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A simple method for the detection of protease activity on agar plates using bromocresolgreen dye

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Abstract

Investigations were conducted to determine the proteolytic activity using bromocresol green dye on substrate agar plates. Two methods were highlighted in this article. The first method was adopted to determine the proteolytic activity by flooding bromocresol green reagent on casein/skimmed milk agar plates. Later, a minimum of 0.0015% of bromocresol green dye was incorporated with the substrate agar plates before autoclaving to detect the proteolytic activity of bacteria. The proteolytic activity appeared as a colourless zone, while the rest of the plates were greenish-blue in colour which was pH dependent. This method was easy to perform and cheap.

Keywords: Bromocresol green, casein agar plates, protease screening.

Introduction

Microbial proteases are one of the important groups of industrially and commercially produced enzymes and the initial screening methods for protease detection are of utmost importance (Kasana et al. 2011). Enzyme substrates have been incorporated in solid culture agar media for protease screening (Di Salvo 1958; Smith and Goodner 1958; Sokol et al. 1979). Many microorganisms were widely screened by using a number of substrates including, skimmed milk agar (Zerdani et al. 2004) and casein-agar (Garriga et al. 1996) for protease secretion. Apart from these direct observations on the substrate agar plates, developing agents such as Trichloro acetic acid (TCA) (Medina and Baresi 2007) and tannic acid (10%) (Saran et al. 2007) were applied for protease activity determination. The hydrolysis zone produced on the casein agar could be related to the amount of protease produced by the organism (Vermelho et al. 1996). In bacteria, serine- and metalloproteases are the principal classes of proteases found in several species such as *Bacillus subtilis*, *B. amyloliquefaciens*, *Pseudomonas* sp., *Lysobacter enzymogenes* and *Escherichia coli* (Fujishige et al. 1992). Identification and characterization of microbial proteases are prerequisites for understanding their role in the pathogenesis of infectious diseases as well as to improve their application in biotechnology. For this aim, rapid and sensitive techniques for the detection and characterization of microbial proteases are highly desirable (Lantz and Ciborowski 1994). In the present study, an attempt was made to use bromocresol green (BCG) to determine proteolytic activity on casein agar plates and used as a developing agent to determine protease activity by well diffusion method. This method may have widespread applications in protease screening, especially alkaline protease.

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Materials and Methods

Bromocresol green (BCG) reagent: composition and concentrations

BCG reagent was prepared by dissolving 0.56% (w/v) succinic acid, 0.1% (w/v) NaOH and 0.028% (w/v) BCG dye. To this reagent, 1% Brij-35 (polyoxyethylene lauryl ether) was added. The pH of the solution was adjusted to 4.15±0.01. This reagent was stored in brown bottle at 2-8°C until further use.

Culture of Bacillus subtilis strain VV *for protease production*

The protease secreting organism (*Bacillus subtilis* strain VV) was isolated from the river (Vijayaraghavan et al. 2012) and was used for protease screening. A loopful culture of *Bacillus subtilis* strain VV was inoculated into a 50 ml nutrient broth (g/L): peptic digest of animal tissue, 5; beef extract, 1.5; yeast extract, 1.5, Sodium Chloride, 5, pH 8.0. The culture was incubated for 72 h at 37°C under shaking condition (150 rpm). The crude enzyme was obtained by centrifugation at 10000 rpm, at 4 °C for 10 min.

Preparation of crude protease extract from the lobster, Panulirus homarus

The lobster, *Panulirus homarus* was collected from the fish landing centres of Kanyakumari, South-Western coast of India. Live lobsters were taken to the laboratory for enzyme assay. The mid-gut gland, hepatopancreas was dissected out and ground separately (50 mM, pH 8.0) using a pestle and mortar. The sample was centrifuged for

10 min at 10000 *g* at 4°C. The resultant supernatant was separated and stored in 0.5 ml vials at -20°C. Suitable dilutions were made before performing enzyme screening (Vijayaraghavan et al. 2011)

Determination of protease activity by well diffusion method

Agar was prepared along with 1% (w/v) casein, skimmed milk, gelatin and poured in petri dishes. The plates were solidified for 30 min and holes (3 mm diameter) were punched. A crude culture supernatant from *Bacillus subtilis* strain VV/ lobster hepatopancrease homogenate extracted from the lobster, *Panulirus homarus* was loaded into the holes. These plates were incubated for overnight at 37°C and photographed (control). To these plates, BCG reagent was flooded and incubated for 20-30 min at room temperature (25–30°C) (experimental plate).

Screening of proteolytic enzyme secreting organism on BCG-Agar plates

For protease screening, the bacterial isolate (*Bacillus subtilis* strain VV) was streaked on casein agar medium (g/l) (peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5.0, agar, 15, casein, 10 and 0. 0015% (w/v) BCG) and incubated at 37°C for 48 h. A zone of proteolysis was detected on the casein agar plates.

Results and Discussion

Casein or skimmed milk agar plate assays allow principally for qualitative determinations of protease activity. The hydrolysis zone produced on the casein agar could be related to the amount of protease produced by the fungus (Vermelho et al. 1996). But some exceptions have been reported, such as the protease produced by *Bacillus licheniformis,* which produces very narrow zones of hydrolysis on casein agar plates inspite of large enzyme production by submerged culture (Aunstrup, 1974). Although protease activity could be readily observed easily, in some cases, without the use of a developing agent, it was very difficult to detect and photograph the narrow zone. BCG dye was effective in determining the narrow zone, and this method is very simple.

In the present study, the clear distinct zone was observed after the addition of BCG reagent on the casein agar plate (Fig. 1b). The zone was distinct and the surrounding was greenish-blue in colour, but the colour of the plates strongly depended on the pH value of the agar medium. The zone was not clear in the control (Fig. 1a). In this study, the pH of the culture medium was maintained as 8.0±0.2, so the plate appeared as blue in colour. Acid media showed yellow-green colouration after the addition of BCG reagent (Figure not shown). BCG reagent sharply increases the colour intensity of the plate, as it binds on unhydrolyzed protein in the plate.

The bacterium, *Bacillus subtilis* strain VV grown on BCG-casein agar plates. These plates were incubated at 37ºC for 48 h for its growth and secretion of protease. BCG did not inhibit the growth and secretion of proteases.

A zone of proteolysis was detected on the casein/skimmed milk agar plates. The proteolytic activity was determined as the clear zone where as the rest of the plates were greenish-blue in colour (Fig. 2). BCG staining is simple and easy to perform. Importantly, this reagent was stable for a period of 1 year when stored between 2-8°C (Thomas 1998).

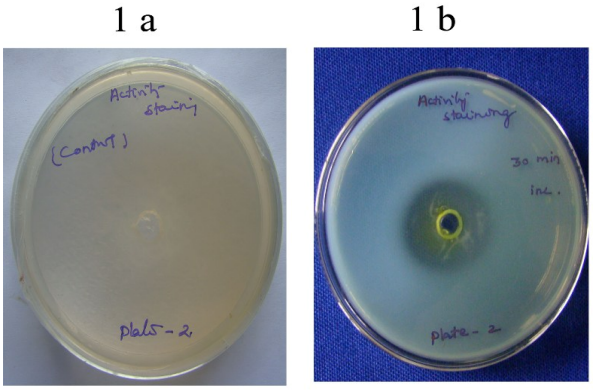

Figure 1: Degradation of the enzyme substrate (casein), incorporated in the agar plate, was seen as the develop- ement of a transparent zone in the plate. (a). Before flooding the BCG reagent on casein agar plate. (b).Casein agar plate flooded with BCG reagent and incubated for 20-30 min.

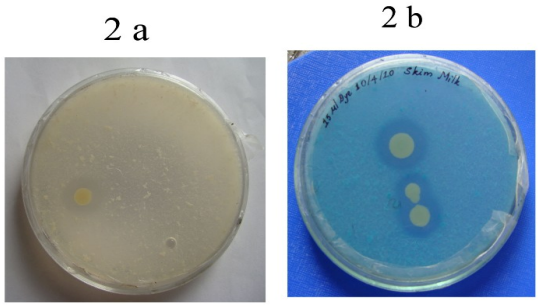

Figure 2: Degradation of the enzyme substrate (casein) by protease secreted by *Bacillus subtilis* was seen as the development of a transparent zone. (a). Control casein agar plate (without BCG dye). (b). Casein agar plate incorporated with BCG dye (0.0015%) and incubated the plates at 37°C for 24 h. A clear zone was formed in the experimental plate.

Conclusion

In conclusion, very few reports have been published to screen proteases. The results indicate that BCG dye is useful to detect extracellular protease produced by bacteria, fungi and other organism. The BCG reagent detects narrow zone on casein agar plates. The technique provides a sensitive, convenient, and inexpensive method for screening various microbial proteases.

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