Journal of
Microbial & Biochemical Technology

Vaishali and Bhupendra, J Microb Biochem Technol 2017, 9:4
DOI: 10.4172/1948-5948.1000361

Research Article Open Access

A Rapid and Efficient Dye Based Plate Assay Technique for Screening of L-Asparaginase Producing Fungal Strains

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Abstract

L-Asparaginase enzyme has received great attention owing to its potential as anticancer drug and food processing agent. L-Asparaginase producing microbes are conventionally screened on phenol red plates containing L-asparagine as the sole nitrogen source for its growth. However, the contrast of the zone in phenol red plates is not very distinct. To overcome this problem, an alternate methodology for the screening of fungal strains producing extracellular L-Asparaginase is required. In the present comparative investigation, an improved method for screening is reported, wherein Alizarin Red S and 4-Nitrophenol are implied as pH indicator. Plates containing Alizarin Red S and 4-nitrophenolare colorless at acidic pH, turns pink and yellow respectively at alkaline pH. Thus, a dark pink and yellow zone is formed around microbial colonies producing L-asparaginase, differentiating between enzyme producers and non-producers. Thus, we report Alizarin red S and 4-Nitrophenol can detect the production of the anticancer enzyme at a much lower dye concentration which appear to be more accurate and distinctive than the conventional method for the screening of fungi producing extracellular L-Asparaginase.

Keywords: L-Asparaginase; Anticancer enzyme; Plate assay method; Alizarin Red S; 4-nitrophenol

Introduction

Along the years enzyme production has emerged as one of the most progressively growing application of microbial technology in the world market [1]. Enzymes have been used in food, beverage, textile, pharmaceutical, leather and cosmetic industries. In today's pharmaceutical market, the manufacture of enzymes as drugs is an important facet. The enzymes in the pharmaceutical industry are promoted due to its properties such as high specificity, economic production and high yield. One such enzyme that has been used for the treatment for childhood Acute Lymphoblastic Leukemia (ALL), Hodgkin's disease, Chronic Lymphocytic Leukemia, Acute Myelocytic Leukemia, Melanosarcoma is L-Asparaginase [2]. L-Asparaginase (EC 3.5.1.1) catalyzes the irreversible hydrolysis of L-asparagine into aspartic acid and ammonia under physiological conditions. L-Asparaginase has been an extensive component in the revolutionary combination chemotherapy protocols used for the treatment of childhood Acute Lymphoblastic Leukemia. In recent years, different studies aimed at finding an alternated source for the enzyme with improved characteristics such as less side effects and longer half-life as compared to L-asparaginase from *E. coli*, with economically viable production as well as causing minimal collateral effects have gained momentum. A different L-Asparaginase source, primarily fungal alternatives, has been proposed to contain less adverse effects and different features, which are advantageous for its application. L-Asparaginase has also been used in the food industry as it can degrade acrylamide found abundantly in the wide range of fried and baked food products [3]. Acrylamide has been a major concern for the health experts as it has been declared a potent neurotoxin and carcinogen [4,5]. Any microbial screening technique needs to be rapid, sensitive, efficient and reproducible. The enzyme had initially been screened in culture filtrates using Nessler's reagent [6,7]. This tedious and time consuming method was soon replaced by plate assay technique which has been reported to work on the principle of increase in pH of the medium on the production of L-Asparaginase [8]. The change in pH has been monitored by the incorporation of a pH indicator in the medium containing L-Asparagine as the sole nitrogen source. Phenol red has been used as pH indicator in the earlier plate based methods for the screening of extracellular L-Asparaginase producing microbes, but the method was found to be disadvantageous as the contrast between yellow and pink is indistinguishable [9]. Recently pH indicators such as Bromothymol blue, Methyl red and Bromocresol purple has been used for the detection of the enzyme production [10-12]. In the present investigation, a rapid plate assay method utilizing Alizarin Red S and 4-nitrophenol (p- nitrophenol) for the screening of fungal isolates is reported. In addition to the plate assay, L-Asparaginase titer in the culture filtrates was also quantified.

Materials and Methods

Sample collection, isolation and identification of fungal strains

Soil samples from the sub surface were collected from different sites of the Guru Ghasidas Vishwavidyalaya, Bilaspur campus. Fungi were isolated on selective medium containing streptomycin sulphate (100 $\mu g\,ml^{-1}$) of Potato Dextrose Agar (PDA) medium. The soil suspensions were serially diluted and plated on the above selective medium so as to inhibit bacterial growt [13]. The isolated fungal strains were stained using Lactophenol cotton blue and identified taxonomically based on their morphological characters [14].

Screening of L-Asparaginase producing fungal strains

For the screening of L-Asparaginase producing fungal strains modified Czapek-Dox agar medium containing (g L^{-1}): 2 g glucose; 10 g L-asparagine; 1.52 g KH_2PO_4; 0.52 g $MgSO_4{\cdot}7H_2O$; 0.52 g KCl; 20 g

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Received June 09, 2017; **Accepted** July 12, 2017; **Published** July 19, 2017

Citation: Vaishali P, Bhupendra NT (2017) A Rapid and Efficient Dye Based Plate Assay Technique for Screening of L-Asparaginase Producing Fungal Strains. J Microb Biochem Technol 9:162-168. doi: 10.4172/1948-5948.1000361

agar and trace $CuSO_4$, $ZnSO_4 \bullet 7H_2O$, $FeSO_4 \bullet 7H_2O$ [10]. The media was supplemented with varying concentrations (0.000-0.009% v/v) of Alizarin Red S dye (Hi-Media) and 4-nitrophenol (Hi-Media). Plates with dye were inoculated with test cultures and incubated at 28 ± 2°C for 96 h, whereas the plates without dye served as positive control. Similarly, the culture broth supplemented with 0.001% to 0.009% v/v of Alizarin Red S dye and 4-nitrophenol were inoculated with test cultures and incubated at 28 ± 2°C for 24 to 96 h whereas broth without dye was set as control.

Enzyme titer studies for the quantitative estimation of L-Asparaginase production

The modified Czapek Dox broth was used as the production medium for quantitative estimation of L-Asparaginase. The mycelia were harvested at an interval of 12 h from the culture broth by filtration using Whatman no. 1 filter paper. The culture filtrate was used as the crude enzyme source for L-Asparaginase assay. The activity of L-Asparaginase enzyme in the crude extract was determined by the estimation of liberated ammonia from the hydrolysis of L-Asparagine with slight modifications [6]. The 0.1 ml of crude extract was added to 0.9 ml of 0.05 M phosphate buffer (pH 7.8) and 0.5 ml of 0.04 M L-Asparagine and the volume was made up to 2 ml with distilled water. The resulting suspension was incubated at 37°C for 30 min followed badding 0.5 ml of 1.5 M Trichloro acetic acid (TCA) to stop the enzymatic reaction. In the next step, 0.1 ml of the above solution was diluted with 3.5 ml of distilled water and 0.4 ml of Nessler's reagent. After 10 min of incubation at room temperature the absorbance was recorded at 450 nm with a UV-Spectrophotometer (Shimadzu spectrophotometer, UV-1800). The standard graph of ammonium sulphate was prepared for the estimation of the sample concentration. For the blank preparation, the crude extract was added after TCA. Enzyme activity was expressed in international units (IU) ml^{-1}, which is defined as the amount of enzyme that catalyzes the formation of 1 µmol of NH3 min^{-1}.

Results

Isolation and identification of fungal isolates

A total of 50 fungal strains were isolated from the soil samples using serial dilution method by incubating the plates of PDA at 30 ± 2°C for 96 h, of which *Aspergillus* spp., *Fusarium* spp. and *Penicillium*

S. No.	Isolate Name	Microscopic Morphological characters	Taxonomic Classification
1	BV–A	**Hyphae**: Septate **Conidiophore**: unbranched **Philaides**: Flask shaped covering the vesicles **Conidia**: Round	**Kingdom**: Fungi **Phylum**: Ascomycota **Class**: Eurotiomycetes **Order**: Eurotiales **Family**: Trichocomaceae **Genus:** *Aspergillus* spp.
2	BV–B		
3	BV–C		
4	BV–D		
5	BV–E		
6	BV–F		
7	BV–G		
8	BV–H		
9	BV–I		
10	BV–J		
11	BV–K		
12	BV–L		
13	BV–M		
14	BV–N		
15	BV–O		
16	BV–P		
17	BV–Q		
18	BV–R		
19	BV–S		
20	BV–T		
21	BV–U		
22	BV–V		
23	BV–W		
24	BV–X		
25	BV - Y		
26	BV - Z		
27	BV–A1	**Appearance of thecolonies:** Wooly **Septa:** Smaller and fewer **Microconidia:** Present in chains or beads **Spores:** Multishaped	**Kingdom:** Fungi **Phylum:** Ascomycota **Class:** Sordariomycetes **Order:** Hypocreales **Family:** Nectriaceae **Genus:** Fusarium spp.
28	BV–B1		
29	BV–C1		
30	BV–D1		
31	BV–E1		
32	BV–F1		
33	BV–G1		
34	BV–H1		
35	BV–I1		
36	BV–J 1		

37	BV–K1	**Mycelium:** Highly branched multinucleate **Septa:** Small **Hyphae:** Colorless **Conidiophores:** Branched **Conidiospores:** Individually constricted	**Kingdom:** Fungi **Phylum:** Ascomycota **Class:** Eurotiomycetes **Order:** Eurotiales **Family:** Trichocomaceae **Genus:** *Penicillium* spp.
38	BV–L1		
39	BV–M1		
40	BV–N1		
41	BV–O1		
42	BV–P1		
43	BV–Q1		
44	BV–R1		
45	BV–S1		
46	BV–T1		
47	BV–U1		
48	BV–V1		
49	BV–W1		
50	BV–X1		

Table 1: Morphological identification of fungal isolates (Alexopolous et al. [14]).

spp. were 26, 10 and 14, respectively (Table 1). The morphological characterization of the fungal isolates was done by Lactophenol cotton blue staining and the morphological characters were recorded leading to the identification up to genus level using taxonomic key [14].

Screening of L-Asparaginase producing fungal strains

All the isolated strains were screened for the production of L-Asparaginase enzyme on modified Czapek Dox agar medium with the optimized dye concentration. Altogether 20 strains out of 50 selected showed positive results for the production of L-Asparaginase enzyme (Table 2). The change in pH of the production medium from 5.0 to 8.5 due to the hydrolysis of L-Asparagine to Aspartic acid and ammonia was indicated through a visible colour change in the course of experiment. The media was colourless at acidic pH and turned dark pink and dark yellow at alkaline pH after the incorporation of Alizarin Red S and 4-Nitrophenol, respectively, thus forming a dark collared zone around the fungal colonies producing L-Asparaginase, whereas, no colour change was observed around the fungal colonies lacking L-Asparaginase in the control plates. The filtrates of the culture broths not supplemented with the two potential dyes also showed a change in pH from 5.0 to 8.5. The result suggested that the dyes do not affect the growth of the fungal isolates and the enzyme production.

Comparative effect of optimized dye concentrations on the screening of L-Asparaginase producing fungal strains

The present study was carried out with the objective to improve the plate assay method for the screening of fungus producing extracellular L- Asparaginase using 4-nitrophenol and Alizarin Red S as potential screening dyes. For this purpose, the fungal isolate *Aspergillus* spp. (BV–C) showing maximum colour contrast was selected. The change in color is attributed to the release of ammonia which increases the pH during the hydrolysis of L-Asparagine [15]. Studies with different concentrations of the dyes revealed that the enzyme production was directly proportional to the change in colour intensity. However, Alizarin Red S has the advantage of the development of sharp colour contrast between the unhydrolyzed and hydrolyzed L-Asparagine at a much lower concentration of 0.001% (Table 2). Also, the colour change in case of Alizarin Red S from colourless to dark pink was found to be distinctive as compared to colourless to dark yellow for 4-nitrophenol. It was also observed that the dye did not affect the growth of test fungus which was measured in terms of enzyme activity and dry weight of the fungal biomass. It was also observed that the enzyme titter studies in presence and absence of dye as the fungal biomass and pH of the culture remained the same.

Enzyme titer studies for the quantitative estimation of L-Asparaginase production

The culture filtrates of fungi grown in modified Czapek Dox broth were used as enzyme source and L-Asparagine served as the substrate. Enzyme activities were determined by direct nesslerization of NH3 and were expressed as international units (IU) ml^{-1} [16]. After 96 h of incubation, the release of ammonia was indicated by the increase in the pH of the culture medium. The formation of sharp and intense colour contrast suggested the production of L-asparaginase as compared with the relatively less contrasting zone formed by 4-nitrophenol. The enzyme titter experiments clearly differentiates the course of L-Asparaginase on the basis of colour change at an optimized dye concentration of 0.001 (Figures 1, 2a and 2b) and 0.003% (Figures 3a and 3b) for both the dyes over the period of 96 h.

Discussion

The present investigation was carried out to improve the plate assay method used for the screening of fungal strains using a more sensitive dye instead of phenol red or Bromothymol blue in L-asparagine-containing medium. 50 fungal strains were isolated on a streptomycin containing PDA selective medium which were microscopically identified using taxonomic key [9,10,14]. These strains were further screened on Modified Czapek Dox medium containing Alizarin Red S and 4-Nitrophenol for the production of L-Asparaginase. The objective of incorporating a dye is to detect a change in the color of the medium due to release of ammonia after the hydrolysis of L-Asparagine to L-Aspartic acid. The previously reported dyes such as Phenol red Bromothymol blue, methyl red and Bromocresol purple were used at higher concentrations viz., 0.009%, 0.007%, 0.008% and 0.005%, respectively [9-12]. However, Alizarin red S and 4-nitrophenol show optimum intensity in colour change at a much lower dye concentration of 0.001% and 0.005%, respectively (Table 3). The zone diameter of colour change in the plate assay was found to be directly proportional to the enzyme activity in quantitative assay for few fungal isolates as the control plates having no indicator also showed prominent colony formation but no zone formation. The study of fungal biomass along with L-Asparaginase in the absence of dye confirmed that the dyes did not affect the growth of the fungus proving it to be a safe alternative to the present dyes (Supplementary table).

S. No.	Isolate Name	L-Asparaginase production	Genus
1	**BV–C**	**++++**	*Aspergillus* spp.
2	BV–K	+++	
3	BV–M	+++	
4	BV–N	+++	
5	BV–Q	+++	
6	BV–R	+++	
7	BV–S	+++	
8	BV–U	+++	
9	BV–X	+++	
10	BV–Z	+++	
11	BV–B	++	
12	BV–D	++	
13	BV–E	++	
14	BV–F	++	
15	BV–G	++	
16	BV–H	++	
17	BV–P	++	
18	BV–T	++	
19	BV–V	++	
20	BV–Y	++	
21	BV–A	+	
22	BV–I	+	
23	BV–J	+	
24	BV–L	+	
25	BV–O	+	
26	BV–W	+	
27	BV–A1	+++	*Fusarium* spp.
28	BV–C1	+++	
29	BV–E1	+++	
30	BV–F1	+++	
31	BV–H1	+++	
32	BV–I1	+++	
33	BV–J 1	+++	
34	BV–B1	++	
35	BV–D1	++	
36	BV–G1	++	
37	BV–K1	+++	*Penicillium* spp.
38	BV–T1	+++	
39	BV–U1	+++	
40	BV–X1	+++	
41	BV–L1	++	
42	BV–M1	++	
43	BV–N1	++	
44	BV–Q1	++	
45	BV–S1	++	
46	BV–V1	++	
47	BV–W1	++	
48	BV–O1	+	
49	BV–P1	+	
50	BV–R1	+	

Table 2: Fungal isolates and their respective L-Asparaginase production potential (++++ - high production of L-Asparaginase; + - lowest production of L-Asparaginase).

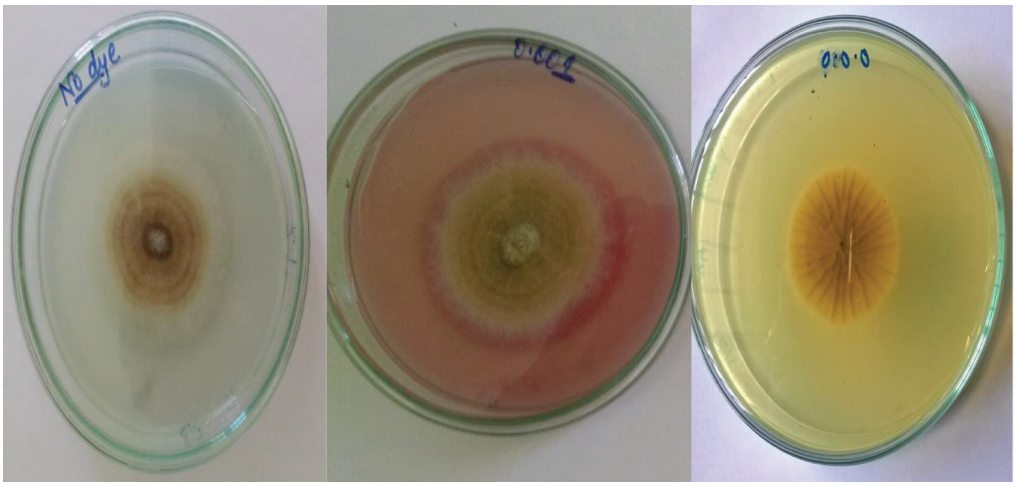

Figure 1: Plates incorporated with no dye, supplemented with Alizarin Red S and 4-Nitrophenol (Left to right).

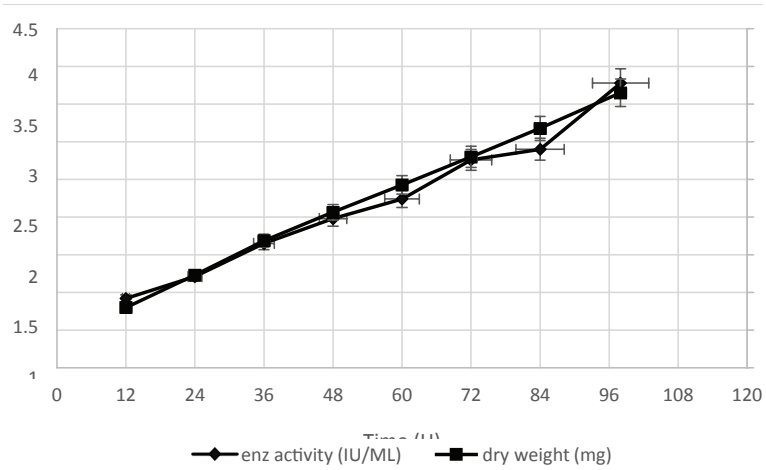

Figure 2a: Profile of enzyme production and growth in terms of dry weight (mg) by the fungal culture in presence 0.001% of Alizarin Red S dye for *Aspergillus* spp. (BV–C).

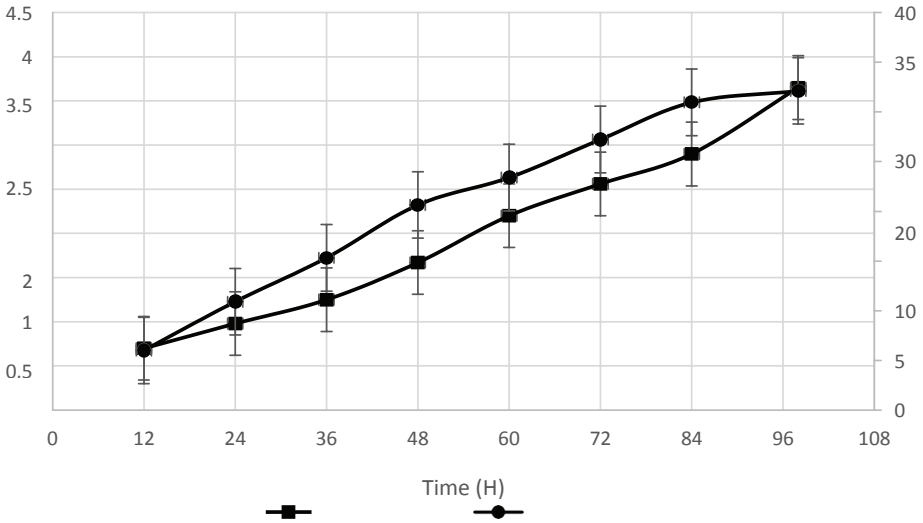

Figure 2b: Profile of enzyme production and growth in terms of dry weight (mg) by the *Aspergillus* sp. culture (BV–C) in absence of dye.

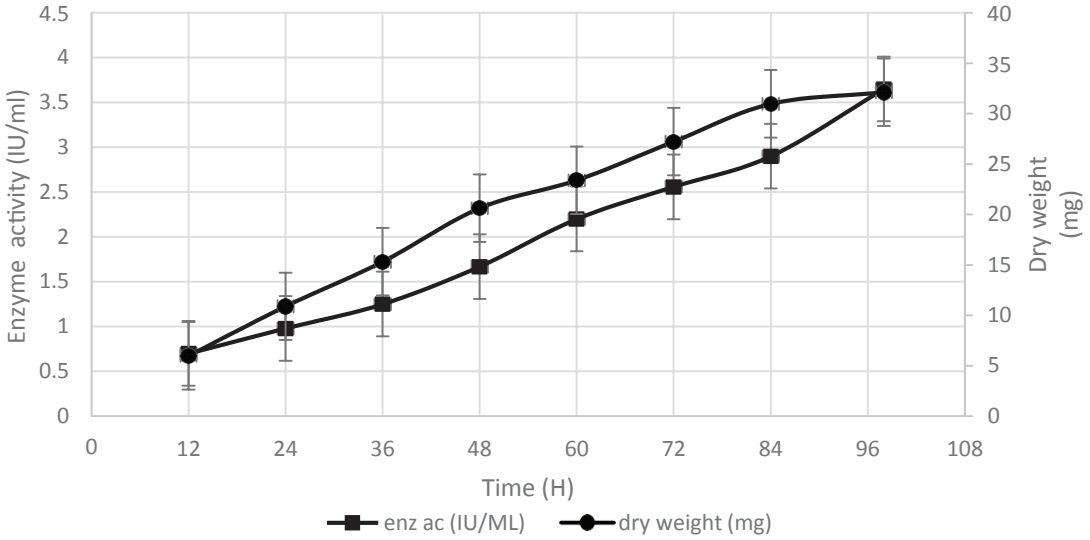

Figure 3a: Profile of enzyme production and growth in terms of dry weight (mg) by the *Aspergillus* sp. culture (BV–C) in presence 0.007% of 4-nitrophenol.

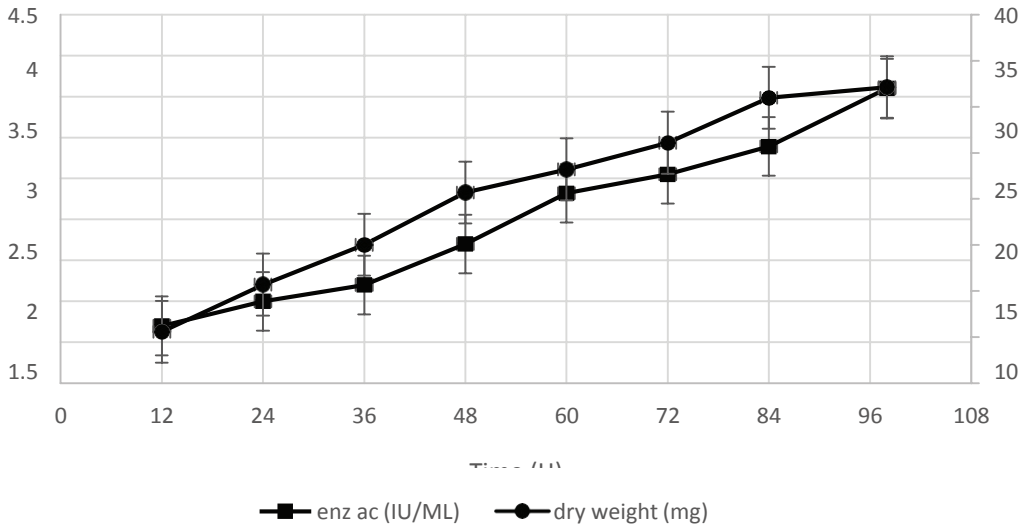

Figure 3b: Profile of enzyme production and growth in terms of dry weight (mg) by the *Aspergillus* sp. culture (BV–C) in absence of dye.

Concentration of dyes (% v/v)	Alizarin Red S		4-nitrophenol	
	Colony diameter (cm) ± S.E*	Zone diameter (cm) ± S.E	Colony diameter (cm) ± S.E	Zone diameter (cm) ± S.E
Control	4.5 ± 1.21	Nil	3.0 ± 1.32	Nil
0.001	4.5 ± 1.04	9.00 ± 2.12	4.6 ± 1.23	9.00 ± 0.98
0.003	4.6 ± 0.09	9.00 ± 0.10	5.2 ± 0.08	9.00 ± 0.07
0.005	4.7 ± 0.34	9.00 ± 0.23	6.3 ± 0.05	9.00 ± 0.14
0.007	4.7 ± 0.49	9.00 ± 1.09	7.6 ± 0.95	9.00 ± 0.34
0.009	4.8 ± 0.04	9.00 ± 1.32	7.6 ± 0.04	9.00 ± 0.45

*S.E: Standard error of the mean

Table 3: Effect of concentrations of Alizarin Red S and 4-nitrophenol on L-asparaginase production by a representive fungal isolate *Aspergillus spp.* (BV–C).

Conclusion

In the present study, Alizarin Red S at a lower concentration (0.001%) showed intense color contrast indicating the production of L-Asparaginase than 4-nitrophenol concentration (0.005%). Moreover, Alizarin Red S and 4-Nitrophenol did not affect the enzyme production

and growth of fungi used in the study, suggesting the non-toxic nature of the pH indicators. Based on the overall results with the pH indicator dyes Alizarin Red S is proposed as a better indicator than 4-Nitrophenol in terms of sensitivity for the screening of Fungal L-Asparaginase enzyme due to the clarity of the contrasting color zones.

Acknowledgement

The authors acknowledge with thanks the financial assistance from the Department of Biotechnology, Ministry of Science and Technology, Government of India under BUILDER Project (Sanction Order No. BT/PR7020/INF/22/172/2012).

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