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A Comparative Study on Screening Methods for the Detection of Protease Activity Containing Bacteria

Niranjana J[1], Bavithra P.S[1]

Dr. N.G.P. Arts and Science College (Autonomous), Coimbatore, Tamil Nadu, India

Abstract: ***Investigations were carried out to detect the proteolytic activity of the bacteria inoculated on substrate agar plates using different screening methods. This article focuses on the three methods for screening protease activity. The first method involves the flooding of bromocresol green reagent (BCG) on casein milk agar plates. Second, a minimum of 0.0015% of BCG reagent was incorporated with casein milk agar plates and third, gelatin clearing zone plates flooded with mercuric chloride solution. As an outcome, the plates screened with BCG reagent showed clear zone with green-blue background was effective in determining protease activity containing bacteria when comparing to the plates that were colorless.***

Keywords: Protease screening, Bromocresol green reagent, Casein milk agar, Gelatin agar plates

1. Introduction

Microbes are known to play a vital role in the production of intracellular and extracellular enzymes on an industrial scale. For any reactions, enzymes act as the catalyst and it is essential for life. Among these enzymes, Microbial proteases are among the most important hydrolytic enzymes and have been considered extensively since the advent of enzymology[1]. It consists of larger industrial applications in clean, efficient, environment friendly and cost effective biotechnological processes making it as commercial enzymes. Protease is used in textile industries for removing stiff and dull gum layer of sericin from the raw silk fiber leading to its brightness and softness. Also proteolytic enzymes support the natural healing process by removing necrotic material in skin ulceration and it is an important component of biopharmaceutical products such as enzymatic debriders and contact lens cleaners. The protease producing microbes are been isolated from various natural resources where *Bacillus* species are obligate aerobes or facultative anaerobes and include both free-living and pathogenic species and they act as the main producers of extra-cellular proteases. Therefore, industrial sectors frequently use *Bacillus subtilis* for the production of various enzymes.

For initial screening of protease producing microbes, enzyme substrates have been incorporated in solid culture agar[2]. Many microbes are widely used for screening of protease by using different substrates such as skimmed milk agar, casein-agar and gelatin-agar[3]. Apart from these direct screening plate methods, some developing agents are also used including Trichloro acetic acid (TCA)[4], tannic acid (10%)[5] in determining protease activity. The hydrolysis zone produced on the substrate – agar plates can be related to the amount of protease produced by the organism.

Protease being an essential enzyme, proper screening of protease activity containing bacteria is important. The present study is an attempt to determine the effective screening method for the detection of protease producing bacteria by comparing three different screening methods.

2. Materials and methods

2.1 Sample source

The soil sample for this study was collected from domestic-waste dumped region of Ganapathy, Coimbatore, India. About 10g of soil was obtained from the depth of 2-5cm and stored in sterile polythene bag until further studies.

2.2 Bacterial culture preparation for protease production

Bacterial colonies for the production of protease enzyme was isolated from the soil sample using serial dilution method and were inoculated on nutrient agar medium with an incubation period of 24 hours at 37°C (spread plate method). The grown distinct colonies of interest were inoculated in nutrient broth, pH 8.0. The culture was incubated for 48 hours at 37°C under shaking condition.

2.3 Bromocresol green (BCG) reagent

BCG reagent was prepared by dissolving 0.56% (w/v) succinic acid, 0.028% (w/v) BCG dye and 0.1% (w/v) NaOH. 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.

3. Protease Screening Methodology

3.1 Casein agar plate flooded with BCG (well diffusion method)

Agar was prepared with 1% casein (w/v) and poured into Petri dishes (control and experimental). The plates were solidified and holes of 3mm diameter were punched. Bacterial culture filtrate was dispensed aseptically into holes. These plates were incubated overnight at 37°C. To the experimental plate, BCG reagent was flooded and incubated for 20-30 minutes at room temperature.

3.2 Casein- BCG agar plates

The bacterial isolate was streaked on casein agar medium (g/L) containing peptone (5.0); beef extract (1.5); yeast

extract (1.5); sodium chloride (5.0); agar (15); casein (10); and 0.0015% (w/v) BCG dye and incubated at 37°C for 48 hours. The zone of proteolysis was observed on casein-BCG agar plates.

3.3 Gelatin-agar clear zone method

The isolates for protease screening were streaked on gelatin agar plates containing (g/L) glucose (1.0); peptone (5.0); 1% gelatin (15.0); agar (15); K_2HPO_4 (2.0). The plates were incubated for 24 hours at 37°C and flooded with 15% of $HgCl_2$ prepared in HCL solution (15g $HgCl_2$ and 20 ml 4N HCL). This is referred as gelatin clear zone method[6].

4. Results and Discussion

Casein or skimmed milk agar and gelatin agar plate assays are commonly used for primary screening protease secreting microorganisms. The proteolytic zone formed on the substrate –agar plate could be related to the amount of protease produced by the bacteria or fungus[7]. However it is known that certain protease producing microorganisms such as *Bacillus licheniformis* showing proteolytic activity are negletected due to its narrow zones of hydrolysis on casein agar plates inspite of its efficacy to produce larger quantity of enzyme by submerged culture [8]. Protease being an important industrial enzyme, it is necessary to have an efficient screening method to detect proteolytic activity containing bacteria to support the production of enzyme in large-scale. Even though, proteolytic activity can be readily observed, in certain cases, it is difficult to detect and retrieve the colonies.

In the present study, on comparing with the three screening method for detecting proteolytic activity containing bacterial isolates, the transparent zone photograph the narrow proteolytic zones without any developing agent[9].was prominent on Casein-BCG agar plate (Fig. 2a) in which BCG dye was in-corporated with the Casein.

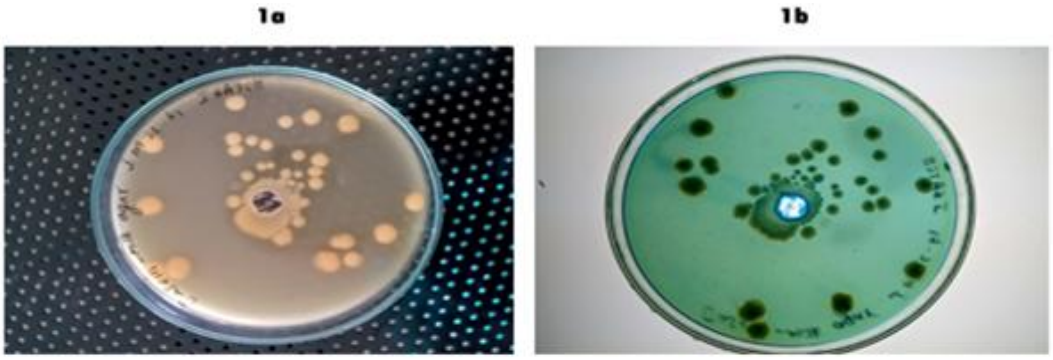

Figure 1: Casein agar plate flooded with BCG (well diffusion method) - Proteolytic activity of the enzyme secreted by bacteria was seen as transparent zone showing the degradation of the enzyme substrate (casein). (a) Control plate before flooding with BCG reagent (b) Experimental plate after flooding with BCG reagent ,incubated for 20-30 minutes

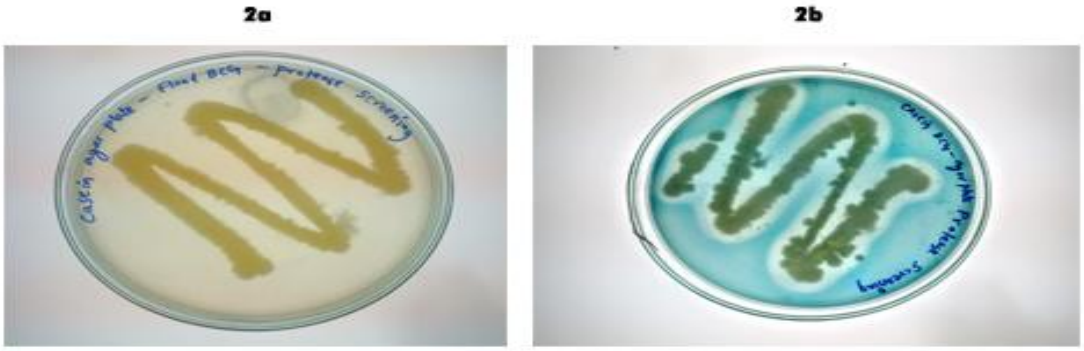

Figure 2: Casein- BCG agar plate- Proteolytic activity of enzyme secreted by bacteria was seen as a development of clear zone showing the degradation of enzyme substrate (casein). (a) Control casein agar plate (without BCG dye) (b) Casein agar plate with BCG dye incubated for 24 hours at room temperature . The clear zone was prominent in Casein-BCG agar plate.

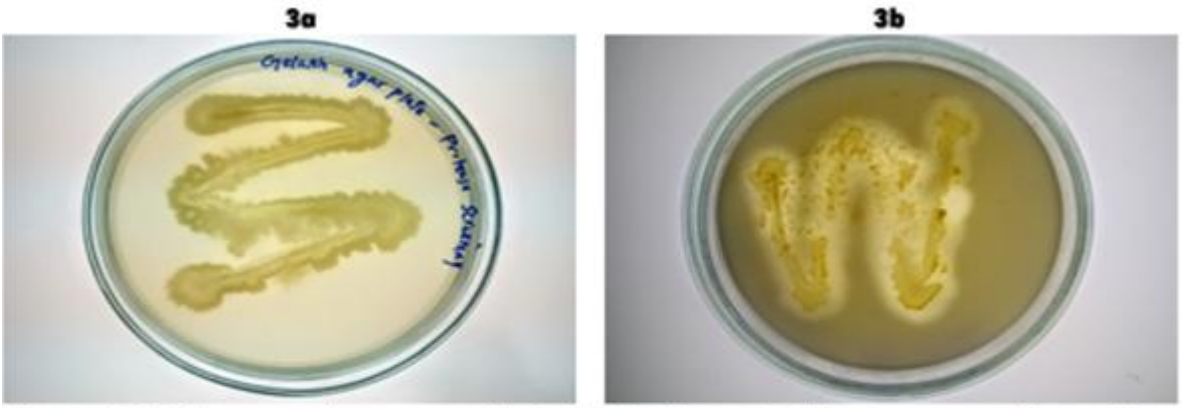

Figure 3: Gelatin - agar clear zone method- The development of clear zone was observed by flooding with 15% of $HgCl_2$ solution showing proteolytic activity of bacterial isolate on gelatin-agar plate. (a) gelatin-agar plate before flooding with 15% $HgCl_2$ solution (b) gelatin-agar plate after flooding with $HgCl_2$ solution.

The casein-agar plate incorporated with BCG shown clear zone with blue background (Fig. 2a). The plate flooded with BCG reagent showing zone with greenish-blue background strongly depends on agar medium (Fig. 1b). In this study, the pH of culture medium was maintained as alkali with the range of 8.0±0.4 and this lead to greenish-blue appearance of the medium. It is known that acid media showed yellow-greenish coloration after the addition of BCG reagent[9] and this reagent was stale for a period of one year when stored between 2-8°C[10].

The zone of proteolytic activity was detected on gelatin-agar plate after flooding it with 15% $HgCl_2$ solution. However, the zone was not prominent and lead to colony distortion. Also the isolate from $HgCl_2$ flooded plate on nutrient agar medium with incubation period of 24 hours at 37°C shown suppressed growth of the colony when compared to the isolate from casein-BCG agar plate (Figure not shown).

5. Conclusion

The result indicates that BCG dye is useful to detect the proteolytic activity of micro-organisms exhibiting protease production. It shows prominent zone that clearly aids the selection of the protease producing isolates. The casein agar incorporated with BCG dye provides sensitive, inexpensive and convenient method for screening various microbial proteases.

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