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EVALUATION OF AGRICULTURAL BYPRODUCTS FOR THE PRODUCTION OF BETAGLUCOSIDASE BY *ASPERGILLUS NIGER* MBT-2 USING SOLID STATE FERMENTATION

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Abstract

The experiment was conducted to isolate and screen fungal strain and optimization of solid-state fermentation conditions for enhanced production of β-glucosidase. Different fungal cultures were isolated and screened for β-glucosidase production. The physicochemical and nutritional parameters were optimized for enhanced production of β-glucosidase from higher producer. Among all the isolates the isolate which exhibited highest β-glucosidase potential was identified and assigned the code as *Aspergillus niger* MBT-2. The optimum β-glucosidase production was obtained in M5 medium containing wheat bran after 72 hrs of incubation at 40°C, pH 6 and 20 ml of moisture contents. In addition to this 2% fructose and 2% yeast extract proved to be best carbon and nitrogen sources, respectively and gave maximal enzyme productivity. The exploitation of agricultural by products as a substrate reduced the production cost of enzyme and makes the process economical. The *Aspergillus niger* MBT-2 has promising potential of bioconversion of low-cost material into valuable product like β-glucosidase.

Introduction

β-glucosidase (EC 3.2.1.21) is one of the important enzymes. It breaks the β 1, 4-glycosidic bond of cellobiose and ultimately results in the production of glucose from the non-reducing ends (Raza *et al.* 2011). This enzyme plays many vital roles in many physiological phenomenon. The most important role in microorganisms is the breakdown of cellulose, gene induction of cellulase and the recycling of carbon (Ahmed *et al.* 2017). β-glucosidase are also biologically active that speed up the glycosyl transfer reaction between oxygen nucleophiles. This reaction causes the cleavage of beta-glucosidic bond occurs between the sugar residues present in aryl and alkyl beta glucosides, cyanide releasing glucosides, and short chain oligosaccharides (Singhania *et al.* 2013). β-glucosidase has enormous range of applications in different industries including cosmetics, cotton, grain wet milling, food, diagnostics and pharmaceutical etc. In addition to this it is also used as an additive in animal feed, tobacco and food products, and synthesis of organic chemicals (Leite *et al.* 2007).

β-glucosidase can be produced from various sources such as fungi and bacteria. Mostly, filamentous fungi are used for the β-glucosidase production at a large scale. It is due to their enlarged hyphae which penetrate into the crystalline structure of cellulose and produce pressure due to which enzyme is produced in large amounts (Amouri and Gargouri 2006; Zang *et al.* 2018). The industrial residues that are used as a substrate proved to be beneficial for the filamentous fungi. The morphological characteristics of filamentous fungi help them to integrate their mycelia structure into the tough surface. So, the industrial raw material, which is considered as a waste product, utilized as a substrate thus reducing the pollution and enhancing the enzyme production (Bhargav *et al*. 2008). Nowadays, industrial enzymes are produced in a more renewable and an economically cheaper method which ultimately requires the pursuit of raw materials. In solid state

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fermentation (SSF), the conditions required for growth are simple because these growth conditions resemble the natural habitat of many microorganisms' particularly filamentous fungi. The energy consumption is low, gives higher productivity, and the complex and sophisticated controlling system is not required to perform the solid state fermentation (Garcia *et al.* 2015).

Materials and Methods

The experiments were carried out in Department of Biotechnology, LCWU. Different fungal strains were isolated from various samples including soil, animal dung, bark according to Gupta *et al.* (2015) using plates containing Berg medium. The pretreatment of substrate like sugarcane bagasse was carried by alkali treatment method. The substrate is chopped in small pieces and sundried. The air-dried substrate was soaked in 1N NaOH solution for 24 hrs. After this duration washes the soaked sugarcane bagasse with the tap water and oven dry for 24 hrs at 50ºC (Maeda *et al.* 2011).

The solid-state fermentation was carried out using 10 g of sterilized substrate moistened with 10 ml of minimal medium. One ml of conidial inoculum was added in sterilized substrate. All the flasks were placed in an incubator for 72 hrs at 30ºC. After fixed duration 100 ml of phosphate buffer (pH 7) was added in each flask to extract the enzyme and flasks were placed in shaking incubator for 60 min. The suspension was filtered through muslin cloth and filtrate was further centrifuged at 6000 rpm for 15 min in order to obtain clear supernatant. The supernatant was used for the determination of β-glucosidase.

Following fermentation media were tested for the production of beta-glucosidase using solid state fermentation. The pH of all media was maintained at 6. M1: 5 g of rice straw and 5 g of wheat bran moistened with 10 ml of mineral culture medium containing (g/l): 3g $NaNO_3$, 0.1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g $KCl.2H_2O$ (Sherief *et al.* 2010). M2: 10 g of pretreated saw dust moistened with 10 ml of mineral salt media (MSM) containing (g/l) 0.8 g NaCl , 0.8 g KCl, 0.1 g $CaCl_2$, 2.0 g Na_2HPO_4 , 0.2 g $MgSO_4$, 0.1 g $FeSO_4$, 8.0 g glucose, 2.0 g NH_4Cl , 1000 ml distilled water (Khan and Singh 2011). M3: 10 g of corn straw moistened with 10 ml of nutrient solution containing 1 g ammonium sulfate, 1 g magnesium sulfate heptahydrate and 1 g ammonium nitrate (Santos *et al.* 2016). M4: 10 g of pretreated sugarcane bagasse was moistened with 10 ml of mineral requirement media containing (g/l): 1.4 g $(NH_4)_2SO_4$; 2.0 g KH_2PO_4; 0.34 g $CaCl_2$. $2H_2O$; 0.30 g $MgSO_4.7H_2O$; 0.005 g $FeSO_4.7H_2O$; 0.0016 g $MnSO_4.H_2O$; 0.0014 g $ZnSO_4.7H_2O$ and 0.002 g $CoCl_2.6H_2O$ (Ng *et al.* 2010). M5: 10 g of wheat bran moistened with 10 ml of mineral solution containing g/l: 5.0 g KH_2PO_4, 2.0 g NH_4NO_3, 4.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 2.0 g peptone, 2.5 g trisodium citrate and 1.0 g yeast extract (Bhatti *et al.* 2013). M6: 10 g of wheat straw moistened with 10ml of nutrient solution containing (g/l): 1 g ammonium sulfate, 1 g magnesium sulfate heptahydrate and 1 g ammonium nitrate (Garcia *et al.* 2015).

β-glucosidase activity was determined according to Rajoka and Malik (1997). One unit of β-glucosidase is defined as "the amount of enzyme required to yield 1.0 µM of *p*-nitrophenol within one minute under standard assay conditions" (Garcia *et al.* 2015). Total protein was estimated according to Bradford method (Bradford 1976).

All the data were tabulated and subjected to statistical analysis. Post Hoc multiple comparison test was used under one-way ANOVA. The software used for statistical analysis was SPSS version 23.

Results and Discussion

The choice of appropriate strain plays pivotal role in the success of any fermentation process. For this purpose 20 different fungal strains were isolated and screened for β-glucosidase in solid state fermentation (data not shown). Among all the fungal strains the strain which exhibited

highest β-glucosidase productivity (7.09 IU/ml/min) along with 0.30 mg/ml total protein was selected. The selected strain was identified according to Diba *et al*. (2007). The selected strain was identified as *Aspergillus niger* and given the code *Aspergillus niger* MBT-2. The choice of suitable medium is a significant factor in the production of BG. Six different fermentation media were evaluated for the β-glucosidase (Fig. 1a). All other media gave less β-glucosidase production as compared to M5 (16.1 IU/ml/min). The reason might be that the capability of wheat bran for the maximal production of enzyme based on its chemical composition because it consists of significant amount of carbohydrates, fats, proteins and fiber which are necessary for the growth of fungi as well as BG production. In contrast the decline in BG production in other media was either due to the shortage of certain substances in the media that were crucial for growth of fungi as well enzyme productivity (Singhania *et al.* 2011).

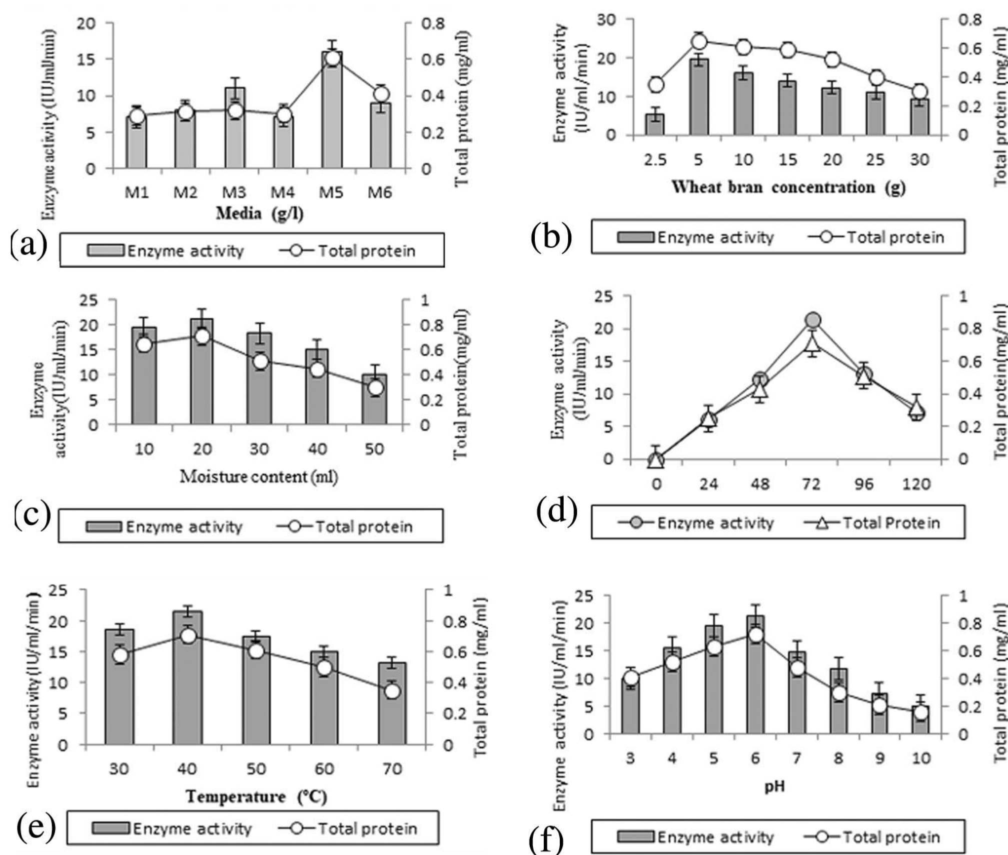

Fig.1. Impact of different physical parameters on the production of β-glucosidase. (a) Fermentation media, (b) substrate concentration, (c) moisture content, (d) incubation time and (e) incubation temperature.

Substrate concentration plays a crucial role for the enhanced and better production of enzyme under optimal conditions. The influence of varying concentration (2.5 - 30 g) of wheat bran was noted (Fig. 1b). The optimal production of beta-glucosidase was obtained when 5 g of wheat bran was used. The greater amount of substrate concentration reduces the enzyme production because substrates molecules were very closely associated around the molecules of enzyme. They might be

attached to the regions on the enzyme, which might not be the active site or blocked the active site, hence, enzyme production stopped (Dixon 1971). The varying moisture content ranging from 10 - 50 ml was tested. The highest production of β-glucosidase was obtained in the presence of 20 ml moistening agent (Fig. 1c). Above or below this level reduction in the production of enzyme was recorded. At higher moisture level substrate particles stick together which cease proper supply of oxygen in substrate for the growth of microorganism (Sharanappa *et al.* 2011).

The fermentation was carried out from 0 - 120 hrs (Fig. 1d). The maximal production of the β-glucosidase was found at 72 hrs. Further rise or fall resulted decline in enzyme production. This is due to the reason that during the early hours of incubation, spores have the capability to germinate and form mycelia. The increase in mycelia, may lead to the better enzyme production (Tu *et al.* 2007). The influence of varying temperature greatly affects the enzyme activity. The impact of variation in incubation temperature (30 - 70ºC) was evaluated (Fig. 1e). The optimal β-glucosidase production (21.4 IU/ml/min) along with 0.71 mg/ml total protein content was obtained at 40ºC. The gradually decline in enzyme production was noted with the increase or decrease in incubation temperature. Likelihood the reason for less productivity of BG above the optimal point is evaporation of moisture resulting in decline of moisture contents; which ultimately result decrease in fungal growth as well as enzyme production (Fawole and Odunfa 2003).

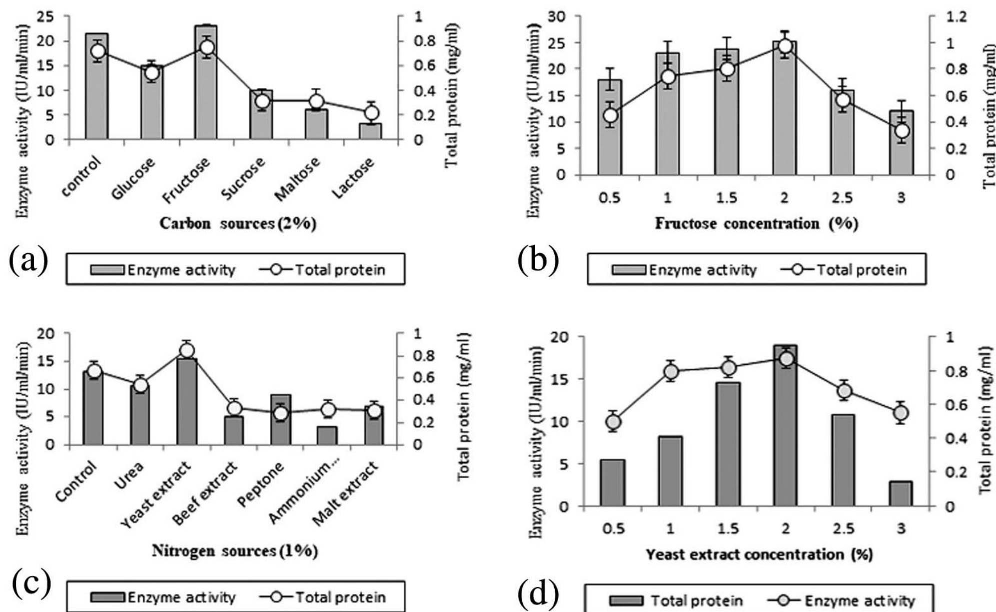

Fig. 2. Impact of different nutritional parameters on the production of β-glucosidase. (a) Carbon sources, (b) fructose concentration, (c) nitrogen sources and (d) yeast extract concentration

In order to optimize the process parameters, pH was found to be the very critical parameter for the better enzyme production. The different pH values (3 - 10) were evaluated for the β-glucosidase production. The enzyme productivity increases gradually from 3 - 6 pH (Fig. 1f). The enzyme production was found to move towards the decline after the optimum pH 6.0. The same results were reported by Olajuyigbe *et al.* (2016) who found that the enzyme production increases

by fungi form pH 3 - 6. Perhaps the reason was that the ionic state of substrate changes which greatly affect the growth of microorganisms.

Carbon is the important component present in all living organisms. The breakdown process of carbon sources release energy. This released energy is then utilized by the microorganisms for their better growth and development**.** Impact of different carbon sources on β-glucosidase production was evaluated (Fig. 2a). The carbon sources screened at 1% level include glucose, fructose, sucrose, maltose and lactose. The maximum β-glucosidase production was obtained when fructose was added in the medium. So, different concentrations (0.5 - 3.0) of fructose were tested. Two Per cent fructose gave optimal β-glucosidase productivity (Fig. 2b). The present findings are similar to the results reported by Shahzadi *et al.* (2014) who reported fructose as a best carbon source for beta-glucosidase production. The influence of different nitrogen sources including urea, yeast extract, beef extract, peptone, ammonium sulfate, malt extract was evaluated on the beta-glucosidase production (Fig. 2c). Two per cent yeast extract proved to be the best nitrogen source for maximal beta-glucosidase production (Fig. 2d). The present findings are similar to the results of Shahriarinour (2011 who reported that yeast extract was best nitrogen source for beta-glucosidase production.

From the present study it may be concluded that the exploitation of agricultural by products as a substrate lessens the production cost of enzyme and makes the process economical. The *Aspergillus niger* MBT2 has promising potential of bioconversion of low-cost material into valuable product like β-glucosidase.

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