

A rapid, efficient and sensitive plate assay for detection and screening of L-asparaginase-producing microorganisms

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Abstract

L-Asparaginase-producing microbes are conventionally screened on phenol red L-asparagine-containing plates. However, sometimes the contrast of the zone obtained (between yellow and pink) is not very sharp and distinct. In the present investigation, an improved method for screening of the microorganisms producing extracellular L-asparaginase is reported wherein bromothymol blue (BTB) is incorporated as pH indicator in L-asparagine-containing medium instead of phenol red. Plates containing BTB at acidic pH are yellow and turn dark blue at alkaline pH. Thus, a dense dark blue zone is formed around microbial colonies producing L-asparaginase, differentiating between enzyme producers and non-producers. The present method is more sensitive and accurate than the conventional method for screening of both fungi and bacteria producing extracellular L-asparaginase. Furthermore, BTB gives a transient green colour at neutral pH (7.0) and dark blue colour at higher pH 8.0–9.0, indicating the potency of the microorganism for L-asparaginase production.

Introduction

L-Asparaginase is a hydrolytic enzyme (EC3.5.1.1) globally known for its potential as chemotherapeutic agent, particularly in the treatment of acute lymphoblastic leukaemia (ALL) in children, various neoplasms of mouse and cat and certain canine lymphosarcomas (Wriston & Yellin, 1973). Its anti-leukaemic effect results from the depletion of L-asparagine circulating in the blood, which is essential for malignant lymphoblastic cells (Ohnuma *et al.*, 1970). L-Asparaginase irreversibly catalyses the conversion of L-asparagine to L-aspartate and ammonium under physiological conditions (Lubkowski *et al.*, 1996). Degradation of acrylamide is another important application of L-asparaginase. The formation of acrylamide in a wide range of fried and oven-cooked foods, especially in potato chips, has been a major concern in the modern world due to its neurotoxic (Rosen & Hellenas, 2002) and carcinogenic effects (Tareke *et al.*, 2002). The acrylamide formation in heated foods is mainly attributed to the reaction of free asparagine and reducing sugars (Ciesarova *et al.*, 2006).

The enzyme, L-Asparaginase interrupts the interaction of L-asparagine with reducing sugars, thus preventing acrylamide formation (Ciesarova *et al.*, 2006). L-Asparaginase is widely distributed in animal, plant and microbial sources. The naturally occurring plants and animal sources of enzymes are often insufficient for therapeutic and other industrial applications. The principal sources of commercial L-asparaginase are *Escherichia coli* and *Erwinia carotovora* (Bonetti *et al.*, 1969). However, the enzyme produced by these microbes arise low in yield and intracellular, making extraction and purification of enzyme difficult. Thus, there is an urgent need for rapid screening of microbes which produce this enzyme extracellularly and in high yields. The earlier phenol red plate assay method for screening of extracellular L-asparaginase-producing microbes was reported by us (Gulati *et al.*, 1997). In the present investigation an improved, sensitive, and contrasting modified rapid plate assay method for the screening of extracellular L-asparaginase-producing microbes using bromothymol blue (BTB) is reported.

Materials and methods

Chemicals

Nessler's reagent was purchased from Fluka (Buchs, Switzerland). L-Asparagine, BTB and phenol red were purchased from Spectrochem (India). Other chemicals used were of analytical grade from Hi-Media (India).

Culture preparation

The microorganisms employed in the present study were soil isolates identified on the basis of 16S RNA gene and gene sequence analysis (Midilabs) and were maintained on nutrient agar (NA) and potato dextrose medium

(PDA), respectively. The cultures of (1) *Bacillus licheniformis* and *Bacillus subtilis*; and (2) *Aspergillus niger* were used as test organisms.

Test plate preparation

Modified Czapek Dox medium (Gulati *et al.*, 1997) was prepared, containing: 6 g L⁻¹ Na₂HPO₄; 2 g L⁻¹ KH₂PO₄; 0.5 g L⁻¹ NaCl; 20 g L⁻¹ L-asparagine; 2 g L⁻¹ glycerol; 0.2 g L⁻¹ MgSO₄·7H₂O; 0.005 g L⁻¹ CaCl₂·2H₂O; pH 5.5. This medium was supplemented with 2% agar, 0.009% phenol red and 0.007% BTB, respectively (obtained after optimization of concentration of dye) for preparation of plates. Plates were inoculated with test cultures and incubated at 37 °C for 24 and 72 h, respectively. Uninoculated plates were set as control.

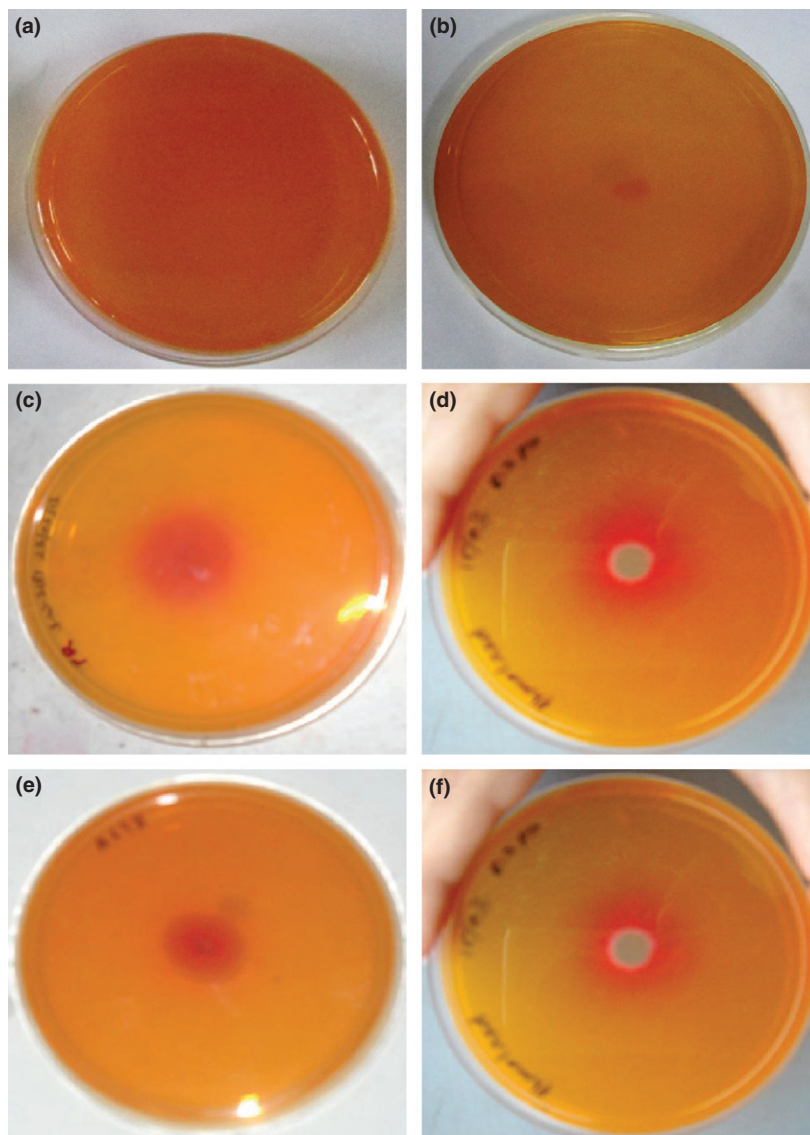


Fig. 1. Detection of L-asparaginase producers on plates supplemented with phenol red dye: (a) uninoculated plates; (b) plate without L-asparagine inoculated with *Bacillus licheniformis*; (c) plate inoculated with *B. licheniformis*; (d) plate inoculated with culture filtrate of *B. licheniformis*; (e) plate inoculated with inoculated with *Aspergillus niger*; (f) plate inoculated with culture filtrate of *A. niger*.

Broth studies

Medium broth supplemented with 0.009% phenol red and 0.007% BTB were inoculated with test cultures and incubated at 37 °C for 18 and 48 h, respectively. Uninoculated broth and the medium without asparagine (substrate) were set as control.

Enzyme titre studies

The modified Czapek Dox broth was used as the production medium for quantitative estimation of L-asparaginase. The samples were withdrawn at interval of every 3 h and checked for enzyme activity as per the nesslerization procedure (Shifrin *et al.*, 1974) using 189 mM L-asparagine as substrate in 50 mM Tris buffer (pH 8.6), reading the absorbance at 436 nm.

Results and discussion

The present study was carried out to improve the plate assay method used for screening of microorganisms using BTB dye instead of phenol red in L-asparagine-containing medium. In the test plate experiments where 0.009% phenol red was used on the plates inoculated with bacterial culture (*B. licheniformis*), its culture filtrate (Fig. 1c and d) and fungal culture (*A. niger*) and its culture filtrate (Fig. 1e and f), it was observed that the intensity and the contrast of the zone was very low. It was difficult to differentiate between the pink (enzyme lysed zone) and orange yellow (unhydrolyzed asparagine) zone on the plates. On the other hand, when 0.007% BTB dye was used in the plates in place of 0.009% phenol red, a sharp, distinct, clear and prominent zone of enzyme hydrolysis (dark blue) was observed in the inoculated plates with

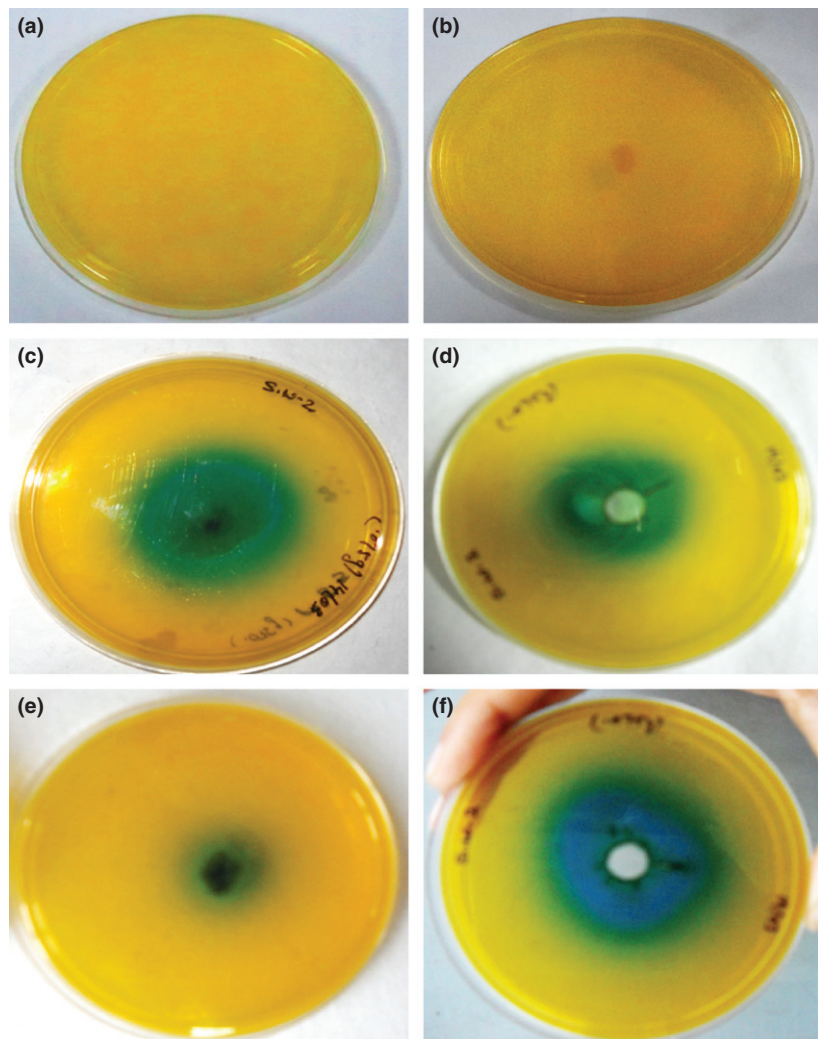


Fig. 2. Detection of L-asparaginase producers on plates supplemented with BTB dye: (a) uninoculated plates; (b) plate without L-asparagine inoculated with *Bacillus licheniformis*; (c) plate inoculated with *B. licheniformis*; (d) plate inoculated with culture filtrate of *B. licheniformis*; (e) plate inoculated with inoculated with *Aspergillus niger*; (f) plate inoculated with culture filtrate of *A. niger*.

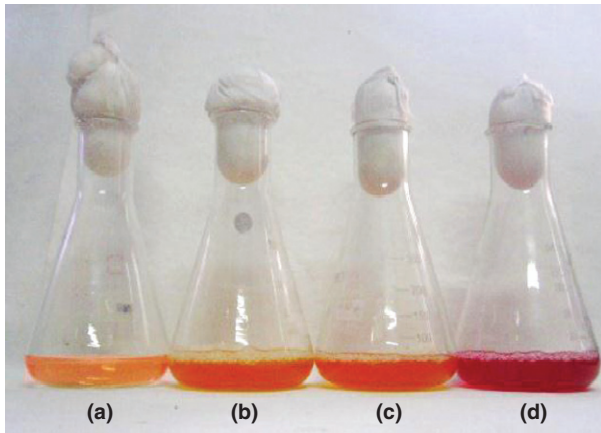


Fig. 3. Effect of phenol red (0.009%) by different L-asparaginase producers after 18 h of incubation: (a) uninoculated medium (pH 5.5); (b) medium without L-asparagine inoculated with *Bacillus licheniformis* (pH 5.7); (c) low asparaginase production from *Bacillus subtilis* (pH 7.26); (d) high asparaginase production from *B. licheniformis* (pH 8.9).

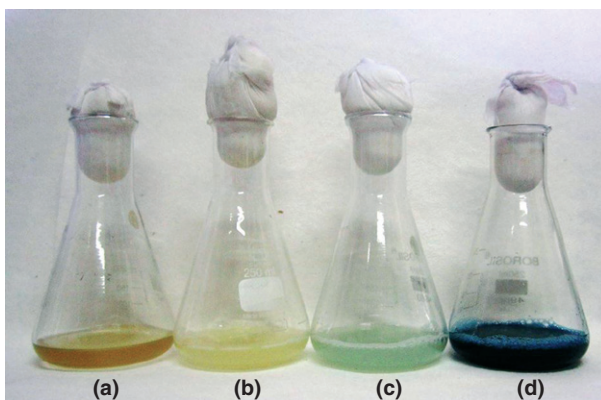


Fig. 4. Transient effect of BTB (0.007%) by different L-asparaginase producers after 18 h of incubation: (a) uninoculated medium (pH 5.5); (b) medium without L-asparagine inoculated with *Bacillus licheniformis* (pH 5.7); (c) low asparaginase production from *Bacillus subtilis* (pH 7.2); (d) high asparaginase production from *B. licheniformis* (pH 8.92).

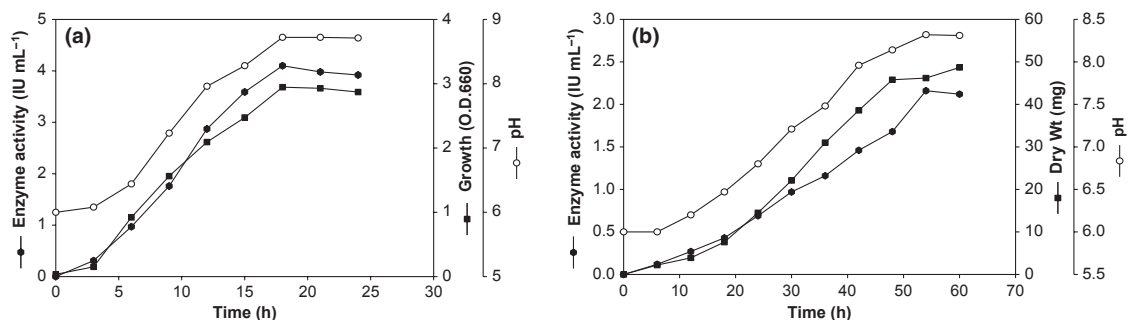


Fig. 5. Profile of enzyme production by the test cultures: (a) *Bacillus licheniformis* (growth presented in terms of OD); (b) *Aspergillus niger* (growth presented in terms of dry weight).

the test microbial cultures (viz. *B. licheniformis* and *A. niger*) and their respective culture filtrates (Fig. 2c–f), while uninoculated plates with both the dyes remained bright yellow in colour (Figs 1a and 2a). The control plates having no L-asparagine also showed prominent colony formation but no change in colour (Figs 1b and 2b).

In test broth experiments (Fig. 3a–d) clearly shows that the transient effect of phenol red dye was not very evident for L-asparaginase production. However, the broth supplemented with BTB dye (Fig. 4a–d) clearly differentiates the low L-asparaginase production (pH 7.2; Fig. 4c) by *B. subtilis* and high L-asparaginase titres (pH above 8.9; Fig. 4d) in the case of *B. licheniformis*. It can also be observed that 0.007% BTB has no adverse effect on the growth of the microorganism [optical density (OD) 3.0; Fig. 5a]. It is also evident from the results that the medium supplemented with dye but with no asparagine showed a good growth of *B. licheniformis* but no change in colour, evidence that change in colour is due solely to L-asparaginase production.

The modified Czapek Dox medium was used for production of L-asparaginase using the test organisms. It can be shown from the production profile presented in Fig. 5a that production began at 4 h in the bacterial cultures and reached a peak of 4.1 IU mL⁻¹ at 18 h with an increase in pH from 5.5 to 8.92 in *B. licheniformis*. In the case of *A. niger*, production started after 12 h of inoculation and reached a peak of 2.3 IU mL⁻¹ in 48 h with an increase in pH from 6 to 8.3 (Fig. 5b). In *B. subtilis* a low enzyme yield of 0.8 IU mL⁻¹ of L-asparaginase was obtained after 18 h of incubation.

L-Asparaginase hydrolyzes the L-asparagine into L-aspartic acid and ammonia. Release of ammonia leads to an increase in the pH of the production medium (Mahajan *et al.*, 2012). The dye BTB is yellow at acidic pH and turns blue at alkaline pH. Due to the formation of ammonia, the pH of the culture filtrate becomes alkaline and the dye changes its colour to blue. Both the assays devised for screening use the same principle of

incorporating the pH indicator phenol red and BTB in the medium containing L-asparagine. However, the advantage of BTB is that it gives a sharp colour contrast (dark blue and bright yellow) between the hydrolyzed and unhydrolyzed asparagine as compared with the relatively less contrasting zone indicated by phenol red (Gulati *et al.*, 1997). The BTB assay has an advantage over the phenol red assay as it can give a marked dark green colour at neutral pH (when the enzyme titres are low) as compared with the dark blue colour at alkaline pH (Fig. 4).

In conclusion, the use of BTB remarkably increases the clarity of the zone of hydrolysis, making the process, easy, rapid and effective for the screening of large numbers of microorganisms producing L-asparaginase.

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