+
Curvularia sp. 1	566	*Sargassum wightii*	Thallus	Sundramadam	+
Curvularia sp. 3	577	*Sargassum wightii*	Thallus	Sundramadam	-
Curvularia tuberculata	606	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Drechslera sp.	610	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Emericella nidulans	541	*Portieria hornemonii*	Thallus	Kizhakkari	+
Emericella nidulans	560	*Halymenia* sp.1	Thallus	Kizhakkari	+
Emericella nidulans	567	*Sargassum wightii*	Thallus	Sundramadam	+
Fusarium-like	542	*Gracillaria edulis*	Thallus	Mandapam	-
Fusarium sp.	595	*Turbinaria* sp.	Thallus	Kodiyakkarai	+

Table 1 (Continued) L-asparaginase activity of some marine derived endophytic fungi isolated from seaweeds (Plate Assay).

Fungus	Isolate code	Algal host	Organ	Location	Enzyme activity
Fusarium sp.	612	*Sargassum* sp	Bladder	Kodiyakkarai	+
Fusarium sp. 3	580	*Sargassum wightii*	Thallus	Sundramadam	+
Nigrospora sp.	594	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Nigrospora sp.	627	*Ulva fasciata*	Thallus	Pondicherry	+
Nigrospora sp.	538	*Jamia adherens*	Thallus	Mandapam	-
Nigrospora sp.	546	*Ulva lactuca*	Thallus	Mandapam	+
Nigrospora sp.	573	*Gratelouipa lithophila*	Thallus	Mandapam	+
Paecilomyces sp. 1	609	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Paecilomyces sp. 1	534	*Caulerpa scalpelliformis*	Thallus	Mandapam	+
Penicillium sp. 1	563	*Portieria hornemonii*	Thallus	Kizhakkari	-
Penicillium sp. 1	536	*Caulerpa scalpelliformis*	Thallus	Mandapam	+
Penicillium sp. 1	521	*Caulerpa racemosa*	Thallus	Mandabam	+
Penicillium sp. 1	527	*Caulerpa sertularioides*	Thallus	Mandabam	+
Penicillium sp. 1	552	*Halimeda macroloba*	Thallus	Mandapam	+
Pestalotiopsis sp.	574	*Sargassum wightii*	Thallus	Sundramadam	+
Phaeotrichoconis sp.	590	*Turbinaria* sp.	Thallus	Kodiyakkarai	+
Phoma sp.	605	*Sargassum* sp.	Bladder	Kodiyakkarai	+
Phomopsis sp.	562	*Portieria hornemonii*	Thallus	Kizhakkari	-
Pithomyces sp.	604	*Sargassum* sp.	Axis	Kodiyakkarai	+
Sterile form 1	618	*Ulva fasciata*	Thallus	Kovalam	-
Sterile form 2	530	*Caulerpa racemosa*	Thallus	Mandapam	+
Sterile form 2	565	*Portieria hornemonii*	Thallus	Kizhakkari	-
Sterile form 2	571	*Sargassum wightii*	Thallus	Sundramadam	-
Sterile form 2	614	*Ulva fasciata*	Thallus	Kovalam	+
Sterile form 3	617	*Ulva fasciata*	Thallus	Kovalam	+
Sterile form 3	619	*Chaetomorpha* sp.	Thallus	Kovalam	+
Trichoderma sp.	568	*Sargassum wightii*	Thallus	Sundramadam	-

Note: + : positive enzyme activity, - : no enzyme activity

Effect of culture age and pH

Fusarium sp. 3 (high producer – Fig. 2) was chosen to study the effects of incubation time and pH of the culture medium on enzyme activity. The fungus was grown in Czapek Dox medium amended with L-asparagine (pH 6.2) and assayed for the enzyme at intervals. Enzyme activity was observed on the first day of incubation and it increased as time progressed to reach a maximum value on the fifth day (Fig. 3). A similar observation was made by Khamna et al. (2009) for an actinomycete isolated from soil. Maximum enzyme production was related to the dry weight of the mycelium of the fungus (Fig. 3). Earlier studies with prokaryotes have also demonstrated that the growth of the producer organism is positively correlated with enzyme production (Savitri & Azmi 2003, Khamna et al. 2009). The optimum pH for L-asparaginase production from prokaryotes is reported to be from pH 7.0 – 7.5 (Koshy et al. 1997, Narayana et al. 2008, Khamna et al. 2009,). In the present study, we found that a pH of 6.2 was optimum for L-asparaginase production by *Fusarium* sp. 3, although maximum production occurred at a lower pH (Fig. 4).

Effect of carbon source

Glucose as a carbon source in the medium is known to inhibit L-asparaginase synthesis in bacteia (Cedar & Schwartz 1968, Heinemann & Howard 1969). In *Fusarium* sp. 3 enzyme activity increased with decreasing concentration of glucose in the growth medium, suggesting that in this fungus also a high concentration of glucose may not be suitable for L-asparaginase production (Fig. 5).

Effect of nitrogen source

L-asparagine as a nitrogen source in the medium is known to stimulate the production of enzyme activity (Lapmak et al. 2010). In *Fusarium* sp. 3 enzyme activity increased with increasing concentration of L-asparagine in the medium (Fig. 6), suggesting that in this fungus also a high concentration of asparagine is

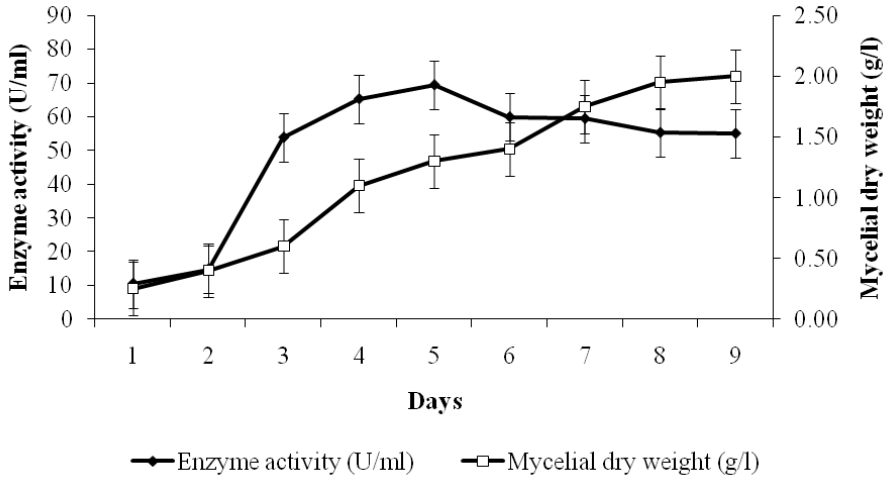

Fig. 3 – L-asparaginase enzyme activity and mycelium dry weight of *Fusarium* (VIG 580) (Bars represent standard error).

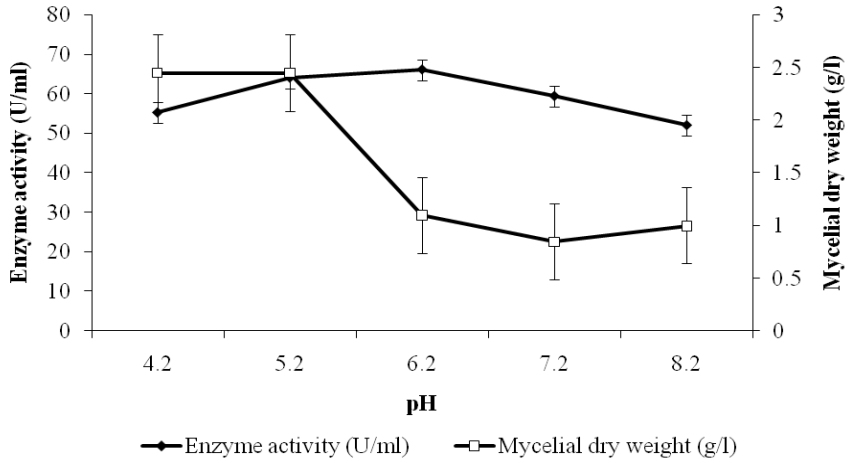

Fig. 4 – Effect of pH of growth medium on L-asparaginase activity of *Fusarium* (VIG 580) (Bars represent standard error).

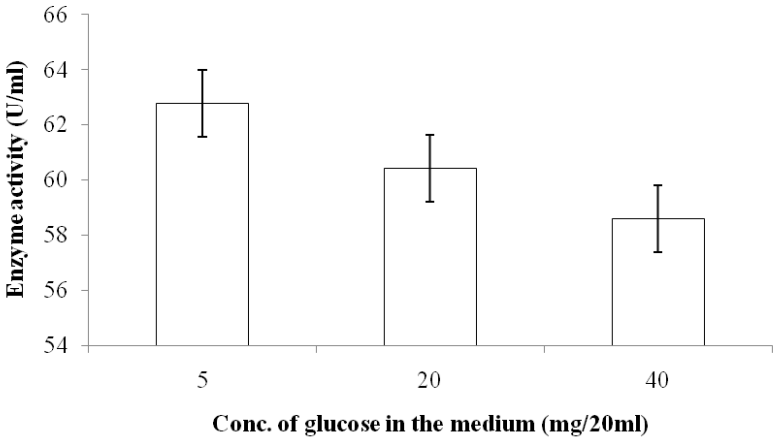

Fig. 5 – Effect of glucose (carbon source) concentration of growth medium on L-asparaginase enzyme activity and amount of glucose in the medium of *Fusarium* (VIG 580) (Bars represent standard error).

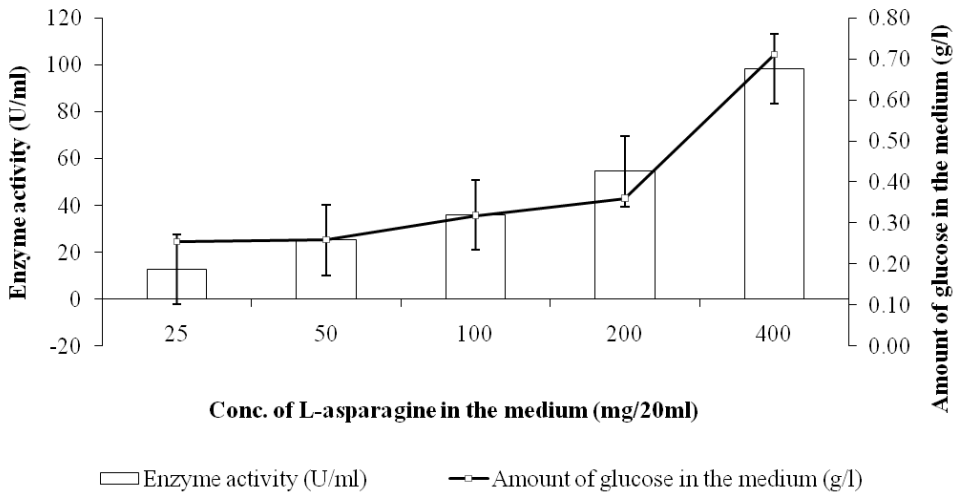

Fig. 6 – Effect of substrate (nitrogen source) concentration of growth medium on L-asparaginase enzyme activity and amount of glucose in the medium of *Fusarium* (VIG 580) (Bars represent standard error).

suitable for L-asparaginase production.

The L-asparaginase which is currently used clinically is from a bacterial source (Schrey et al. 2010). Although L-asparaginase is antineoplastic and has been used for treating acute lymphoblastic leukemia for nearly 30 years (Narta et al. 2007), its use in chemotherapy is fraught with difficulties. The administered enzyme remains only for a short period in the body and, being a large protein, it induces immune response leading to allergic reactions (Goodsell 2005). Bacterial asparaginase induces in some patients toxicity symptoms such as fever, nausea and vomiting, weight loss, and affects the liver and pancreas (Oettgen et al. 1970) and can cause cerebrovascular complications (Keislich et al. 2003). Modifications of the enzyme molecule such as PEG-L-asparaginase may prove to be more effective than the native enzyme (Narta et al. 2007). As a search for a less toxic and more efficient enzyme is warranted, it would be worthwhile studying the enzyme from an eukaryotic source such as fungi that may be less toxic to the human system. Although more than a hundred species of bacteria produce L-asparaginase (Peterson & Ciegler 1969), only a few filamentous fungi such as species of *Aspergillus*, *Penicillium* and *Fusarium* have been studied for this enzyme. It now appears that other genera of fungi, especially the endophytic ones could be a good source of this enzyme. However, the clinical suitability of the enzyme elaborated by endophytes has to be established in future studies. Our study clearly indicates that, apart from the fungal genera such as *Aspergillus*, *Penicillium* and *Fusarium* that have been shown to produce L-asparaginase, several endophytic fungal species are a good source of this therapeutic enzyme.

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