

### PRODUCTION AND OPTIMIZATION OF GROWTH CONDITIONS FOR INVERTASE ENZYME BY *ASPERGILLUS SP.*, IN SOLID STATE FERMENTATION (SSF) USING PAPAYA PEEL AS SUBSTRATE

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#### ABSTRACT

Invertase enzymes are produced mainly by plants, some filamentous fungi, yeast and many other microorganisms which finds applications in food industries, confectionaries, pharmaceuticals, etc., The present work deals with the production of Invertase by *Aspergillus sp.*, isolated from various soil samples in solid state fermentation using papaya peel waste as substrate. Enzyme activity was checked using Fehling's reagent and assay was carried out by DNSA method. The results of optimized conditions showed that the invertase activity was high in the SSF using papaya peel as substrate, incubated for 6 days at temperature of 35°C, pH 7, with 2.25gms/100ml of Ammonium nitrate as nitrogen source and 10gms/100ml of sucrose as carbon source. Hence the agro wastes from industries can be recycled by using it as substrate in SSF for high invertase enzyme production which finds applications in many fields.

**Keywords:** Invertase, *Aspergillus sp.*, papaya peel substrate, solid state fermentation (SSF), optimization

#### INTRODUCTION

Sucrose, a disaccharide composed of  $\alpha$ -D-glucose molecule and  $\beta$ -D-fructose molecule linked by an alpha 1,4 glycosidic bond is hydrolyzed in the presence of enzyme invertase to give an equi molar mixture monosaccharide's of D-glucose and D-fructose is called invert sugar (Rubio et al., 2002).



Invertases [  $\beta$  - fructo furanosidase ( EC.3.2.1.26)] are members of the GH32 family of glycoside hydrolases which include more than 370 enzymes of plant and microbial origin (Alberto et al., 2004). As a result of enzymatic action, the effective net rotation of the plane polarized light changes from right to left due to higher levo rotatory action of fructose. Since the rotation of the plane polarized light is inverted from right to left, this enzyme is called invertase.

The enzymatic activity of invertases has been characterized mainly in plants ( Alberto et al., 2004; Hussain et al., 2009), Several filamentous fungi such as those from *Aspergillus sp.* (Ashok Kumar et al., 2001, Nyugen et al., 2005 and Guimaraes et al., 2009), in different yeast strains such as *Candida utilis* (Belcarz et al., 2002), *Pichia anomela* (Rodriguez et al., 1995) and *Rhodotorula glutinis* (Rubio et al., 2002) and many other organisms such as *Neurospora crassa*, *Fusarium oxysporium*, *Phytophthora megasperma*, *Aspergillus niger*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Schwanniomyces occidentalis* produce invertase (Silveira et al., 2000).

*Aspergillus niger*, a cosmopolitan, widely distributed fungus, is an important industrial source for citric acid and enzymes such as amylase, invertases and enjoys a GRAS (Generally Regarded As Safe) status (Martens - Uzunova et al., 2006). Hence it is the choice of organism for invertase production.

Invertases are intracellular as well as extra cellular (Nakano et al., 2000) and are mainly of two types,  $\alpha$ -D-glycoside glucohydrolase [EC 3.2.1.20],  $\beta$ -D-fructo furanosidases fructo hydrolases [EC 3.2.1.26], according to whether the fructose or glucose end, respectively of the molecule is cleaved (Neuberg & Mandl, 1950).  $\alpha$ -glucosidases are found in animals and  $\beta$ -fructo furanosidases are found in plants and microorganisms (Quaroni and Semenza., 1976).

SSF is characterized by development of microorganisms in a low aqueous content on a non-soluble material that can act as physical support and in sometimes also as nutrient sources (Vandenbergh et al., 2000). Many microorganisms are capable of growing on solid substrates but only filamentous fungi can grow to a significant extent. Among them, three classes have gained practical importance in SSF and they include Phycomycetes such as the genera *Mucor* and *Rhizopus*, the Ascomycetes such as the genera *Aspergillus* and *Penicillium* and the

Basidiomycetes especially the white rot fungi among them are certain edible mushrooms.

The objective of our study was production of invertase by *Aspergillus sp.*, in SSF using papaya peel as substrate and to optimize its growth conditions including temperature, pH, different carbon and nitrogen sources.

#### MATERIAL AND METHODS

##### Isolation and Screening

*Aspergillus sp.*, was isolated from different soil samples by serial dilution method followed by spread plate technique using Rose Bengal agar plates and identified by colony morphology and microscopy (Lacto phenol cotton blue mount). The isolates were cultured in Czepak Dox medium containing 50% sucrose. Screening for invertase activity was done by using Fehling's reagent.

##### Substrate

Papaya peel waste was processed, dried and sterilized at 121°C for 20 minutes and used as substrate for solid state fermentation.

##### Solid state fermentation

20ml of Feeding solution 1 (1gram Sucrose, 0.2gram Yeast extract in 100ml of distilled water) & Feeding solution 2 (4.5 grams of Ammonium sulphate, 2.3 grams of Potassium Di hydrogen Phosphate, 0.01 gram Ferrous sulphate, 0.7 gram of Magnesium sulphate, 5.0 grams of Sucrose, 1.1 gram of Urea , 0.5 gram of Yeast extract in 100ml of distilled water) were added to 10 grams of processed papaya peel substrate in a 250 ml flask separately and sterilized at 121°C for 20 minutes, inoculated with the isolated *Aspergillus* spore suspension of  $10^6$  in sterile distilled water and incubated at room temperature for 5 days.

##### Enzyme Extraction

*Aspergillus sp.*, isolate screened for invertase (1ml of spore suspension of  $10^6$  in sterile distilled water) was inoculated into solid state fermentation with papaya peel as substrate and incubated at room temperature for 5 days. Crude enzyme extract was obtained by addition of 100ml sterile distilled water and homogenized the contents in a shaker for 1 hour followed by filtration using Whatman No.2 filter paper.

**Enzyme Assay**

Enzyme assay was carried out by DNSA method described by **Sumner and Howell 1935** using sucrose as substrate. To the test tubes, added 1.4ml of distilled water, 0.5ml of Sodium Acetate buffer (pH-4.8) and 0.1ml of enzyme extract (filtrate from SSF) were added. Then to the content in the test tubes 1ml of 0.3mM sucrose was added. The test tubes were then placed in a boiling water bath for 10minutes at 80°C. The tubes were then cooled and the reaction was stopped by adding 2ml of DNSA and kept in the water bath for 5minutes. The tubes were then taken out cooled rapidly and absorbance was measured at 540nm using blank zero (reagent blank was prepared using 0.1ml distilled water instead of enzyme filtrate).

**Optimization**

Optimization of growth parameters was done at different incubation period from 4<sup>th</sup> day till 8<sup>th</sup> day, pH ranging from 3 to 11, temperatures from 25°C to 65°C, different carbon sources including glucose, maltose, lactose, sucrose, Raffinose, and different nitrogen sources including Sodium nitrate, Ammonium sulphate, Ammonium chloride, Ammonium nitrate, Potassium nitrate, Silver nitrate and Urea. Different concentrations of carbon sources (ranging from 5 grams, 10 grams up to 50 grams) and nitrogen sources (ranging from 0.25grams, 0.5grams up to 2.5grams) were also checked to obtain maximum enzyme yield.

**RESULTS AND DISCUSSION**

**Isolation of *Aspergillus sp.*,**

On observing Rose Bengal Agar plates, the colonies were found to be thick with good sporulation that were dark green in color, and were completely spread on the plate. Under Lacto phenol cotton blue mount, observed under high power objective (45X), the aerial mycelia form of fungi was found to be clear with sterigmata and dispersed conidia. With the microscopic observation and sporulation pattern, the organism was identified as *Aspergillus sp.*

**Screening for invertase activity**

On addition of Fehling’s reagent to the 5 days incubated culture in Czepak Dox medium with 50% sucrose, formation of orange to reddish precipitate indicates a positive reaction for invertase production (Figure 1).

**Solid state fermentation**

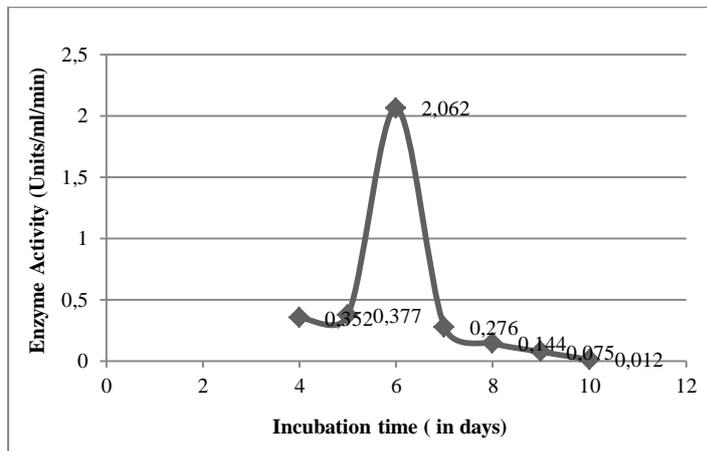
Good growth was observed in solid state fermentation flasks inoculated with *Aspergillus sp.*, isolates in papaya peel substrate. Enzyme activity was found to be high with the medium inoculated with Feeding solution 2 used to enhance the enzyme production (Figure 2).



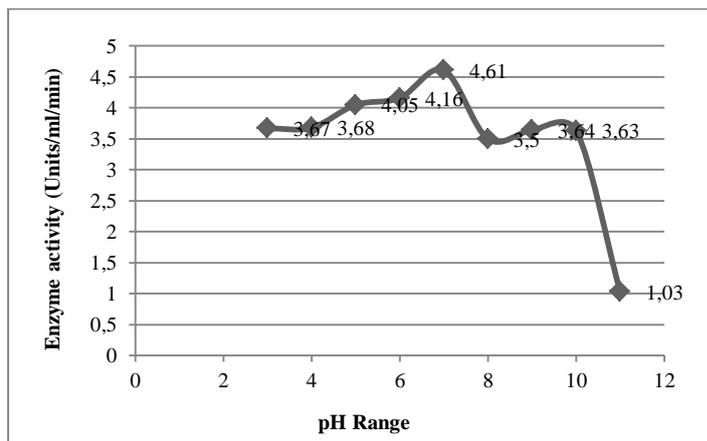
**Figure 1** Screening invertase activity using Fehling’s reagent **Figure 2** SSF using papaya peel substrate

**Enzyme Assay and Optimization**

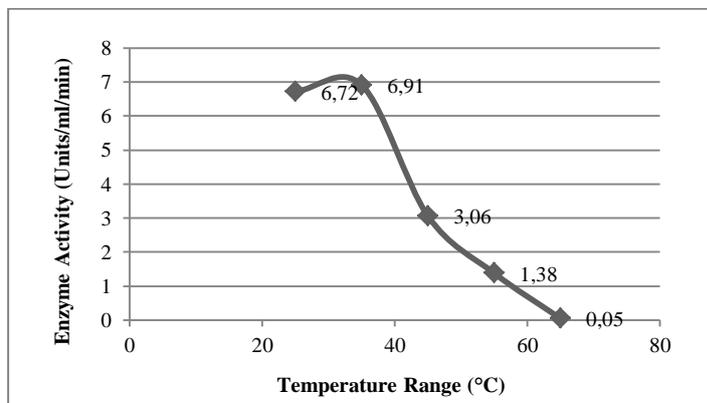
Enzyme assay was carried out by DNSA method and the growth conditions were optimized and the results are as follows: Maximum Invertase activity was obtained on the 6<sup>th</sup> day of incubation (Figure 3) with pH of 7(Figure 4), at temperature of 35°C (Figure 5), 10 grams /100ml of Sucrose as carbon source (Figure 6, Figure 7) and 2.25 grams/100 ml of Ammonium nitrate as nitrogen source (Figure 8, Figure 9).



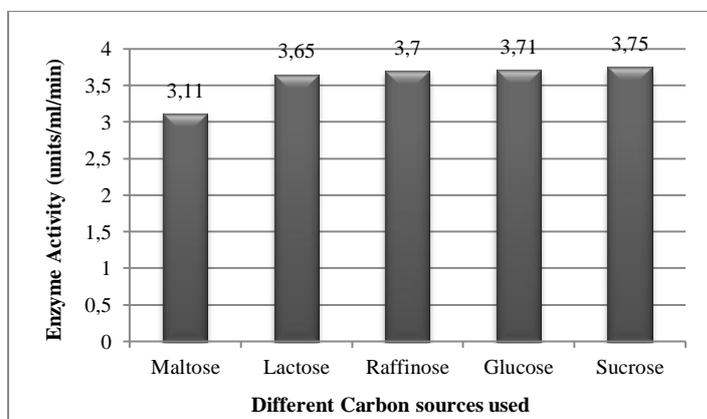
**Figure 3** Effect of Incubation time on Invertase production.



**Figure 4** Effect of pH on Invertase production.



**Figure 5** Effect of Temperature on Invertase production.



**Figure 6** Effect of different Carbon sources on Invertase production.

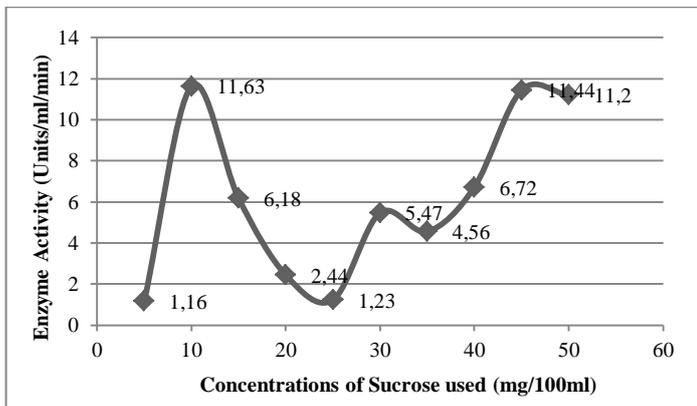


Figure 7 Effect of different concentrations of Sucrose on Invertase production.

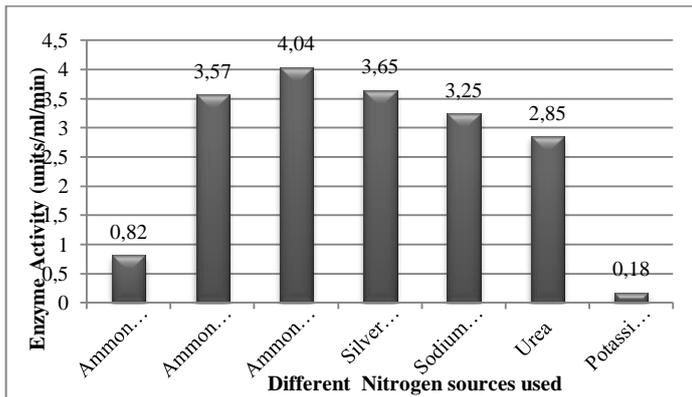


Figure 8 Effect of different Nitrogen sources on Invertase production.

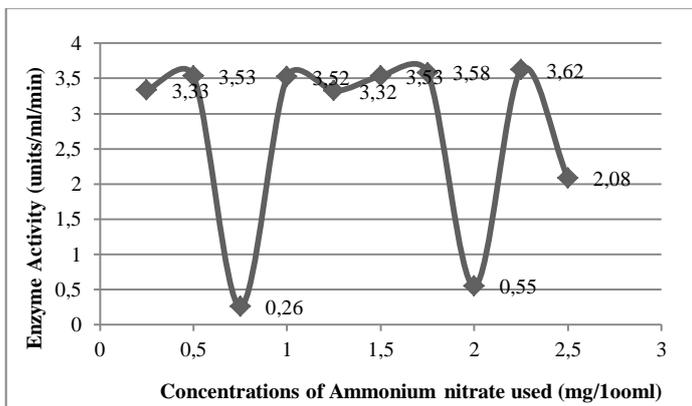


Figure 9 Effect of different concentrations of Ammonium nitrate on Invertase production.

Invertase enzyme production by *Aspergillus sp.*, in solid state fermentation was studied by inoculating  $10^6$  spores/ml of fermentation medium containing the fruit peel waste as substrate and the growth parametric conditions were optimized.

To determine the optimum incubation period for invertase enzyme production, fermentation flasks were incubated for different time duration (4 - 10 days). Enzyme activity was analyzed at every 24 hrs time intervals. Maximal titers of enzyme were reached at 6<sup>th</sup> day with the fungal tested (Figure 3) after which the rate declined; this might be on the basis of consumption of nutrients.

The invertase enzyme from fungal strain using papaya peel waste as substrate was examined under different conditions of temperature {25-65 °C} and at varying pH {3-11}. The enzyme activity was high at the temperature of 35°C and at pH of 7 (Figure 4, 5) but at high temperature the enzyme activity was not significant, because of high temperature denaturation of enzyme active site (Russo P et al., 1996). This shows that enzyme is not stable towards alkaline and acidic conditions so the sucrose inversion efficiency is also affected in direct way (Balasundaram B and Pandit AB., 2001).

Different carbon sources such as glucose, maltose, sucrose, lactose and Raffinose at different concentration were selected for the invertase production. For all the carbon sources tested, sucrose at concentration of 10 grams / 100ml gave the best result (Figure 6). The results was supported by the findings of Cairns et al., 1995 who reported that invertase production in some other fungi was induced by sucrose. Glucose and fructose are not involved in the induction of the synthesis of β-D- fructo furanosidase in *A. niger* (Rubio et al., 2006).

The effect of different nitrogen sources were tested by incorporating various nitrogen sources like Ammonium Chloride, Ammonium Sulphate, Ammonium Nitrate, Sodium Nitrate, Silver nitrate, Urea, Potassium Nitrate into the fermentation medium. Production was more pronounced by the addition of 2.25grams of Ammonium Nitrate per 100 ml of fermentation medium. Different organic nitrogen sources and their concentrations have a major effect on the ability of yeast to synthesize fructo furanosidase (Nakano et al., 2000). Our result differs from the observation of Shafiq et al., 2002 who reported that among all the nitrogen sources peptone gave maximum production of invertase activity using *Saccharomyces cerevisiae* under the temperature of 30<sup>o</sup> C and pH 6.0 and agitation rate 200 rpm.

## CONCLUSION

Increasing concern about pollution that occurs from agricultural and industrial wastes has stimulated interest in converting waste materials into commercially valuable products. Beside their pollution and hazardous aspects, in many cases, food processing wastes might have potential for recycling raw materials or for conversion into useful product of higher value.

From the above research, it was found that maximum invertase activity can be obtained from SSF with papaya peel waste as substrate with the above optimized growth conditions, which has advantages over Submerged fermentation, as higher productivity fermentation, absence of contaminant organisms, concentrated product formation and use of agro industrial residues as substrates (Ashok Kumar et al., 2001).

Hence the data from this work will be useful for industrial production of invertase enzyme in SSF using fruit peel waste as substrate by *Aspergillus sp.*, that finds applications in many fields including the preparation of jams and candies, preparation of invert sugar and high fructose syrup (HFS), for manufacture of artificial honey, plasticizing agent used in cosmetics and paper industries as well as enzyme electrodes for the detection of sucrose, fermentation of cane molasses into ethanol, manufacture of calf feed and food for honey bees (Sanchez et al., 2001) and in pharmaceuticals (Ashok Kumar et al., 2001).

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