INVERTASE PRODUCTION BY SPORES OF *ASPERGILLUS NIGER* IN SUBMERGED FERMENTATION

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Abstract
Production of fructose is important especially in the food industries where fructose is used as a sweetener in preference to sucrose. This work studied some cultural conditions for the production of invertase by Aspergillus niger. In a preliminary experiment, all isolated fungal spores were incubated in 0.2 M sucrose solution and the spores of Aspergillus niger produced the highest invertase activity of 72.8U/mg protein and was therefore selected for further work. Inversion of sucrose by spores of Aspergillus niger was then studied after various incubation periods. Incubation for 6 hours with shaking was best for enzyme production at which 94.6 U/mg protein of the enzyme was produced. Enzyme yields at 1 and 2 h of incubation were very low while incubation for elevated periods of 7 and 8 h resulted in moderate enzyme yields of 68.3 and 60.4 U/mg protein respectively. Lower sucrose concentrations of 0.3 and 0.4 M were almost completely inverted while higher concentrations showed decreases in enzyme activities. Invertase from Aspergillus niger was incubated at various temperatures and it was found that at 10°C, only 26.5 U/mg protein of the enzyme activity was produced. This level increased gradually with increases in temperature until 30°C at which the highest enzyme activity of 72.8 U/mg protein was produced. This level reduced progressively with increases in temperature until it reached a temperature of 50°C at which very low activity was obtained. It was shown that pH 5.0 was the best for invertase production by the test fungus.

Keywords: Invertase, *Aspergillus niger*, Sucrose, Temperature, pH, Time variable

Received: 13.07.2020 Reviewed: 26.11.2020 Accepted: 21.12.2020

INTRODUCTION

Sucrose is a disaccharide composed of an D-glucose molecule and a D-fructose molecule which is linked by an 1,4-glycosidic bond. Sucrose is hydrolyzed by invertases (EC 3.2.1.26) into equimolar mixture of D-glucosse and D-fructose (Zhou et al., 2016). Invertases are also referred to as β-fructofuranosidase are in the class of glucoside hydrolase (Nguyen et al., 2005). These enzymes are important in research and in industrial and food sectors and are used in the preparation of invert sugar, high fructose syrup, jams, candies, chocolates creams powder milk for infants artificial honey and beverages (Nadeem et al., 2015). The inverted sugar is sweeter than sucrose and easier to incorporate into industrial preparations and it does not show crystallization problems of its precursor in highly concentrated solutions and it has more added value than sucrose (Martinez et al., 2014).

Enzymes are biological catalysts found in living cells that speed up the rate of biological reactions. They act as bio–catalysts facilitating metabolic reactions. Because of its potential in biotechnology and its application in the food and beverage industries, invertase is one of the most used in the food industry, where fructose is preferred to sucrose, especially in the preparation of jams and candies because it is sweeter and does not crystallize easily (Maria de Lourdes, 2016). There is a wide range of commercial applications of the invertase including the production of confectionery with liquid or soft centers, chocolate and in the fermentation of cane molasses into ethanol and in pharmaceutical industry as digestive aid tablets, powder milk or infants' foods, as calf feed preparation, assimilation of alcohol in fortified wines and in manufacture of inverted sugars as food for honeybees (Uma et al., 2010).

All fungi are not able to produce invertase and that is why not all fungi have the ability to utilise sucrose as carbon and energy source. Some of the fungi with the ability to produce invertase include *Saccharomyces cerevisiae*

(Kulshrestha et al.,2013). *Neurospora crassa, Candida utilis, Fusariumoxy sporium, Phytophthora meganosperma, Aspergillus niger, Schizosaccharomyces pombe* (Nyugen et al., 2005), *Neurospora crassa, Fusariumoxy sporium, Schwanniomyces occidentalis, Aspergillus caespitosus, Aspergillus japonicas, Aspergillus flavus,* and *Paecilomyces variotii* among others (Alegre et al.,2009). Invertase activities have also been reported in plants (Ru et al., 2017; Bergareche et al., 2018; Wan et al., 2018; Shen et al., 2019).

Invertase produced by fungi hydrolyzes the D-fructofuranoside linkage between the glucose and fructose units of sucrose to yield glucose and fructose residues. Temperature, pH, carbon source, metal ions are some the factors that affect the rate of production of invertase by fungi (Rustiguel et al., 2015). The work is aimed at assessing different fungal isolates for invertase production and to evaluate the best cultural conditions for optimum yield of the enzyme.

MATERIAL AND METHODS

Isolation of microorganisms

Soil sample was collected from a cassava processing mill in Nsukka into conical flasks. The pH of the sample was read with a hand held pH metre (pHep; Hanna Instruments) and recorded as pH=7.2. The sample was serially diluted and plated on Potato Dextrose agar (Oxoid Ltd. UK) which contained chloramphenicol solution to suppress bacterial contaminants. Petri plates were inoculated with diluted samples and incubated for 48 h at room temperature 30±2^{o}C. Pure colonies of all isolated fungi were picked and stored in slants at room temperature. Identification of the fungal cultures was based on their cultural and physiological characteristics as outlined by Pitt and Hocking (1997).

Spores from 8-day old slant cultures grown on Potato Dextrose agar plates at room temperature were harvested with 0.1% Tween 80 (Difco Laboratories, USA) solution and inoculated into 100 mL medium in 500mL conical flasks each containing 0.2 M sucrose solution. Into the media was added the following compounds in g/L: $(NH_4)_2$ SO_4, 1.0; KH_2PO_4, 2.0; Na_2HPO_4, 0.7. The pH was adjusted to 7.2 and each inoculated with $2x10^7$ spores/ml of each isolated fungal culture and incubated in a Gallenkamp orbital shaker for 5h at 30^{o}C. After the incubation period, each sample was centrifuged at 6000xg for 10 min. Cell pastes were each ground with sterile river sand and centrifuged at 6000xg for 10 minutes. The two supernatants were combined and designated as crude enzyme and then assayed for invertase activities. The fungus with the best invertase activity was identified as *Aspergillus niger* and used for further work.

Effects of time variable on enzyme production

Flasks were each added 0.2 Mol/l sucrose solution and the pH was adjusted to 7.2 and inoculated with 2 x 10^7spores/ml of *Aspergillus niger* then incubated at 30 ^{o}C with shaking for varying periods of time (0, 1, 2, 3, 4, 5, 6, 7 and 8 h). The enzyme was harvested after each incubation period as described above.

Effects of concentrations of sucrose on enzyme production

Conical flasks were each added different concentrations of sucrose solutions (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 Mol/l) and the pH was adjusted to 7.2. The experimental flasks were each inoculated with 2 x 10^7 spores/ml of *Aspergillus niger* and incubated at 30^{o}C with shaking. This was followed by centrifugation. Cell pastes were also harvested as described above and assayed for enzyme activities.

Partial purification of the enzyme

The crude enzyme was dialyzed overnight against 0.1M phosphate buffer (pH 7.2). Solid ammonium sulphate was added to the enzyme extract to 70% saturation, incubated for 10 h with shaking. The solution was centrifuged at 6000 x g for 10 min. and the supernatant was dissolved in 0.1M phosphate buffer (pH 7.2) and dialyzed overnight against the same buffer. The dialysate was used as enzyme solution.

The effect of temperature on invertase activity

The temperature activity profile of the invertase was determined by incubating 2 mL

of enzyme solution with 1 mL of 1% (w/v) sucrose solution prepared in 0.1M phosphate buffer (pH 7.2) and incubated for 5 h at different temperatures (10, 15, 20, 25, 30, 35, 40, 45 and 50^{o}C) in a thermo static water bath (Kottermann, Bremen, Germany). The reaction was stopped by the addition of 2 mL of dinitrosalicylic acid (DNS) solution.

The effect of pH on invertase activity

The effect of pH on invertase activity was determined by using buffer solutions of different pH (acetate buffer, pH 2.0-3.0; citric acid/sodium citrate buffer, pH 4.0-6.0; potassium phosphate buffer, pH 7.0 to 8.0; Tris/HCl buffer, pH 8.1-9.0 and carbonate/bicarbonate buffer (pH 9.0-11.0) for enzyme assay. The pH activity profile of the enzyme was determined by incubating 2 mL of enzyme solution with 1 mL of 1% (w/v) sucrose solution prepared in buffers of different pH values and incubated for 5 h at room temperature. The reaction was stopped by the addition of 2 mL of DNS solution.

Enzyme assay

The invertase assay was based on the reduction of bright yellow coloured solution of 3, 5-dinitrosalicylate (DNS) to dark orange-coloured solution of 3-amino-5-nitrosalicylate resulting from enzymatic hydrolysis of sucrose (Bhalla et al., 2017). The absorbance was recorded at 540 nm and compared with the standard curve of glucose. One unit of invertase activity (U) was defined as the amount of enzyme required to produce one micromole of reducing sugar per min under the assay condition.

Analysis

Protein content was determined by the method of Lowry et al., (1951) using bovine serum albumin (Sigma-Aldrich) as a standard. The concentrations of reducing sugars were determined by the dinitrosalicylic acid (DNS) method of Miller (1959).

RESULTS AND DISCUSSION

Table 1 is a summary of the experiment used to select the best fungal colony for invertase production. Invertase of spores from eight isolated fungi incubated with shaking in 0.2 M sucrose solution showed that *Aspergillus niger* spores produced the highest amount of invertase activity of 72.8U/mg protein. Lowest enzyme activity of 12.9 U/mg protein was produced by *Saccharomyces cerevisiae. Aspergillus niger* has been widely reported for invertase production (Rubio and Maldonado 1995; Nguyen et al., 2005; Goosen et al., 2007; Nadeem et al., 2009). Fungi are important environmental organisms especially in the ecosystem where they are responsible for spoilage, and in some cases desirable bioconversions and they are able to utilize a variety of compounds in secreting a diverse range of enzymes (Hamad et al., 2014).

Isolate	**Invertase activity (U/mg protein)**
Aspergillus flavus	*42.5*
Trichoderma viride	*35.6*
Candida utilis	*16.3*
Candida tropicalis	*18.0*
Aspergillus niger	*72.8*
Aspergillus awamori	*66.5*
Fusarium oxysporium	*68.9*
Saccharomyces cerevisiae	*12.9*

Table 1. Comparison of isolated fungal spores for invertase production

The rate of inversion of sucrose by spores of *Aspergillus niger* was affected by the period of incubation (Figure 1). Incubation for 6 hours was best for enzyme production at which 94.6 U/mg protein of the enzyme was produced. Enzyme yields at 1 and 2 h of incubation were very low while incubation for elevated periods of 7 and 8 h resulted in moderate enzyme yields of 68.3 and 60.4 U/mg protein respectively. Highest invertase production by *Cladosporium cladosporioides* occurred on the fourth day (Uma et al., 2012). Rashad and Nooman (2009) reported maximum invertase production by *Saccharomyces cererisiae* NRRL Y – 12632 on the 4th day of incubation. Guimaraes et al., (2007) demonstrated that invertase production by *Aspergillus ochraceus* occurred after 96 h. Maximum invertase activity by *Aspergillus oryzae* occurred on the 4thday (Shankar and Mulimani, 2007). Maximum amount of invertase was reported for *Saccharomyces cerevisiae* after 48 h incubation while the lowest occurred after 96 h (Shankar et al., 2013).

The fungal spores were tested under various sucrose concentrations ranging from 0.1 to 1.0 M for invertase production. Results in Figure 2 show that two lower sucrose concentrations 0f 0.3 and 0.4 M were almost completely inverted. At higher concentrations of sucrose, there was a decrease in enzyme activities.

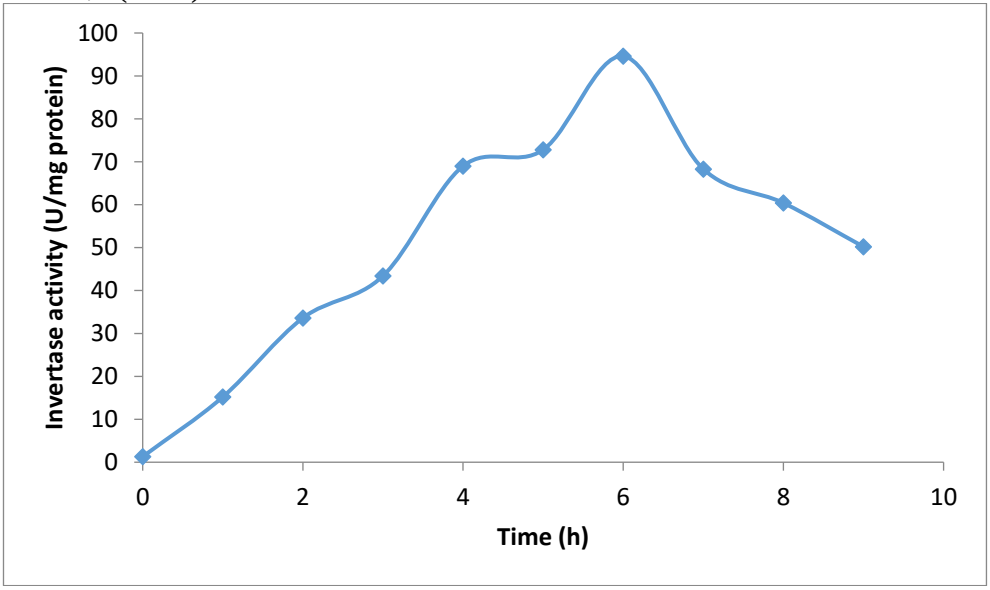

Figure 1. Time variable for the production of invertase by *Aspergillus niger*

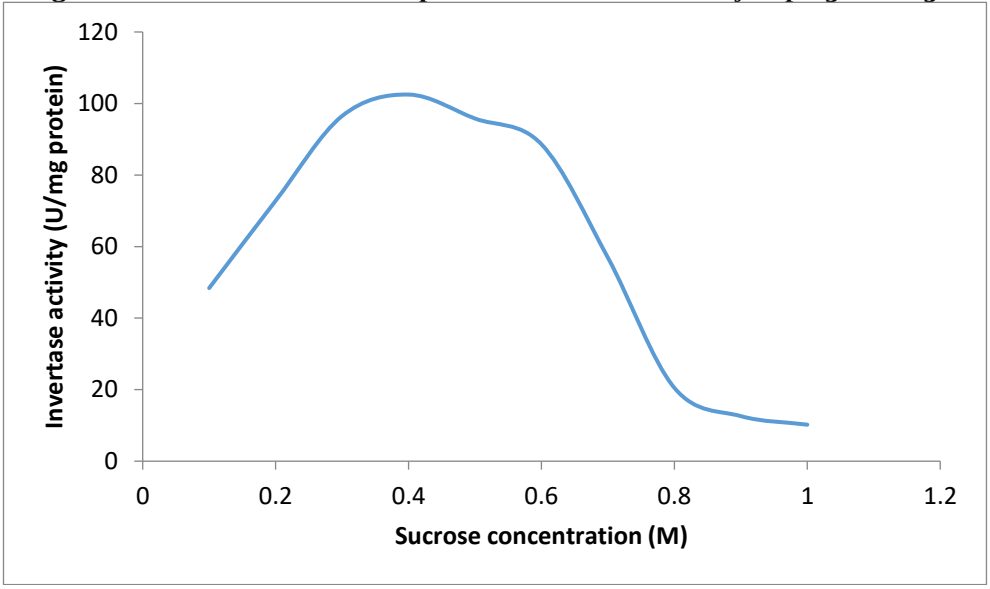

Figure 2. Invertase production by *Aspergillus niger* as affected by sucrose concentration

At a concentration of 0.1M, about 48.4 U/mg protein of the enzyme was produced but at 0.4M, the best enzyme titre was produced (102.5 U/mg protein). This level diminished to 10.2 U/mg protein when the level of sucrose in the medium was 1.0 Molar. Enzymatic hydrolysis of sucrose is usually preferable than acidic hydrolysis in the production of high-quality invert syrups (Krastanov, 1997).A high level of β -fructofuranosidase was produced by *Penicillium brevicompactum* when sucrose was used as a carbon source and yeast extract was used as the nitrogen source (Uma et al., 2011). Shankar et al.(2013) investigated the effect of different concentrations of sucrose on invertase production and the authors reported that maximum amount of enzyme production occurred at 2% sucrose while the minimum enzyme activity occurred at 3.5% sucrose concentration. Commercial invertase from *Saccharomyces cerevisiae* showed approximately 30% of its highest enzyme activity at 2 M sucrose concentration (Vasquez–Bahena et al., 2004). Invertases from a metagenomic library and *Aspergillus niger* showed approximately 50% activity (Du et al., 2010) and 30% activity (Goosen et al., 2007) it 1M sucrose concentration. At 2M sucrose concentration, *Candida guilliermondii* invertases INV3a – N and INV3a –D presented nearly 50 and 10% of highest acitvities respectively (Plascencia-Espinosa et al., 2014). The invertase reported by Zhou et al. (2016) remained approximately 50% of its highest activity in the presence of 2045mM sucrose.

In the present study, the effect of temperature on invertase activity by *Aspergillus niger* was investigated. At temperature of 10^{o}C, only 26.5 U/mg protein of the enzyme activity was produced. This level increased gradually with increase in temperature until 30^{o}C at which the highest enzyme activity of 72.8 U/mg protein was produced. This level reduced progressively with increases in temperature until it reached a temperature of 50^{o}C at which a low enzyme activity of 28.6 U/mg protein was obtained (Figure 3). Temperature is an important parameter which should be controlled during microbial enzyme production. Highest invertase produced with *Cladosporium cladosporioides* occurred at 30^{o}C (Uma et al., 2012). Temperature of 50°C for invertase production was reported as optimum for *Saccharomyces cerevisiae* enzyme (Vrabel et al., 1997; Rashad and Nooman 2009). Purified *Fusarium graminearum* invertase showed optimum activity at 55–60^{o}C (Goncalves et al., 2016). *Mucor geophilus* invertase had optimum activity at 50^{o}C (Qureshi et al. 2012). Guimaraes et al., 2007 reported highest invertase production at 40^{o}C whereas Ul-Haq et al. (2003) reported an optimum temperature of 25^{o}C for β-fructofuranosidase production by *Saccharomyces cerevisiae* GCB-K5.

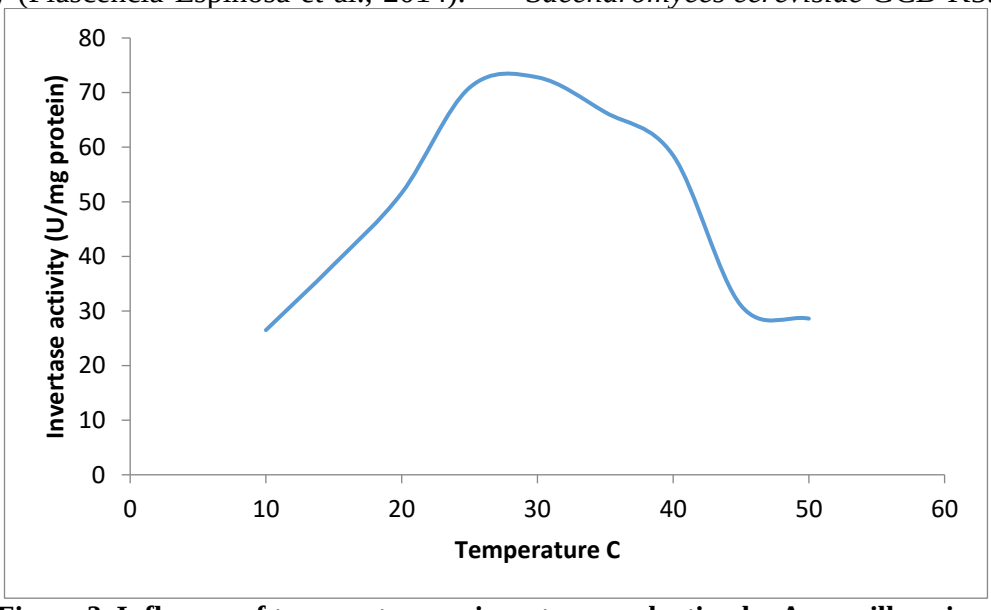

Figure 3. Influence of temperature on invertase production by Aspergillus niger

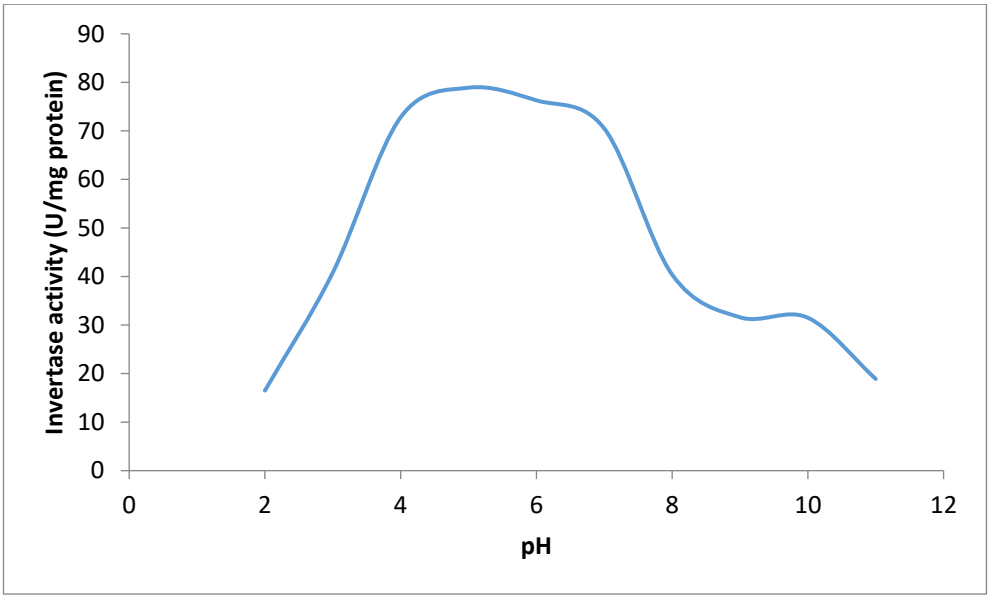

Figure 4. Influence of pH on invertase production by *Aspergillus niger*

Data for invertases of fungi has shown optimum activities at temperature range of 30-37^{o}C which is also their best temperature of growth (Qureshi et al., 2017).

The pH of microbial growth medium is important in inducing morphological and some physiological changes during enzyme production and this affects product stability (Romero – Gomez et al., 2000). *Aspergillus niger* invertase was incubated in broth at different pH values and it was found that pH 5.0 was optimal for enzyme production (Figure 4). At this pH, invertase activity of 78.9 U/mg protein was obtained. Incubation of spores at pH 2.0 resulted in lower enzyme activity while pHs 9 to 11 also resulted in low enzyme production by the fungus. At pH 3.0 and at pH 2.0, there was evidently acid hydrolysis of the sucrose and the enzyme seemed inactive in these pH range. The optimum pH for invertase of *Cladosporium cladosporioides* was found to be of 4.0 (Uma et al., 2012). The invertase activity of free cells was maximum at pH 6.0 and decreased by at least fourfold in the reactions at pH values of 3.0, 4.0 and 8.0 (Martinez et al., 2014). Shankar et al. (2014) also reported maximum invertase activity in citrate buffer by *Saccharomyces cerevisiae* MK. Uma et al. (2012) also found maximum invertase activity at pH 6.0. Qureshi et al. (2012) reported the highest invertase activity at pH 5.0 from *Mucor geophillus*. For *Saccharomyces cerevisiae* invertase, the optimum pH occurred in the 3.5 to 6.0 range (Santana de Almeida et al., 2005). Blanch and Clark (1997) and Chávez et al.(1997) reported optimum pH between 4.5 and 6.0.The activity of the *Rhodotorula glutinis* enzyme was drastically reduced at pH 8.0 (Rubio et al., 2002). Enzyme activity of invertase from *Penicillium brevicompactum* exhibited a broad pH range from 5.0-7.0 with an optimum at pH 6.0 (Uma et al., 2011). Ul-Haq et al. (2003) also specified that the maximum production of invertase was obtained at pH 6.0. Peak invertase production by *Aspergillus fumigatus* was observed at pH 5.0 for all the substrates tested (Uma et al., 2010 b). The optimum pH of activity for extracellular invertase from fungi ranged from 4.0-6.0 (Alegre et al., 2009). Many results show different pH values for each invertase and for each microorganism that produce it (Santana de Almeida et al., 2005).

CONCLUSION

Invertase is one of the most widely used enzymes in the food industry. Invertase was produced from *Aspergillus niger* and used to hydrolyze sucrose to fructose and glucose. The enzyme hydrolyzed sucrose at laboratory scale and this work studied some cultural conditions

which lead to optimal enzyme production by the fungus. From the present study, it could be seen that parameters like sucrose concentration, incubation periods, temperature of incubation and media pH exerted some effects on enzyme production. These cultural conditions when optimized will result to higher yields and make the invertase suitable for versatile applications.

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