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A SIMPLE AND EFFICIENT DYE-BASED TECHNIQUE FOR RAPID SCREENING OF FUNGI FOR L-ASPARAGINASE PRODUCTION

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ABSTRACT

Three hundred and sixty four (364) isolates of tropical soil fungi were screened for L-asparaginase production by rapid plate method using modified Czapek-Dox agar containing L-asparagine, and either bromo cresol purple or phenol red dye as an indicator. Results of the study revealed that total one hundred and thirty five (135) isolates showed positive reaction for L-asparaginase production as indicated by the color change in and around the colonies between 48-72 hours of incubation at 28^0C. A comparative study of the two indicator dyes with varying concentrations showed bromo cresol purple is a better and efficient indicator for L-asparaginase screening than phenol red. Quantitative estimation of L-asparaginase in the selected fungi showed *Aspergillus* sp. and *Fusarium* sp. were good candidates for L-asparaginase production.

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1 Introduction

Enzymes produced by microorganisms have been used in industries for various applications. One of the enzymes L-asparaginase, (L-asparagine amido hydrolase E.C.3.5.1.1), belongs to amidase group and catalyzes the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. It is used as a therapeutic agent for the treatment of acute lymphocytic leukemia (mainly in children), Hodgkin's disease, acute myelocytic leukemia, chronic lymphocytic leukemia, and for the treatment of lymphosarcoma and melano sarcoma (Stecher et al., 1999; Verma et al., 2007). L-asparaginase also has significant applications in the food industry to reduce the formation of carcinogenic acryl amides in deep fried potato recipes (Friedman, 2003; Pedreschi., 2008). Free asparagine in food was found to be the primary source for acrylamide into which it is converted in the presence of reducing carbohydrates at temperatures above 120^0C (Mottram et al., 2002).

This enzyme is widely distributed in animals, plants and microorganisms. Microbes are the better source for the large scale production of L-asparaginase, because they can be cultured easily and the extraction and purification of L-asparaginase from them are also convenient. Extensive use of L-asparaginase from bacterial origin has been reported to cause pancreatitis, liver dysfunction, neurological seizures that may guide to intracranial thrombosis or haemorrhage and hypersensitivity leading to allergic reactions and anaphylaxis. (Moola et al., 1994). In view of the fact that L-asparaginase from eukaryotic microorganisms is known to have less adverse effects (Sarquis et al., 2004) combined with the fact that fungi are attractive candidates owing to their protein secretion capability, our laboratory was interested in screening primarily fungal isolates from tropical soil for the production of L-asparaginase.

Any successful microbial screening program requires a rapid, sensitive, reliable, reproducible and relatively inexpensive screening assay. In present study, the selection of fungal isolates for L-asparaginase production, various assays and dye-based screening was tried and obtained promising result. The highest producers of L-asparaginase among the screened fungi are *Fusarium* spp. Although *Aspergillus* species were also showing dominancy among the fungal isolated but the production of L-asparaginase was low when compared to *Fusarium* spp.

2 Materials and Methods

2.1 Sample collection and isolation

Soil samples (sub surface) were collected from tropical areas of different places of Karnataka, Tamil Nadu, Andhra Pradesh, Kerala and Maharashtra. Fungal strains were isolated from the collected soil samples using Potato Dextrose Agar (PDA) medium by serial dilution method (Booth, 1971).

2.2 Rapid screening of fungal isolates for L-asparaginase production

2.2.1 Design for screening medium

The rapid assay to screen for L-asparaginase producing fungi was performed using pH indicator dyes, bromo cresol purple and phenol red (Gulati et al., 1997). The medium used was modified Czapek-Dox agar medium containing, Glucose (2.0g/l); L-asparagine (10.0g/l); KH_2PO_4 (1.52g/l); KCL (0.52g/l); $MgSO_4.7H_2O$ (0.52g/l); $Cu(NO_3)_2.3H_2O$ (trace); $ZnSO_4.7H_20$ (trace); $FeSO_4.7H_20$ (trace); Agar (18.0g/l), initial pH 5.6 supplemented with 0.009% (v/v) of either bromo cresol purple or phenol red dye (Saxena & Sinha, 1981). Czapek-Dox agar medium either without the dye alone or L-asparagine (1%) served as control.

2.2.2 Screening for L-asparaginase producing fungal isolates

Czapek-Dox agar medium amended with either bromo cresol purple or phenol red was yellowish in color at pH 5.6. L-asparaginase produced by fungal colonies catalyzes the hydrolysis of L-asparagine present in the medium to L-aspartic acid and ammonia, which in turn increases the pH of the medium surrounding the L-asparaginase producing fungal colony. The increase in the pH leads to color change, from yellow to purple in bromo cresol purple amended medium or yellow to pink in phenol red amended medium. This color change was used as an indicator for L-asparaginase producing fungal isolates (Gulati et al., 1997).

2.3 Optimization of the dye-based screening assay

Modified Czapek-Dox liquid medium was prepared with varying concentrations of the pH indicator dyes which ranged from 0.001% through 0.009% with an increment of 0.002%. Fungal disc of 5mm diameter was placed on modified Czapek-Dox agar (Acuna et al., 1994) containing different concentrations of pH indicator dyes 0.001% - 0.009% (Gulati et al., 1997). After incubation at 28°C for 96 hrs, those concentration of the pH indicator dyes that resulted in purple and pink zones, respectively on bromo cresol purple and phenol red were chosen as the indicator of L-asparaginase production. A concentration at which there was an apparent color change indicating the production of L-asparaginase, but without causing any changes in the morphology or growth was considered as an optimal concentration and selected for routine screening.

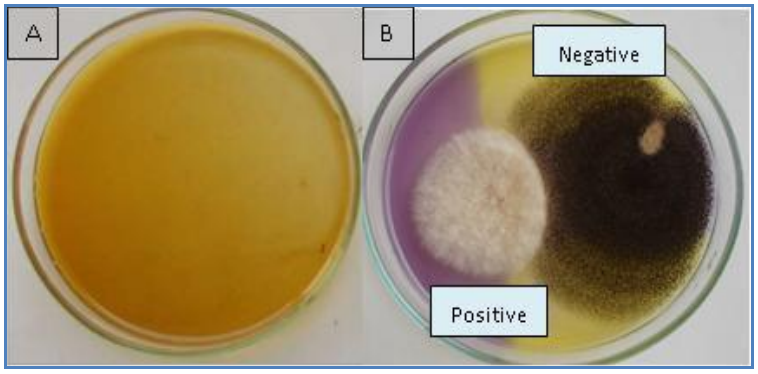


Figure 1 Screening for L-asparaginase with bromo cresol purple amended medium A= control, B= Plate with Bromo cresol purple dye (Positive= color change from yellow to purple indicates L-asparaginase production. Negative= no color change indicates no L-asparaginase production).

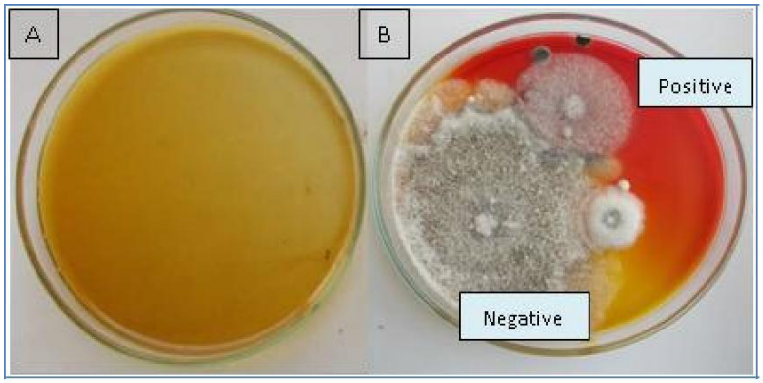


Figure 2 Screening for L-asparaginase with phenol red amended medium: A= control, B= Plate with Phenol red dye (Positive= color change from yellow to pink, indicates L-asparaginase production. Negative= no color change, indicates no L-asparaginase production).

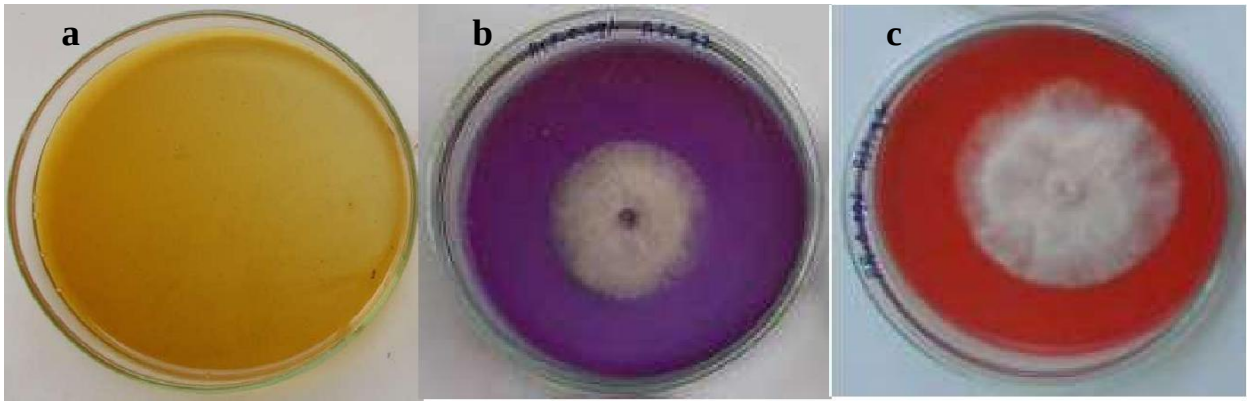
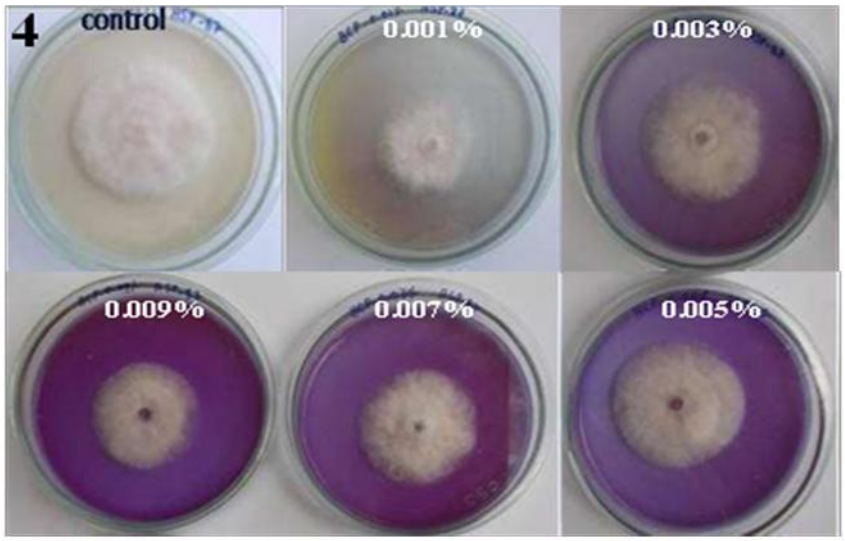
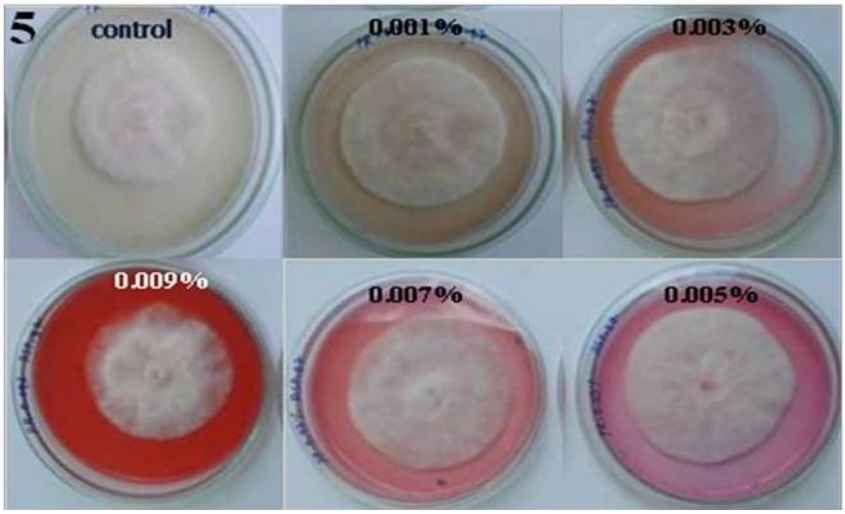


Figure 3 Preliminary screening for L-asparaginase production. A: Control, B: assay plate with bromo cresol purple dye, C: assay plate with phenol red dye.



(A)



(B)

Figure 4.A and 4.B Comparison of color change with bromo cresol purple in 48 hrs (4.A) and with phenol red in 72- 96 hrs (4.B) as indicator for L-asparaginase production in *Fusarium* sp. (concentration of dye from 0.001% to 0.009%).

2.4 Quantitative screening for L-asparaginase activity

The fungal isolates that showed maximum zone of color change in the plate assay was chosen for further evaluation in the liquid medium to quantify L-asparaginase by spectrophotometric assay (Imada et al., 1973). The selected isolates were grown in 100 ml of modified Czapek-Dox liquid medium. The cultures were incubated at 120 rpm at 28°C for four days. At the end of the incubation period, the cultures were harvested and mycelia were separated from the culture broth by filtration through Whatman no. 1 filter paper and the clarified culture filtrate was used as the crude enzyme source for L-asparaginase assay.

2.5 Assay of L-asparaginase

The level of L-asparaginase enzyme in the culture filtrates was determined using the method of Imada et al. (1973). In this way, the rate of hydrolysis of L-asparagine was determined by measuring the ammonia released using Nessler's reagent. A mixture of 0.5ml of enzyme extract, 0.5ml of 0.04M L-asparagine, 0.5ml of 0.05M Tris-HCl buffer (pH 7.2) and 0.5ml of distilled water was incubated at 37^0C for 30 min. The

reaction was stopped by the addition of 0.5ml of 1.5M trichloroacetic acid (TCA).

The ammonia released in the supernatant was determined spectrophotometrically by adding 0.2ml of Nessler's reagent into tubes containing 0.1ml of supernatant and 3.7ml of distilled water and incubated at room temperature for 20 min. The absorbance was read at 450 nm. One international unit (IU) of L-asparaginase activity is defined as the amount of enzyme required to produce 1μmol of ammonia per min under the standard assay conditions.

3 Results and Discussion

3.1 Isolation and identification of fungi

Tropical soil samples were screened for fungal isolates by plating the serially diluted samples on PDA medium and incubating for 04 days at 28°C. A total of three hundred and sixty four (364) fungal cultures that were morphologically distinct were isolated from tropical soil and purified. Based on the morphological and microscopical observation (colony morphology, conidiophores, conidial head and conidia), most of the isolated fungi belonged to the genera *Aspergillus* (96), *Fusarium* (68), *Penicillium* (22) and *Cladosporium* (05). Furthermore, 29 species of non-sporulating fungi were also isolated.

3.2 Rapid screening for L-asparaginase production

All three hundred and sixty four fungal isolates were screened for L-asparaginase production using modified Czapek-Dox medium amended with pH indicator dyes. Among the three hundred and sixty four (364) fungal isolates screened for L-asparaginase production, one hundred and thirty five (135) isolates showed positive reaction for asparaginase production as indicated by the color change between 48-72 hours of incubation at 28°C (Figure 1). It was observed that L-asparaginase production is accompanied by an increase in the pH of the culture filtrate from the initial pH 5.6 to 8.1 after 96 hours of incubation. At acidic pH, the medium amended with either phenol red or bromo cresol purple is yellow (Figure 1a) and at alkaline pH turns purple whereas phenol red is yellow at acidic pH and at alkaline pH turns pink, thus, a purple or pink zone is formed around the colonies when fungal isolates produce L-asparaginase. The change in color of the dye amended plates was specific to L-asparaginase producing colonies. No change in color was observed in uninoculated control plates (Figure 1a and Figure 2a) or surrounding the fungal colonies that are negative for L-asparaginase (Figure 1b and 2b) production. In addition, there appeared to be a direct positive correlation between dye color change and L-asparaginase production for the majority of the isolates screened. The L-asparaginase positive and L-asparaginase negative strains were subjected to quantitative assay for L-asparaginase, and the results obtained (Table 2) were in concordance with the results of the rapid screening method.

3.3 Comparison of two pH indicator dyes for L asparaginase production

One hundred and thirty five (135) fungal isolates which showed positive for L-asparaginase production were further screened by disc plate method (Acuna et al., 1994) among which fifty two (52) strains were selected for further studies which showed change of color above1.5cm-4.0cm (Figure 3). It was observed that the intensity of the color change is directly proportional to the concentration of dye. Among the two dyes tested, bromo cresol purple was found to be a better indicator of L-asparaginase production than phenol red, as the change in coloration occurred even at the concentration of 0.005% within 48 hours of incubation. (Table 1, Figure 4.A and 4.B). The diameter of zone of color change in plate assay was directly proportional to the enzyme activity in quantitative assay for few fungal isolates (Table 2), such finding was reported by earlier workers Gulati et al. (1997), Balakrishnan et al. (2013) and Anamika et al. (2013). However, for some isolates, the zone of color change was not proportional to enzyme activity similar to that reported by Holker et al. (2004) and Lee et al. (2005).

This lack of direct correlation between color change to that of enzyme activity is not totally surprising considering the fact that the color change of the indicator dye depends on the secreted L-asparaginase and that fungi vary in their capabilities to secrete enzymes.

Table 1 Effect of concentrations of bromo cresol purple and phenol red on L- asparaginase production in *Fusarium* sp. after 48 hrs of incubation with bromo cresol purple dye and 96 hrs of incubation with phenol red dye.

Concentration of dyes(%v/v)	With bromo cresol purple		With phenol red	
	Colony diameter(cm)	Zone of color change in diameter(cm)	Colony diameter(cm)	Zone of color change in diameter(cm)
0.001	1.51	3.02	2.32	1.06
0.003	2.20	3.94	2.30	2.24
0.005	2.16	4.00	2.32	3.57
0.007	2.06	4.00	2.35	3.81
0.009	2.12	4.00	2.41	4.00
control	1.92	Nil	1.92	Nil

Table 2 Rapid screening of fungal isolates for L-asparaginase activity.

Serial No	Culture code	Colony diameter (cm)	Zone of color change (cm)	Enzyme activity (U/ml)
1	**ASP-04**	1.0	4.0	0.30
2	**ASP-09**	1.7	3.2	0.15
3	**ASP-14**	1.3	3.0	0.08
4	**ASP-20**	1.4	2.9	0.12
5	**ASP-24**	1.2	2.3	0.05
6	**ASP-27**	1.3	2.7	0.05
7	**ASP-28**	1.3	4.0	0.20
8	**ASP-34**	1.8	4.0	0.16
9	**ASP-35**	1.5	3.0	0.13
10	**ASP-36**	1.0	2.5	0.08
11	**ASP-37**	0.7	3.2	0.10
12	**ASP-41**	1.6	2.0	0.01
13	**ASP-44**	1.8	2.6	0.09
14	**ASP-48**	0.8	1.8	0.05
15	**ASP-50**	0.7	4.0	0.20
16	**ASP-52**	0.8	4.0	0.25
17	**ASP-53**	1.0	4.0	0.38
18	**ASP-54**	1.8	3.2	0.11
19	**ASP-56**	1.7	4.0	0.45
20	**ASP-58**	1.0	3.6	0.18
21	**ASP-60**	1.3	4.0	0.21
22	**ASP-61**	1.0	3.2	0.15
23	**ASP-64**	2.2	1.7	0.02
24	**ASP-68**	1.5	4.0	0.52
25	**ASP-72**	1.2	2.0	0.02
26	**ASP-74**	1.6	2.5	0.02
27	**ASP-75**	0.3	3.1	0.11
28	**ASP-77**	1.2	1.5	0.01
29	**ASP-79**	1.4	2.5	0.03
30	**ASP-80**	0.7	3.0	0.14
31	**ASP-84**	0.5	1.5	0.06
32	**ASP-87**	1.3	4.0	0.50
33	**ASP-88**	1.4	2.2	0.02
34	**ASP-89**	1.0	2.5	0.08
35	**ASP-92**	1.6	3.4	0.12
36	**ASP-93**	0.5	2.3	0.08
37	**ASP-94**	1.4	3.0	0.05
38	**ASP-95**	0.6	3.4	0.16
39	**ASP-97**	1.5	4.0	0.15
40	**ASP-99**	2.0	4.0	0.15
41	**ASP-102**	0.8	3.4	0.10
42	**ASP-105**	1.3	2.0	0.01
43	**ASP-108**	2.1	4.0	0.33
44	**ASP-110**	1.5	2.5	0.05
45	**ASP-113**	1.0	2.1	0.05
46	**ASP-117**	1.4	1.5	0.02
47	**ASP-123**	0.8	3.0	0.08
48	**ASP-126**	1.5	2.1	0.04
49	**ASP-129**	1.2	4.0	0.21
50	**ASP-130**	2.0	2.8	0.07
51	**ASP-132**	1.2	1.8	0.03
52	**ASP-135**	0.9	1.7	0.05

Enzyme activity is expressed in U/ml

Owing to the importance of L-asparaginase in pharmaceutical and food industries in general and favorable characteristics of L-asparaginase derived from fungal sources; there has been a constant search for microorganisms capable of producing L-asparaginase. The method utilized in this study for screening L-asparaginase producing fungal isolates based on pH indicator dye is relatively faster, sensitive, reproducible and inexpensive which takes advantage of the change in the color of the medium which was brought about by change in pH resulting from the formation of ammonia.This method is advantageous as it is brought to the product of L-asparaginase and thus enabling the identification of fungal colonies producing L-asparaginase that is active directly on the screening of plates without performing time consuming liquid cultures and assays (Wade et al., 1971; De- Jong, 1972; Arima et al., 1972; Imada et al., 1973; Gulati et al., 1997; Rani et al., 2012). As early Gulati et al., (1997) reported that phenol red is an useful indicator for the deduction of L-asparaginase in fungi. Subsequently, this dye was used by other workers (Sarquis et al., 2004; Siddalingeshwara and Lingappa 2011; Soniyamby et al., 2011). However, till date no other indicator dye with possible superior screening characteristics was evaluated for rapid screening of L- asparaginase in fungi.

Conclusion

In the present study, it was reported that bromo cresol purple is a better indicator in terms of sensitivity for L-asparaginase production than phenol red for the fungal isolates screened. We also observed that bromo cresol purple even at lower concentration (0.005%) showed better color change with high intensity indicating the production of L-asparaginase than phenol red. Although previous studies have utilized single pH indicator dye, our screening with two indicators side-by-side for L-asparaginase production has enabled the identification of the best-suited dye for screening purposes. So far, this is the first report demonstrating that bromo cresol purple could be used as a suitable indicator for the screening and selection of an organism producing L- asparaginase. Out of all fifty two L-asparaginase producing fungi isolated, 35% of isolates were characterized as species belonging to the genus *Aspergillus* and 26 % belonged to the genus *Fusarium* which are the subject of current and future studies aimed at developing potential strains for L-asparaginase production as well as biotechnological application.

Conflict of interest

The authors declare no conflict of interest.

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