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A Rapid Sensitive Detection Method by Plate Assay for Catalase Activity from Bacterium *Acinetobacter calcoaceticus* AV6.

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ABSTRACT

Bacterium exhibiting extra-cellular catalase activity was isolated from lime stone soil. Catalase is an essential component of the cell to cope up with oxidative stress. This study deals with simple detection method by plate assay for catalase activity from bacterium *Acinetobacter calcoaceticus* AV-6. Rapid assay method involves plating the supernatant on the substrate bound agar with the dye. This assay gave result within 60 s, producing a zone around the well. This enzyme was extracted from stationary phase at 36 h. The molecular weight of the protein was determined to be approximately 60 kDa. Sequencing studies were carried out and the strain *Acinetobacter calcoaceticus* AV6 was partially sequenced. The results imply the significance of this detection method in industries.

INTRODUCTION

The enzymes which have been isolated from a broad range of prokaryotic and eukaryotic micro-organisms [1]. Catalases are evolved to decompose hydrogen peroxide efficiently and rapidly to oxygen and water [2]. According to properties of enzymes bacterial catalase has been classified into three categories; heme-containing monofunctional catalases, heme-containing bifunctional catalase-peroxidases, and non-heme-containing catalases [3]. Since 1990's catalases described are tetramers made of subunits each containing protoheme as prosthetic group with molecular masses ranging from kDa 220-270. Multiple catalases have been found in almost all bacterial species, including *Bacillus subtilis* [4] and *Escherichia coli* [5]. There have been several reports regarding catalases from halophiles [6,7], thermophiles [8], and psychrophiles [9,10].

Hydrogen peroxide (H_2O_2) is used commercially in industries like paper, semiconductor, food, textile industries for bleaching and as microbicidal agent due to its powerful oxidant activity. However, due to its toxicity to environment and human health, hydrogen peroxide has to be eliminated from industries after processes. Thus, catalases are utilized in those industrial sectors to eliminate hydrogen peroxide [12].

MATERIALS METHODS

Sample collection and screening

The soil sample was collected from 1 cm depth with help of sterile spatula in a sterile plastic bag from the lime stone area at Kovil patti (longitude of 77.9 and latitude of 9.2.) in Tamil nadu. The collected soil sample was brought to the laboratory and 0.25 g of sample was weighed and suspended in 25 ml of Luria Bertani broth medium and incubated for 1 hour on an orbital shaker at 30 ºC. From this, 0.2 ml sample was spread on Luria agar plates and incubated at 30 ºC for 24 - 48 h [12].

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Multiple cell colonies were tested for catalase activity by adding 0.003% H_2O_2 (Merck) and oxygen generation by visual estimation [13]; highly active colonies were selected and subcultured. The procedure was repeated until a single colony was isolated. The isolated colony (AV-6) was sub cultured in Luria Bertani agar medium at pH 7.2 at 25 °C for 24 h and was further subjected to the enzyme assay. This culture was maintained on Luria Bertani agar slant at 4 ºC for further experiments.

Identification of strain

Identification of strain AV6 was done by 16S ribosomal RNA gene sequencing. A partial DNA fragment of the ribosomal gene was amplified by PCR using the sense and anti-sense primer (forward: 5'-AGA GTT TGA TCC TGG CTC AG-3' reverse: 5'ACG GCT ACC TTG TTA CGA CTT-3') thus partially 900 bp was sequenced and submitted to gene bank. (ACCESSION NO HM130705)

Culture condition

Bacterium was cultivated as described [14] on modified of LB medium containing 1.0 g of Peptone, 0.5 g of Beef extract and 0.5 g of NaCl in 100 ml Distilled water (10 ml preculture in 1000 ml) in 2L Haffkine flasks, on a orbital shaker at 200 rpm at 30 ºC. The culture was harvested at the end of the stationary (36th hours) phase. Then the harvested culture was collected by centrifugation at 12000×*g* for 15 minutes at 4 °C, from which supernatant was collected. Then the collected supernatant was considered as a crude extract for further studies and stored at 4ºC.

Enzyme assay

Enzyme assay was performed using the supernatant. Catalase activity was determined by measuring the absorbance during the conversion of H_2O_2 to oxygen and water [15]. Catalase-catalyzed decomposition of H_2O_2 was monitored by the decrease in the absorbance at 240 nm, utilizing the extinction coefficient of 39.4 $M^{-1}cm^{-1}$. The activity was calculated using a standard curve generated by different concentrations of H_2O_2 monitored at 240 nm. One unit of activity was given as the amount of enzyme that catalysed the consumption of 1 μmol of H_2O_2 per min [16].

Confirmative test for catalase-Plate assay method

Two gram of agar was added to 100 mL of glass distilled water (2%) in a conical flask and boiled until it completely dissolved. To the molten agar 3% of H_2O_2 (30% MERCK) was added. About 20 mL of the agar-H_2O_2 mixture was poured on to Petriplate and kept aside for 10 min. Wells were bored using sterile cork borer and 50 μl of sample was loaded in the well. The plates were incubated at 28 ºC for 6 h aseptically.

Fresh solution (staining solution) containing 2% each of $K_3Fe(CN)_6$ and $FeCl_3.6H_2O$ in 30 ml of sterilized distilled water was prepared. Petri plate containing the sample was flooded with the staining solution. The Petri plate was steadily shaken until a green colour appeared, staining solution drained, rinsed and filled with distilled water [17].

Polyacrylamide Gel Electrophoresis method

Catalase activity staining

Non denaturing discontinuous gel electrophoresis was performed with 10 % polyacrylamide gel without Sodium lauryl sulfate (SDS) and β-mercaptoethanol (18). Protein bands were stained with Coomassie brilliant blueR-250.Catalase bands were visualized by staining using procedure of Woodbury et al. (19) with 10 μl of crude catalase per well. The bands were determined by the procedure of Gregory and Fridovich (20) for catalase activity.
2.6.2 SDS- PAGE electrophoresis

SDS-gel electrophoresis was run as described by Laemmle (21). Polyacrylamide gels containing 10% acrylamide and 0.1% SDS were loaded with 5 to 10μl of crude protein sample which is treated with 5% of 2-mercapto-ethanol and 2% SDS for 10 min at 100°C. Protein staining was performed with Coomassie brilliant blue R-250.

RESULTS

To observe the results for the catalase activity the supernatant, 3 Petri plates were taken one served as control another contained crude sample and third one contained partially purified enzyme sample condensed 50μl of the culture supernatant loaded in the 7mm well respectively. (Fig 1. a, b & c). After 6 hours incubation, 30 ml mixture of 2% of potassium ferric cyanide and ferric chloride was flooded on the incubated plate for few seconds and a zone of 5cm diameter was observed.

While the Native gel was run the same samples were detected based on plate assay (Fig 2. b), SDS PAGE was run to check the presence of protein and stained with Coomassie brilliant blueR-250 (Fig 2 c). Fig 2a is Standard Protein marker Geni from Bangalore 2.5 lμ).

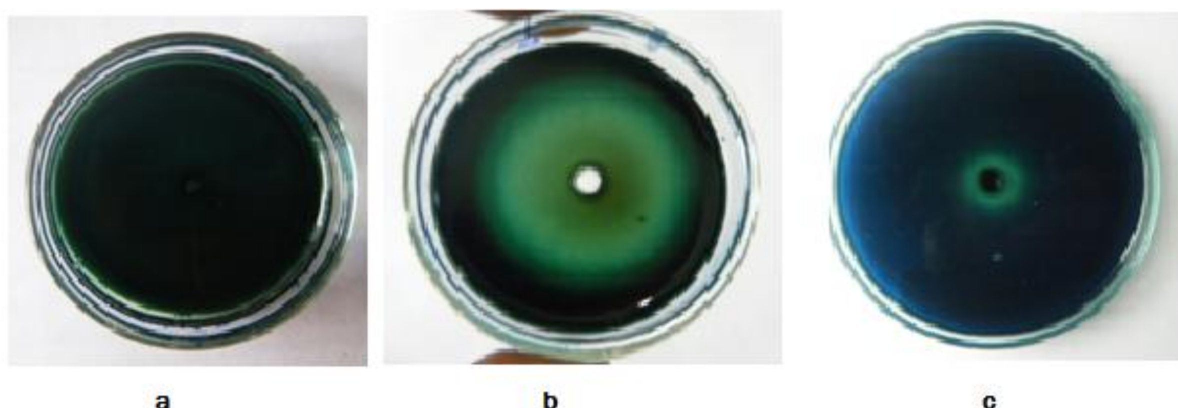

Figure 1: a. Control, b. Zone showing the catalase activity at 50 μl of crude sample, c. Zone showing the catalase activity at 50 μl crude lyophilized sample

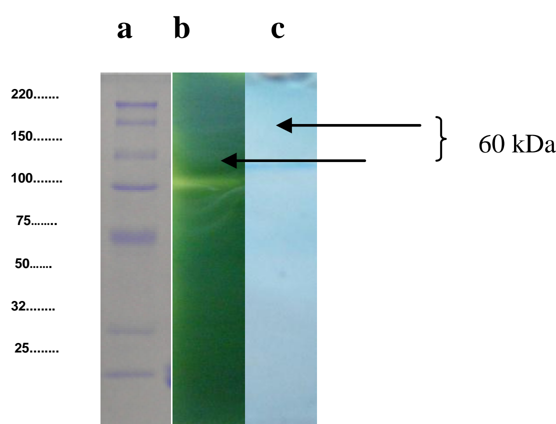

Figure 2: a. Protein standard marker 5 μl from Genie Bangalore, b. Showing the presence of Protein band for catalase on SDS PAGE stained c. Showing the presence of catalase band on Native PAGE stained using Ferric cyanide negative stain

DISCUSSION

This is the first report of a convenient and rapid assay to detect extracellular catalase production by bacteria (*Acinetobacter* sp. AV6 and other bacteria). Although microbial catalase is well studied only a few data of such enzyme from extremophiles have been reported. Moreover the plate assay method has been reported for other enzymes such as proteases (48 h of incubation) [22], lipases (48 to 16 h) [23] and polyurethanases (18 – 20 h) [24].

But our present study showed results of plate assay method for catalase detection (6th h 30 C) .Also I have quantified the catalase using this method (data not given).

The plate assay method is a replica of the native – PAGE staining method [14], which uses 2% potassium ferricyanide and 2% ferric chloride. The Ferric chloride (High pH) binds to the substrate H_2O_2 producing a dark green colour. On the other hand the enzyme breaks down the H_2O_2 to H_2O and O_2, thus the dye is unable to bind to that region around the well and a clearance zone is produced within 1-2 min.

This method also allows the quantification of enzyme activity in the culture supernatant in relation with the diameter of the zone of clearance. Thus this report is an innovative and rapid method for the detection and quantification of extracellular catalase from bacteria.

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This research finding allows screening of multiple organisms at the same time for industrial purposes. This method allows us to visualise and compare the activity of the enzyme of multiple organisms at the same time when compared to the convention effervescence method which has to be done individually to compare activity. It also allows the rapid quantification of the catalase enzyme without the use of conventional Spectrophotometric method and hence is more advantageous and cheap method for detection of the catalase enzyme producing bacteria. This method will be useful in industries which screening for catalase regularly using conventional method.

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