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ARTICLE

A novel and sensitive agar plug assay for screening of asparaginase-producing endophytic fungi from *Aegle marmelos*

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ABSTRACT Twelve strains of asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) producing filamentous fungi were isolated from the bark and leaves of *Aegle marmelos* Linn. In the present study, a new method of isolation and selective screening of asparaginase producing endophytic fungi was developed. Asparaginases producing filamentous fungi were screened by a novel rapid dye based agar plug assay. Agar plug screen is a novel method for efficient screening of large number of fungal isolates. All endophytic fungi could grow on modified Czapek Dox agar medium with phenol red, a dye indicator that changes from yellow (acidic condition) to pink (alkaline condition). This method is not only rapid and cost effective but also less labor intensive and could be efficiently used for isolating high yielding strains.
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KEY WORDS

Asparginase
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endophytic fungi
dye indicator

Asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) belongs to an amidase group that hydrolyzed the amide bond in L-asparagine to produce aspartic acid and ammonia (Ghasemi et al. 2008). Asparagine is a nutritional requirement of normal cells as well as cancer cells. Normal cells have enzyme asparagine synthetase for the synthesis of asparagine from asparatic acid whereas cancer cells have low levels of this enzyme (Theantana et al. 2007). Therefore, they obtain the required asparagine from circulating pool. For this reason, free enzyme is intravenously injected to decrease the L-asparagine concentration in blood and thereby, selectively depriving the cancer cells of aspargine (Sarquis et al. 2004). Hence, L-asparaginase, an antitumor enzyme, is biomedically important group of therapeutic enzymes that accounts for about 40% of the total world-wide enzyme sales and contributes one third of the global requirements of anti leukemic and antilymphoma agents (Warangkar and Khobragade 2009). The FDA and WHO organizations have approved that L-asparaginase can be effectively used in treating Acute Lymphoblastic Leukemia (ALL) and Lymph sarcoma. L-asparaginase production by using microbial systems is beneficial owing to the cost-effectiveness and eco-friendly nature. Commercially, L-asparaginase is mainly obtained from bacterial sources. However, fungal asparginase is more promising than the bacterial asparginase as it is safe and non allergic. Conventionally asparginase producing fungi are isolated from soil. However, medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of medicinal and pharmaceutical importance (Strobel et al. 2004). Endophytes are the "microbes which colonize living internal tissues of plants without causing any immediate, overt negative effects" (Bacon and White 2000). Many of the endophytes are known to produce bioactive compounds that can be used by the host plant for their defense against different phytopathogens. Some of these compounds have been proven for novel drug discovery (Guo et al. 2008). They live in intracellular spaces of the host plant like roots, stems, leaves, and flowers. In most cases they are viewed as having a symbiotic relationship with the host, but, depending on conditions, they may also eventually revert to some form of pathogenesis (Petrini 1991; Strobel 2002). The most frequently isolated microbes from plant tissues are fungi, but bacteria, including the actinomycetes, can exist as endophytes. Although these microbes can be easily obtained from most plant sources, the effectiveness of their recovery is not known and thus many remain non-cultured (Verma et al. 2004).

In the present study, we have made an attempt to isolate asparginase producing fungi from the leaves and bark of an indigenous medicinal plant *Aegle marmelos*. We have also developed a new method for rapid screening of asparginase producing filamentous fungi. The agar plug method is not only rapid and cost effective but also less labor intensive and could be routinely used in isolating high yielding strain for L- asparaginase production.

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Materials and Methods

Plant material

The plant specimen (leaf and bark of *Aegle marmelos*) were obtained from Toranmal forests, dist. Nandurbar, MS, India and authenticated by Dr. D. A. Patil. The samples were stored in freezer bags at 4°C. (Waksman et al. 1916). Endophytic fungi were isolated from the leaves and the bark of indigenous plant *Aegle marmelos* as per previously known procedures (Garry et al, 2007).

Isolation and culture of Endophytic fungi

The fresh leaves were cleaned under running tap water. The samples were surface sterilized successively with 70% ethanol for 1 min. and then rinsed with sterile water. Mercuric chloride ($HgCl_2$, 0.1%, w/v) was used to sterilize the tissues for 3 min, and again rinsed with sterile water five times. The surface-sterilized leaves were cut into about 0.5×1×0.5 cm (length × width × thickness) pieces and placed on new Petri dishes with potato dextrose agar (PDA) medium to which 0.1% (w/v) gentamicin was added (Vega et al. 2005). These Petri dishes were incubated at 28°C for 7 days. Pure fungal cultures were obtained by the methods described by Lacap et al. (2003) and Promputtha et al. (2005). The strains were preserved on PDA slants and stored at 4°C.

Culturing of isolates on agar plugs and bioassay for asparginase

Asparaginase producing endophytic fungi was screened by a rapid dye based agar plug technique that is carried out by using modified Czapek Dox agar medium. Different concentrations of the phenol red dye were supplemented in modified Czapek Dox agar medium. A 2.5% stock of the phenol red indicator was prepared in 70% ethanol and the pH was adjusted to 7.00 using 0.1 mol/L NaOH (Jayaramu et al. 2010). The stock solution of dye was added to 100 ml of modified Czapek Dox's agar medium to get final dye concentrations of 0.001 – 0.009 mg/L (R. Gulatii et al. 1997). The media was autoclaved and poured into pre-sterilized flat bottomed glass Petri plates (Borosil, India) and allowed to solidify. The agar plugs were prepared using a sterile 10mm diameter cork borer. Three agar plugs were then transferred to each glass slide (3 ×1 inch) in the sterile Petri plate containing Whatmann filter paper no 1. The spores collected from the 10 day old slant of isolates were scrubbed in sterile 2% tween 80 using a wire loop to form a spore suspension. The agar plugs were then inoculated by dipping into spore suspension using sterile forceps and placed on the surface of the glass slide. To prevent drying of the agar plugs, the humidity was maintained by adding 5mL sterile distilled water to the Whatmann filter papers in the Petri dishes and incubated in a humidity chamber at 28ºC for 4 days. (Ichikawa et al. 1971; Kleinheinz et al. 1997; Sitaramkumar et al. 2000; Patil et al. 2011). Production of L- asparaginase on agar plug causes colouring of agar plugs (yellow to pink). The pink color indicates the hydrolysis of L- asparagine into L- aspartic acid and ammonia by L- asparaginase. Release of ammonia causes change in pH which turns phenol red from yellow to pink and thus indicates asparginase activity.

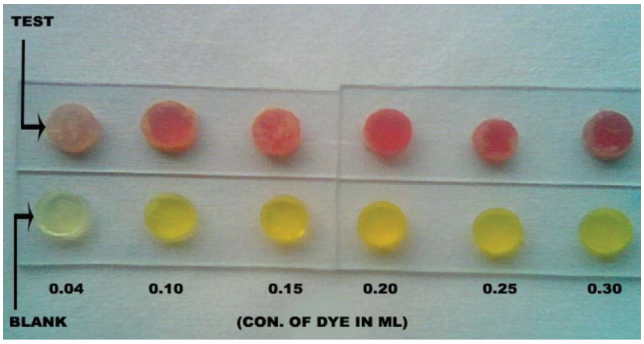

Figure 1. Screening of asparginase producing fungi using agar plug assay (Yellow plug: Negative test, pink plug: asparginase production).

Results and Discussion

In the present study, twelve fungal strains were isolated from the leaves and bark of the *Aegle maremelos.* Out of 12 strains, 3 strains showed highest color intensity in agar plugs. The agar plugs turned yellow to pink as a function of asparginase activity (Fig. 1). To ensure whether or not the coloring of agar plugs in bioassays was due to asparaginase activity; the agar plugs of positive fungi were subjected for production of asparaginase. It was observed that color intensity is directly proportional to asparaginase activity at shake flask level (data not shown).

All endophytic fungi could grow on modified Czapek Dox agar medium with phenol red, a dye indicator that changes from yellow (acidic condition) to pink (alkaline condition). Asparginase causes the hydrolysis of L- asparagine into L-aspartic acid and ammonia. Release of ammonia results in the change in pH. The indicator used in the bioassay, Phenol red, at acidic pH has pale yellow color, as the pH changes to alkaline; it turns to pink and thus leading to the pink coloration of the agar plugs. Conventionally, the asparaginase producers are screened by dye based plate assay. The strains exhibiting high asparaginase activity are cultivated in broth a culture which is tedious, time consuming, requires larger quantity media and is expensive. The agar plug assay used in the present study is not only sensitive but also rapid in comparison with earlier studies.

There is a continuous need for new, safe and useful compounds to control human, plant and animal diseases. Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of medicinal and

pharmaceutical importance. Therefore, we selected various parts of this plant (A. marmelos) for study and isolated few asparginase producing fungi. Many species of bacteria produce L-asparaginase (Peterson and Ciegler 1969); but only a few filamentous fungi such as species of *Aspergillus*, *Penicillium* and *Fusarium* have been reported to produce this enzyme. It has also been understood that, metabolites from the microbial endophytes are likely to be less toxic since they are harbored by eukaryotic host.

Conclusion

The new method developed in the present study can be advocated as rapid, sensitive, and reproducible methods for screening of asparginage producing fungi. The method is particularly helpful in strain improvement protocols where many isolates have to be tested simultaneously for asparginase activity.

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References

Bacon CW, White JF (2000) Microbial endophytes. Marcel Dekker Inc NY.

Friedrich J, Zuzek M, Bencina M, Cimerman A, Strancar A, Radez S (1995) High-performance liquid chromatographic analysis of mevinolin as mevinolinic acid in fermentation broths. J Chromatogr A 74:363-367.

Ghasemi Y, Ebrahiminezhad A, Amini SR, Zarrini G, Ghoshoon MB, Raee MJ, Morowvat MH, Kafilzadeh F, Kazemi A (2008) An optimized medium for screening of L-asparaginase production by *Escherichia coli*. Amer J Biochem Biotechnol 4(4):422-424.

Gulatii R, Saxena RK, Gupta R (1997) Rapid plate assay for screening of L-asparaginase producing microorganism. Letters in applied microbiology 24:23-26.

Guo B, Wang Y, Sun X, Tang K (2008) bioactive natural products from endophytes: A review. Appl Biochem Microbiol 44:136.

Ichikawa T, Date M, Ishikura T, Ozaki A (1971) Improvement of kasugamycin-producing strain by Agar Piece Method and the Prototroph Method. Folia Microbiol (Prague) 16:214-218.

Jayaramu M, Hemalatha NB, Rajeshwari CP, Siddalingeshwara KG, Mohsin SM, Dutt PLNSNS (2010) A novel approach for detection, confirmation and optimization of L-asparaginase from *Emericella nidulans*. Cur Pharma Res 1(1):20-24.

Keating MJ, Holmes R, Lerner S (1993) L-asparginase and PEG Asparginase-past present and future, Leukolymphoma 10:153-157.

Kleinheinz GT, Bagley ST (1997) A filter-plate method for the recovery and cultivation of microorganisms utilizing volatile organic compounds. J Microbiol Methods 29(2):139-144.

Lacap DC, Liew ECY, Hyde KD (2003) an evaluation of the fungal morphotype" concept based on ribosomal DNA sequences. Fungal Divers 12:53-66.

Patil RH, Patil MP, Patel SS, Maheshwari VL (2010) Probing the microbial endophytes in a indigenous plant for production of biologically active metabolites. Journal of Advances in Science and Technology 13(3):51-56

Peterson RE, Ciegler A (1969) L-asparaginase production by various bacteria. App Env Microbio 17:929-930.

Petrini O (1991) fungal endophytes of tree leaves. In Microbial Ecology of Leaves. Andrews J Hirano S., eds, Springer-Verlag New York, p. 179.

Promputtha I, Jeewon R, Lumyong S,McKenzie EHC, Hyde KD (2005) Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from Magnolia oliliifera (Magnoliaceae). Fungal Divers 20:167-186.

Sarquis M, Oliveria E, Santos A, Costa G (2004) Production of L-asparaginase by filamentous fungi. Mem Inst Oswaldo Cruz 99(5):489-492.

Sitaramkumar M, Mahendrakumar, Sarnaik HM, Sadhukhan AK (2000) Rapid technique of Lovastatin-producing strains of *Aspergillus terrus* by agar plug and Neurospora crassa bioassay. Journal of microbiological methods 40:99-104.

Soniyamby AR, Lalitha S, Paveesh BV, Priyadarshini V (2011) Isolation, production and anti-tumor activity of L-asparaginase of *Penicillium sp.* Inter J Microbiol Res 2(1):38-42.

Strobel GA (2002) Rainforest endophytes and bioactive products. Critical Reviews in Biotechnology 22:315.

Strobel GA, Daisy B, Castillo UF, Harper J (2004) Natural Products from Endophytic Microorganisms. J Nat Prod 67:257.

Theantana T, Hyde KD, Lumyong S (2007) Asparaginase production by endophytic fungi isolated from some Thai medicinal plants. KMITL Sci Tech J 7:13-18.

Vega FE, Pava-Ripoll M, Posada F, Buyer JS (2005) Endophytic bacteria in Coffea arabica L. J Basic Microb 45(5):371-380.

Verma VC, Kharwar RN, Strobel GA (2004) Chemical and Functional Diversity of Natural Products from Plant Associated Endophytic Fungi. Natural product Communications 4(11):1511.

Waksman SA (1916) Do fungi live and produce mycelium in the soil? Sci 44:320-322.

Warangkar SC, Khobragade CN (2009) Screening, enrichment and media optimization for L-asparaginase production. J Cell Tissue Res 9(3):1963-1968.