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Detection of Chitinase on Chitin Agar Plates

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Abstract: ***Chitin polymer is the second most abundant polysaccharide on earth after cellulose. The linear insoluble structure of chitin is difficult to be degraded in nature. Hydrolysis of chitin by chemicals results in the production of various non-specific products and has been proved unsafe for the environment. Chitinase is the enzyme which hydrolyses chitin polymer into its oligosaccharides and then to its monomeric form i.e. into N-Acetyl glucosamine. Chitinase have received attention due to its applications in the medicine, agriculture, waste management and industries. When chitinase producing bacteria are isolated from various sources, there is a need to detect their chitinase activity by some convenient and efficient methods so that large number of isolates can be screened. In the present study, chitinase production by different bacterial isolates was detected by flooding the inoculated chitin agar plates with four different stains.***

Keywords: Congo red, cotton blue, Gram's iodine, safranin, analysis.

1. Introduction

Chitin is the linear polymer of N-acetyl-D-glucosamine units joined by β-1, 4-glycosidic linkage. It is the second most abundant polysaccharide after cellulose. It forms the structural unit of many fungi, insects, aquatic organisms (such as shrimps, crabs, lobsters). Due to its insolubility, it gets accumulated in nature which may lead to major environmental problems. Its degradation by chemical treatment is very expensive and also leads to the formation of non-specific products which are toxic in nature. However, it can be degraded biologically by using microbial enzymes such as chitinase. Chitinases are glycosyl hydrolases which catalyze the degradation of chitin polymer by breaking the glycosidic bonds into N-acetyl-D-glucosamine units (GlcNAc). Chitinases are classified into two categories based on their mode of action: (1) Endo-chitinases- cleave randomly at internal sites in polymer of chitin generating low molecular weight oligomers (2) Exo-chitinases- cleaves at the external sites giving rise to diacetylchitobiose and N-acetyl glucosamine (GlcNAc) (Shahidi and Abuzaytoun, 2005). Several chitinolytic microorganisms such as bacteria and fungi produce extracellular chitinases and are able to utilize chitin as carbon source. Various bacterial genera like *Streptomyces*, *Serratia*, *Bacillus*, *Aeromonas*, *Vibrio* and *Enterobacter* have been known to produce chitinase (Cody, 1989; Mowlah et al., 1979). Chitinases are attaining prominence in the field of waste management, pest control in agriculture and human health care etc. N-Acetyl glucosamine, produced upon degradation of chitin polymer, is reported to be anti-inflammatory (Aloise et al., 1996).

For detection of chitinase activity many researchers have employed in situ gel methods using fluorescent dyes such as calcofluor white M2R (Trudel and Asselin, 1989), fluorescein isothiocyanate, rhodamine B (Gohel et al., 2005), Coomassie Brilliant Blue G 250 (Liau and Lin, 2008) and observed clear zones against florescent background under UV transilluminator. However these florescent methods hold some drawbacks like bleaching, phototoxicity, mutagenicity (Massaro et al., 1989), hindrance in mobility of enzymes (Gohel et al., 2005). Moreover, these florescent methods are intricate; require extensive labor and instrumentation, so not very convenient to be used for screening of chitinolytic bacteria. A comparatively easy method is the use of chitin agar plates which is also employed by many workers. In the present study, different stains are used to detect the chitinase production by the bacterial isolates on chitin agar plates.

2. Materials and Methods

2.1 Isolation and screening of chitinase producing bacteria

Various samples of soil, garbage and fecal matter were inoculated and incubated at 37ºC for 24-48 hrs on chitin agar plates. Based on their morphology, the discrete colonies which appeared on the plates were named as bacterial isolates NK-1, NK-2, NK-3 & so on, and maintained for further study.

2.2 Detection of chitinase production

All the isolates were analyzed for chitinase production both qualitatively using plate assay method and quantitatively using DNS method.

2.2.1. Plate assay method

All the bacterial isolates were streaked singly on chitin agar plates containing finely grounded 0.1% chitin from shrimps (HiMedia) as sole carbon source. All the plates were incubated at 37ºC for 3 days. After incubation, the plates were observed for the presence of zone of clearance around the streak of growth. The isolates NK-7, NK-8, NK-11and NK-14 showing chitinolytic zones were further tested for the best chitinase producer using different stains to make the zone more clear. All these four chitinolytic isolates were further spotted on the chitin agar plates and after incubation flooded with different staining solutions.

Staining of plates

Aqueous solution of three stains viz. 0.1% Congo red, 0.1 % Cotton blue, Gram's iodine and safranin were prepared. Safranin, the fourth stain was prepared by dissolving 2.5 g safranin in 10 ml of 95% ethanol and then adding to 100 ml distilled water. After incubation, the bacterial isolates on chitin agar plates were flooded with these stains and allowed to stand for 20-30 minutes with gentle shaking. Plates stained with Gram's iodine were washed off with distilled water thrice, whereas plates flooded with Congo red, cotton blue and safranin were washed off thrice with 1.0 M NaCl solution for 15-20 minutes. Then, all the plates were observed for clear zone formation. Extracellular enzyme activity on the chitin agar plate was measured by the method given by Bradneret al(1999). In each plate, the diameter of the colony and the total diameter of zone of clearance were measured in two dimensions at 90° to each other and the average values were noted. The index of relative enzyme activity value of 1.0 or greater were classified as having significant enzyme activity (Duncan et al., 2008) and isolates with such activity were taken as chitinase positive. The index of relative enzyme activity (RA) was calculated by:

$$RA = \frac{\text{Total diameter of clear zone – diameter of colony}}{\text{Diameter of colony}}$$

2.2.2. Quantitative assay method

For quantitative analysis, chitinase enzyme was produced in broth and assayed by DNS method.

2.2.2.1. Production of enzyme: All the four bacterial isolates were inoculated in minimal salt broth containing 1% colloidal chitin and incubated at 120 rpm in a rotary shaker at 37°C for 3 days. After incubation the cultures were harvested, centrifuged at 10000 rpm for 10 min at 4˚C and the supernatant was used as crude enzyme.

2.2.2.2. Enzyme assay: The chitinase activity of crude enzyme was assayed by DNS method (Monreal and Reese, 1969) which measures the amount of reducing sugar released from colloidal chitin substrate. The absorbance of the standard and the samples reaction mixture was measured at 540 nm. One unit of the chitinase activity is defined as the amount of enzyme which yields 1 µmol of reducing sugar as N-acetyl-D-glucosamine (GlcNAc) equivalent per minute.

3. Results and Discussion

Chitinases are a class of enzymes which degrade chitin and its derivatives. Colloidal chitin prepared by treatment with acids is easily degraded by microorganisms like bacteria, fungi and is used as selective medium to detect chitinase production (Roberts and Selitrennikoff, 1988). Upon utilization of chitin as substrate, a clear zone was developed around the growth of bacteria by hydrolytic action of the chitinase enzyme produced by the microorganism but the zone was small and not very clear for screening of the best chitinase positive isolates. So, in the present study, four stains viz. Congo red, cotton blue, Gram's iodine and safranin were used to detect the clear zone for the screening of chitinase producing bacteria on chitin agar plates.

The results of different staining dyes were diverse than expected in each plate (Fig.1). The mechanism of binding and the efficiency of each dye to produce hydrolytic region are different. It was observed that the zone around the colony produced by Congo red and cotton blue is unstable and drives off the stain after sometime on chitin agar plates while zone formation by Gram's iodine is relatively stable and persists for longer duration. Moreover, the duration for the development of clear zone by Gram's iodine was less than 5 minutes whereas other stains took about 50-60 minutes. Among all the stains used Gram's iodine showed the best, most clear and largest zone of clearance (Fig.1c), followed by Congo red (Fig.1a). Safranin did not produce clear zones but it showed light colored zone with less intensity of color around colonies of isolates NK-7, NK-8 and NK-11 (Fig.1d). Similar results with safranin were reported by Gohel et al (2014) for the determination of extracellular cellulase activity on carboxy methyl cellulose.

Cotton blue which is tried in this study has not been previously used for chitinase detection because it is used for staining of fungi due to its property to stain chitinous fungal cell wall. Cotton blue showed very peculiar type of zone of hydrolysis consisting of inner clear zone and outer dark corona of high intensity with cotton blue diffused in agar medium in the hydrolyzed area around the colonies and the total zone formed was comparable to that of Gram's iodine (Fig.1b). Hydrolysis of the polysaccharides results in loose binding of the stain to the agar which results in the formation of clear zone with less intensity indicating chitinase activity.

Different isolates showed different degree of degradation. The degree of degradation by the enzyme depends on the binding efficiency of the stain with the polysaccharides but not mono or disaccharides (Kasana et al., 2008). The zone of clearance produced by chitinase is due to the hydrolysis of chitin polymer into monosaccharides and disaccharides to which these stains could not bind. Since cotton blue is a stain for chitin, the dark outer zone of blue color might be due to chitin oligomers produced by endochitinase and inner zone due to production of monomeric or dimeric forms of chitin by exochitinase.

Among all the dyes used, the development of clear zone on chitin agar plates showed that Gram's iodine and cotton blue were most efficient stains and also produced similar index of relative enzyme activity as compared to Congo red and safranin (Table 1).

Also, Congo red is widely accepted method for observing clear zones produced by hydrolytic enzymes but it is a benzidine-based azo dye which is known to be toxic and carcinogenic (Tanaka et al., 1981). Although, cotton blue is showing the same index but it includes two types of zones as compared to Gram's iodine.

Table 1: Index of relative enzyme activity on chitin agar plate

Sr. no.	*Stains*	*NK-7*	*NK-8*	*NK-11*	*NK-14*
1.	Congo red	2.0	0.4	1.8	1.0
2.	Cotton blue	2.9	0.7	1.5	1.5
3.	Gram's Iodine	2.9	0.5	1.6	1.5
4.	Safranin	1.4	0.6	1.2	0.0

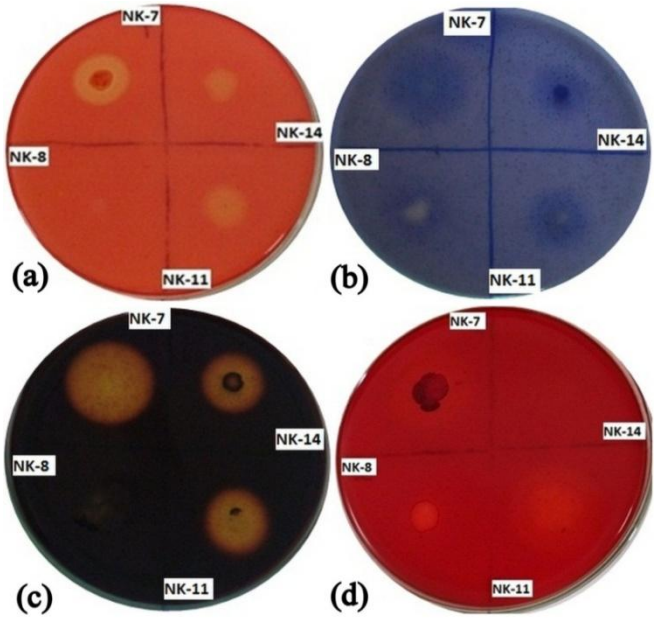

Figure 1: Clear zone by different bacterial isolates NK-7, NK-8, NK-11 and NK-14 on chitin agar plates stained with (a) Congo red (b) Cotton blue (c) Gram's Iodine (d) Safranin.

3.2 Quantitative method

The chitinase activity of the selected bacterial isolates was determined by the DNS method. The chitinase enzyme activity (units/ml) of the bacterial isolates is given in the table 2. Bacterial isolates NK-7 showing largest zone of clearance and maximum index of relative enzyme activity was also found to have maximum chitinase enzyme activity (99.38 U/ml) whereas NK-8 which has minimum index of relative enzyme activity also showed very low chitinase activity (09.06 U/ml). Thus, it is clear that the index of relative enzyme activity observed in the bacterial isolates from chitin agar plate method is corresponding to the chitinase enzyme activity based on quantitative DNS method.

Table 2: Chitinase activity of the selected bacterial isolates

Sr. no.	Bacterial isolates	Chitinase activity (U/ml)
1.	NK-7	99.38
2.	NK-8	09.06
3.	NK-11	77.10
4.	NK-14	66.18

4. Conclusion

Flooding the chitin agar plates with Gram's iodine presented the best results with visibly distinct, stable and clear zone of hydrolysis around the chitinase positive colonies in less than 5 minutes with the production of stable dark brown coloration in the non-hydrolyzed part of the medium. Also, Gram's iodine is non-toxic to the living cells (Kasana et al., 2008). Apparently, screening based on Gram's iodine staining is rapid and efficient qualitative method to determine the chitinase activity. Also, cotton blue which has not been reported earlier could be used efficiently for chitinase detection as one of the staining procedures. However, reasoning of two types of zones produced by cotton blue can give way to more efficient & meaningful use of this stain. The stains used in this study are common in microbiological labs so they offer a convenient method for detection of chitinase activity.

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