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Research Article

Production of Catalase by Solid State Fermentation Using Different Agro and Fruit Peel Wastes as Substrates

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Abstract
In this present study we used Nutrient agar bound H_2O_2 media for isolation of catalase producing bacteria. This media is a selective for enhancing the growth of microorganisms that degraded H_2O_2 in to H_2O and O_2. For further isolation of pure culture we selected large size colonies and subcultured into Nutrient agar medium. Interestingly Bacillus subtilis MO bacteria produced maximum catalase activity hence chosen for the catalase production in Solid State Production (SSF). Various agro wastes were used as substrate and optimized different parameters such as substrate selection, inoculum percentage, and pH of the medium, incubation time, and effect of moisture content of substrates interestingly. The maximum extracellular catalase activity was observed in yellow lentils husk substrate produced 9.13 (U g dt-1) units per gram of catalase activity in dry fermented substrates with the moisture content of 70%. The molecular weight of the protein was determined to be approximately 65 kDa used with 10% of the gel.

Keywords: Catalase, *Bacillus subtilis*-MO, solid state fermentation, substrate, agro waste.

INTRODUCTION

Catalases (E 1.11.1.6) are metalloenzyme which catalyzes the degradation of hydrogen peroxide (H2O2) into dioxygen and water (Shin et al., 2008). This enzyme was isolated from broad range of prokaryotic and eukaryotic micro organisms (Daniela et al., 2003). Solid state fermentation (SSF) is one of the methods for producing catalase enzyme in submerged fermentation, because of its simple low cost investment and also for maximum yield. (Zubeyde et al., 2003 & Mrudula et al 2010). Solid-state (substrate) fermentation (SSF) has been defined as the fermentation process occurring in the absence or near-absence of free water. In SSF processes, generally a natural raw material as carbon and energy source is being used. SSF may also employ an inert material as solid matrix, which requires supplementing a nutrient solution containing necessary nutrients as well as a carbon source. Several agro crops such as cassava, barley and agro-industrial residues such as wheat bran, rice bran, sugarcane bagasse, cassava bagasse, various oil cakes (e.g. coconut oil cake, palm kernel cake, soybean cake, ground nut oil cake), fruit pulps and peels of apple pomegranate, sweet lime and corn cobs, saw dust, seeds of tamarind, jack fruit, coffee husk and coffee pulp, tea waste, spent brewing grains, were commonly used substrates.

In general microbial enzymes are produced mostly by submerged culture, but solid-state fermentation methods also being used (Chisti Y., 1999). If solid-state fermentation variables are well controlled and the purity of the product is better defined, this technology may be a more competitive process than it is commonly thought. Various bio wastes substrates can be used in solid state fermentation for enzyme production of enzymes (Benazir et al., 2011 & Joseph et al., 2006). The present research was undertaken to optimize process condition for the production catalase by solid state fermentation using various such agro wastes such as- yellow lentils husks, wheat bran, rice bran, roasted gram and different fruit peels.

MATERIALS AND METHODS

Sample collection

Soil sample were collected from 1 cm depth with help of sterile spatula in a sterile plastic bag from Chinnakarai village, Thirupur District Tamil Nadu for isolating catalase producing bacteria.

Screening and isolation of micro-organism

0.25 g of samples were suspended in 25 ml of sterile distilled water and agitated for 1h at 30°C. 0.2 ml of this sample was spread on Nutrient agar plates and incubated at 30°C for 24-48 hours (Chaturvedi *et al.*, 2008). Catalase producing colonies were isolated and subcultured. Further catalase positive colonies results were selected and grown to medium containing ($g^{-}l$) peptone 10.0, sodium chloride 5.0, and beef extract 5.0, agar 20.0 at 48 h. 'MO', showed maximum activity was selected and maintained on slants at 4°C and used for further studies. The culture was examined for the various morphological and biochemical characteristics as per Berge's Manual of determinative Bacteriology.

Substrates

Wheat bran, yellow lentils, black gram, roasted gram, rice bran and some fruit peel like sweet lime, orange, pomegranate, were used as substrates. They were procured from a local market of Chennai, India and were washed three times running tap water, then dried at room temperature to reduce the moisture content and ground to the desired size.

Inoculums Preparation

In order to prepare the inoculum, an isolated colony from a freshly grown slant was transferred into a 100 ml conical flask containing 50 ml of soil bacterial broth media (g^{-l} of distilled water) peptone 8, yeast extract 4, NaCl 2, pH 7.2 and incubated at 30°C in a shaking incubator at 150 rpm for 15 h till it reached an OD value of 1.

Media Preparation

10 gm of agro wastes were suspended in 7.5 ml of minimal media in a 250 ml flask and autoclaved at 15 lbs pressure, 121 ºC for 20 minutes. It was cooled before using.

Solid State Fermentation

Further the medium was inoculated with 5 ml of inoculum. After thorough mixing, all the flasks were incubated at desired temperature in a shaking incubator for 48 hours. After a stipulated period samples were drawn and homogenized using a glass rod. Small amount of sample was taken from each flask for extraction and subsequent analysis.

Enzyme Extraction

The crude enzyme from the fermented material (agro wastes) was extracted by simple extraction method. The fermented substrate was mixed thoroughly with 100 ml of phosphate buffer saline (pH 7.0) and then shaking the mixture in a rotary shaker (180 rpm) at 30°C for 2 hrs, by squeezing through a wet muslin cloth followed by centrifugation at 10,000×*g* for 15 min at 4ºC. The clear supernatant (crude extract) was used for enzyme assay.

Enzyme assay

Catalase activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm caused by the decomposition of H_2O_2 (Paar *et al.*, 2001). The reaction mixture with a total volume of 2 mL composed of suitable enzyme solution, 100mM potassium phosphate buffer (pH 7 .0), and 26 mom H_2O_2. The molar absorption for H_2O_2 at 240 nm was assumed to be 39.4 M^{-1} cm^{-1}. One U of catalase activity was defined as the amount of enzyme required to transform 1mmol of H_2O_2 into water and oxygen per minute U/ml (Paar A, *et al.*, 2001).

Optimization of Medium

Selection of substrate, pH of the medium, incubation time and effect of moisture content of substrate were optimized (Pau and Omar 2011).

SDS PAGE and catalase activity staining

According to Yoon-Suk Kang procedure we performed native PAGE and SDS PAGE analysis for ***B. subtilis*** MO bacterium obtained from SSF process (Yoon *et al.*, 2006). After electrophoresis, the proteins were detected via the staining of the gel with Camassive brilliant blue. Further the Catalase activity was observed on native PAGE gel via visualization of band by double staining of ferric chloride-potassium ferric cyanide[13]. Using this staining method, the gels were initially immersed in a solution of 0.003% H_2O_2.The gel was gently shaken for 10 min before H_2O_2 was removed and the gel was further rinsed with distilled water. A freshly prepared solution of 30ml each of 2% $K_3Fe(CN)_6$ and 2% $FeCl_3.6H_2O$ in distilled water was added to a staining tray and the rinsed gel transferred to the mixture. The gel tray was steadily rocked over a light box until a green colour appeared in the gel. The stain solution was the rapidly removed and replaced with distilled water.

RESULTS AND DISCUSSION

Screening and isolation of potential bacteria strain for catalase production

Six catalase producing strains were selected for this study. These isolates were grown in Nutrient broth at pH 7.0 for 48 hrs and the supernatant were assayed for catalase activity after incubating for 48 hours. It was found that the isolate *Bacillus subtilis* MO was found to have highest enzyme production as compared to other isolates. Thus *Bacillus subtilis* MO was taken for further studies (Fig 1b). The growth of the potent strain in the modified nutrient agar medium (Nutrient agar+ H_2O_2) is shown in Fig 2e. Figure 1(a, b and c)

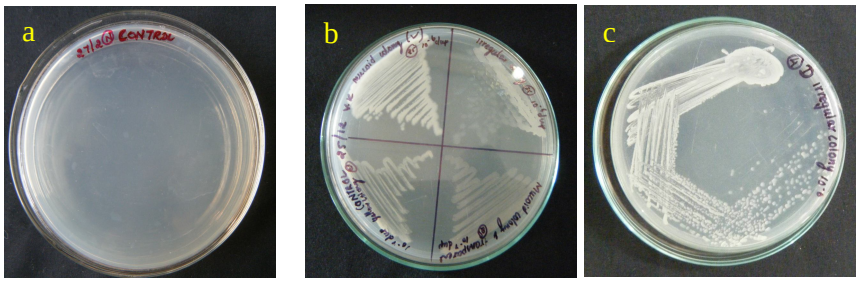

Figure 1: a. Control b. MO, MC, IC & YC c. MO in Nutrient agar medium

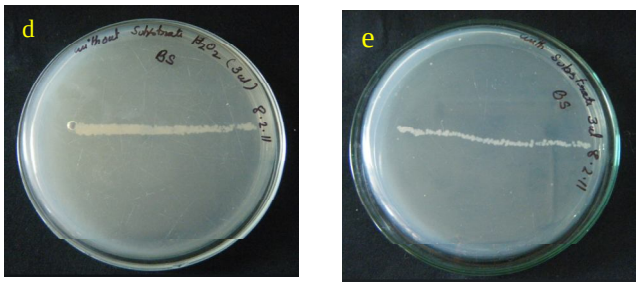

Figure 2: (d & e) *Bacillus subtilis* (MO) without substrate and with substrate (3µl in 100 ml)

Substrate Selection

The maximum catalase activity of 9.13 was observed with Yellow lentils husks. These results were in accordance with observed catalase production from different literatures. Different substrate occupied surface area according to their sizes was an important parameter in solid state fermentation. 10 gram of this substrate yields maximum production of catalase. Due to its easy penetration, the microbial mass of the bacterial culture showed high growth rate with yellow lentils husks (Table 1). The less catalase production at higher level was due to low mass transfer rate and difficulty in penetration of the organism (Rao *et al* 2003). Low production of was observed in roasted gram while sweet lime and pomegranate peels showed no activity (data not shown). Further parameters were checked using yellow lentil as substrate.

S.No	Agro wastes	U g ds^{-1}
1	Roasted gram	8.12
2	Wheat bran	1.01
3	Rice bran	5.07
4	Yellow lentils	9.13
5	Black gram	3.24
6	Orange	3.3

Table 1: Enzyme production in different agro waste/fruit peel waste

Effect of initial moisture content of substrate

Variation in initial moisture content of substrate showed that the enzyme synthesis was related to the availability of moisture. Substrate moisture is a crucial and important factor in SSF for enzyme production. When the initial moisture content was 30 %, catalase yield was 1.24 U g ds^{-1} while the maximum yield 9.13 was at 70% moisture

Moisture Content (%)	Enzyme activity (U g ds^{-1})
10	2.09
20	3.90
30	5.01
60	5.93
70	9.13
100	7.97

Table 2: Effect of different of percentage moisture

Effect of incubation time

Content with 9.13 U g ds^{-1} as listed (Table 2). Higher moisture would lead to decrease porosity, promotes development of stickiness and increases the chances of contamination [7]. The amount of catalase produced was observed from 12 to 76 and the maximum catalase activity was observed at 48 h of fermentation listed (Table 3).

Hours	Enzyme activity (U g ds^{-1})
12	0.69
24	1.19
36	6.66
48	9.13
50	7.11
62	6.16
64	6.01
76	5.90

Table 3: Effect of different of time interval

Effect of pH on enzyme production

pH is one of the most important parameter required for the growth of bacterial culture in respective media. Catalase activity was seen in the basic pH range 6 to 8; this indicates that suitable pH for bacterial growth in the culture media. The data obtained clearly indicates that there is a strong influence of pH on catalase enzyme production. Thus the maximum activity of 6.8 was reported at pH 7 as mentioned in Figure 3).

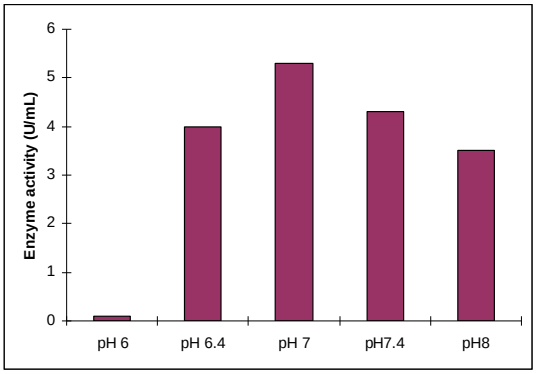

Figure 3: Effect of different pH on enzyme activity.

Effect of percentage of inoculum

Percentage of inoculum plays a vital role in the production of catalase enzyme by SSF. Best results of 0.9 obtained at 2.0 % (Figure 4).

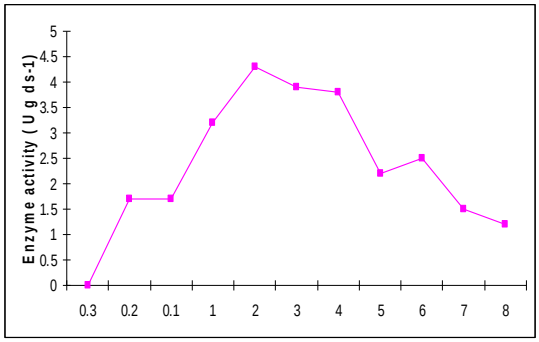

Figure 4: Effect of different percentage inoculum on enzyme activity.

Electrophoresis

Presence of protein was further conformed through SDS PAGE electrophoresis techniques. Interestingly the protein profile was matched with standard marker which is equal to 65 kDa. To our best knowledge this protein is responsible for the catalase producing activity.

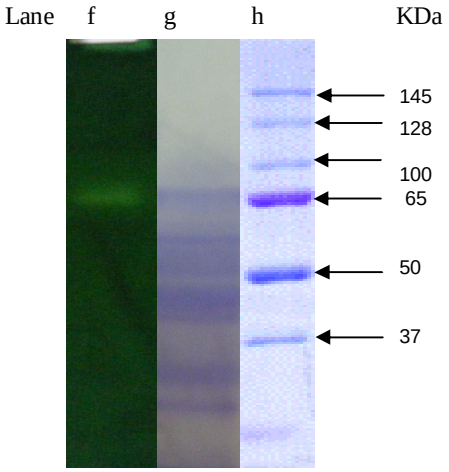

Figure 5. Showing catalase activity of *Bacillus subtilis* in 10 % native PAGE and SDS PAGE with standard marker (5 µl) lane f, g and h respectively

CONCLUSIONS

In this study observed various compositions influenced the enzyme production by the bacteria and it appears that the nature of substrate hard significantly influenced the impact of moisture content and incubation period of over all enzyme yields. Normally physical nature and moisture content of the medium is a critical factor, which supporting the microbial growth in the SSF process. Interestingly maximum catalase activity was observed at 9.13 U/ds-^{1} within 48 hrs in SSF process with the substrate of yellow lentil by using *B.subtilis*-MO bacterium with the moisture content of 70%. Thus this method suitable for optimization of growth parameters in a solid state fermentation medium. This is one of important aspect in industrial microbiology for large scale production of valuable metabolites.

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