Australian Journal of Basic and Applied Sciences, 6(4): 41-54, 2012
ISSN 1991-8178

Production of Glucoamylase by Marine Endophytic *Aspergillus* sp. JAN-25 under Optimized Solid-state Fermentation Conditions on Agro Residues

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Abstract: For effective extraction of glucoamylase from the fermented rice bran by endophytic *Aspergillus* sp. JAN-25 in solid state fermentation, optimized leaching parameters, 1:6 (w/v) of 0.2 M citrate buffer as leaching agent, soaking time with moldy bran 120 min, leaching temperature 45 ºC and leaching pH 4.0 at 150 rpm were found to be the optimum leaching parameters that leached the highest yield of glucoamylase (264.5 U gds^{-1}). Mixed bran of wheat and rice bran (at a ratio of 1:2) as abundant agro-industrial residues in Egypt, stimulated the highest glucoamylase yield as solid substrate in SSF by marine endophytic *Aspergillus* sp. JAN-25 to 398.52 U gds^{-1}. Under the optimized extraction and production parameters (2:50 substrate mass to flask volume ratio; IMC 50-60 %, inoculum size 10^7/ gds; supplementation of bran medium with 0.04 g starch / gds, 0.02 g soybean / gds, 1% $CaCl_2$ and 1% Tween-80 at pH 5.0 and 30 ºC for four days the glucoamylase production was markedly increased to 12.13-fold. Following successive purification steps including, $(NH_4)_2SO_4$ fractionation, gel filtration (sephadex G-200) and DEAE sepharose chromatography, the purified enzyme exhibited 23.34% of the total initial activity and there was a 35.41-fold increase in specific activity (613.30 U/mg) with maximum activity at pH 4.5 - 5.5 and 50 - 60 ºC. Glucoamylase activity markedly decreased to 41 and 52 % at 50 mM of EDTA and EGTA, respectively demonstrated the ion requirements of this JAN-25 glucoamylase activity (metallo enzyme).

Key words: Glucoamylase, Optimization, process parameters, Endophytic *Aspergillus* sp., Solid state fermentation.

INTRODUCTION

Glucoamylase (α-1, 4-glucan-glucohydrolases, EC 3.2.1.3) is exoenzyme of great importance for saccharification of starchy materials and other related oligosaccharides. Glucoamylase consecutively hydrolyzes 1,4-alpha-glycosidic bonds from the non-reducing ends of starch and 1,6-alpha-glucsidic linkages in polysaccharides yielding glucose as the end-product, which in turn serves as a feedstock for biological fermentations (Gupta *et al.*, 2003; Norouzian *et al.*, 2006). Currently, amylases have a great importance in biotechnology with a wide spectrum of applications, such as textile industry, cellulose, leather, detergents, liquor, bread, children cereals, ethanol production, high fructose syrups production and in various strategies in the pharmaceutical and chemical industries such as the synthesis of optically pure drugs and agrochemicals (Pandey and Radhakrishnan, 1993; Gupta *et al.* 2003; Norouzian *et al.* 2006 and Zambare, 2010).

The screening and identification of filamentous fungi capable of secreting extracellular enzymes with biotechnological potential are activities of great importance (Zambare, 2010). Glucoamylase is produced by a variety of fungi but the exclusive production of this enzyme in industry have been achieved mainly by *Aspergillus niger* (Wang *et al.,* 2006), *Aspergillus oryzae* (Biesebeke *et al.*, 2005), *Aspergillus awamori* and *Aspergillus terreus* (Berka *et al.*, 1992) probably because of their ubiquitous nature and non-fastidious nutritional requirements of these organisms. The glucoamylase costs are still too high for the establishment of a cost effective production of energy syrup and one approach to overcome this obstacle is employing solid state fermentation (SSF). In the SSF process, the culture medium is simpler and less expensive often made up of non-refined agricultural residues and these solid substrates not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells to be a low-cost substrate for fungal growth and the production of enzymes of biotechnological interest (Costa *et al.*, 1998; Hölker *et al.*, 2004). The confirmation of the potential for enzyme secretion by a species and the analysis of the conditions of production lead to a possible improvement of the environmental conditions favoring the maximal exploration of this capacity. Therefore, the search for new chemical compounds with biological activity highlights the economical and social importance of this research and its applications in the many areas of interest (Anto *et al.*, 2006). In SSF process, synthesis of glucoamylase depends on the culture conditions and type of nutrients available to the organism (Morita *et al.*, 1998; Morita and Fujio, 2000; Wang *et al.*, 2006; Sun and Zhang, 2007; Zambare, 2010 and Onofre *et al.*, 2011).

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The present study is undertaken to evaluate the production of glucoamylase capacity by the marine endophytic fungus *Aspergillus* sp. JAN-25 in solid state fermentation using agro/agro-industrial wastes due to the utility of marine endophytic fungi has not been investigated for the commercial production of glucoamylase as well as evaluation and optimization of the process parameters, which lead to an improvement of the environmental conditions favoring the maximal exploration of fungal capacity for overproducing of glucoamylase in SSF.

MATERIALS AND METHODS

Microorganism:

The marine endophytic molds were isolated from the marine soft coral *Dendronephtha hamprechii* and maintained on potato dextrose agar (PDA) as previously described by Holler *et al*. (2000) and El-Bondkly and El-Gendy (2010). Screening of fungal isolates for glucoamylase activity was performed by the starch clearing zone technique dopted by El-Safey and Ammar (1994). The selected hyper-amylolytic strain JAN-25 was identified depending on its morphologic and microscopic features according to the keys and studies of Samson (1979); Samson and Gams (1984); Jernejc and Cimerman (2001) and Watanab (2002).

Agro Residues* Used *In Solid State Fermentation:

Different agro residues such as rice husk, potato peel, corncob, rice bran, wheat bran, barely bran, groundnut shell, banana peel, and soybean meal were collected from local suppliers and preserved at room temperature.

Fermentation:

The experiments were conducted in 500 ml Erlenmeyer flasks containing 10 g of the solid substrate moistened to 60 % moisture content with mineral salt solution containing (%) KH_2PO_4, 0.5; urea, 0.3; KCl, 0.1 and $MgSO_4.7H_2O$, 0.1 and then incubated at 30 ºC and pH 5.0 in a stationary condition.

Glucoamylase Assay:

The enzyme activity was determined by incubating (at 50 ºC for 20 min) a reaction mixture containing 0.9 ml of 50 mM citrate buffer (pH 5), 1.0 ml starch solution (1 %, w/v) and 0.1 ml of crude enzyme and then the released reducing sugars were measured with 3, 5-dinitrosalicyclic acid (DNS) reagent (Miller, 1959) using glucose as a standard. One unit (U) of glucoamylase activity is defined as the amount of enzyme that releases one µmole of glucose as the reducing sugar per minute under the assay condition. Enzyme activity is expressed in terms of units per gram dry substrate (U gds^{-1}).

Protein Estimation:

Protein was assayed by determining the absorbance (O.D) at 280 nm using bovine serum albumin (BSA) as the standard (Suntornsuk and Hang, 1997). All the tests have been performed in triplicates.

Optimization Of Leaching Conditions Of Glucoamylase Resulted From SSF:

To optimize the leaching process, different studies were carried out**.** The conditions were optimized by adopting search technique with varying parameters one at the time as described by Shata (2005).

Effect Of The Utilized Leaching Agent, Solvent Volume, Soaking Time, Temperature, pH And Physical State:

The leaching out of glucoamylase from the fermented solids was carried out with different extractants such as tween 80 (0.1%), different salt solutions (NaCl, KCl and $CaCl_2$ at a concentration of 1 %), different buffers (0.2 M citrate buffer pH 4.0, 0.2 M phosphate buffer pH 7.0, and 0.2 M glycine-NaOH buffer pH 10) and different solvents (water, methanol, ethanol, acetone, ethyl acetate and glycerol) at a ratio of 1:1 (w/v) in 500 ml flasks containing 10 g of fermented rice bran at 40 ºC and agitated at 150 rpm for 60 min to find out the most efficient leaching agent for glucoamylase leaching process.

To optimize the other leaching parameters, glucoamylase was leached out from the fermented rice bran by the best extractant at varying solid / liquid ratios (1:1, 1:2, 1:4, 1:6, 1:8 and 1:10, wt. of bran / vol. of extractant), contact time (30, 60, 90, 120, 150 and 180 min), temperature (30, 40, 45, 50, 60 and 70 ºC), pH (3, 4, 5, 6, 7 and 8) and physical state (agitation or stationary conditions). Each parameter once optimized was fixed for subsequent studies. Each extracted solution was filtered through Whatman filter paper number 1 and the filtrate was again centrifuged at 5000 rpm for 15 min. cell free extracts were used as enzyme preparations

Optimization Of Production Conditions Of Glucoamylase Using SSF Technique:

Nutritional and environmental parameters were evaluated and optimized for enhancing the production of glucoamylase by *Aspergillus* sp. JAN-25 in SSF. The fermentation was carried out considering different parameters like suitable solid substrate (rice husk, potato peel, corncob, rice bran, wheat bran, barely bran, groundnut shell and banana peel) during different incubation periods (1-5 days), different mixtures of the best solid substrates (wheat bran and rice bran at a ratio of 1:2, 1:1 and 2:1), solid substrate mass to the flask volume ratio (0.5: 50, 1.0: 50, 1.5: 50, 2.0: 50, 2.5: 50 and 3.0: 50), initial moisture level (40, 50, 60, 70, 80 and 90%, v/w), inoculum size (10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 spore gds^{-1}), various carbons supplementation (glucose, fructose, maltose, lactose, sucrose and starch at 0.04 g/gds (Slivinski *et al.*, 2006), different nitrogen sources supplementation (ammonium sulphate, ammonium nitrate, sodium nitrate, yeast extract, malt extract, peptone, casein and soybean meal at 0.02 g/gds (Slivinski *et al.*, 2006) and mineral additives (NaCl, KCl, $MgCl_2$ and $CaCl_2$), incubation temperature (30, 37, 40, 45 and 50 ºC), initial pH (4, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0 and 9.0) and different detergents at a concentration of 1% (Triton X-100, Tween 80 and SDS). The conditions were optimized by adopting search technique with varying parameters one at the time as described by Pandey and Radhakrishnan (1993). Each parameter once optimized was fixed for subsequent studies. Enzyme extracted and extract suspension was filtered through Whatman filter paper number 1 and the filtrate was again centrifuged at 5000 rpm for 15 min and the solid-free supernatant was used as enzyme source for assaying glucoamylase activity.

Purification of JAN-25 Glucoamylase:

Glucoamylase JAN-25 was purified by fractional precipitation with ammonium sulfate, gel filtration (Sephadex G-200) and ion exchange chromatography (DEAE Sepharose CL-6B) according to kumar *et al.* (2011) with slight modification. All purification procedures were carried out at 4 °C.

Ammonium Sulfate Precipitation:

The concentrated enzyme preparation was subjected to fractional precipitation with ammonium sulphate in concentrations ranging between 20 and 100% according to the method of Gomori (1955). The precipitate of crude enzyme was dissolved in a minimum volume of 0.2 M phosphate buffer (pH 6.0) and dialyzed overnight in a dialysis bag against the same buffer at 4 °C.

Gel Filtration On Sephadex G-200:

The concentrated dialyzed solution was loaded into sephadex G-200 column (Pharmacia, Sweden, 2.5 X 45 cm) and equilibrated and eluted with 0.2 M phosphate buffer (pH 6.0) containing 0.2 M NaCl. 5 mL fractions were collected at a flow rate of 60 ml h^{-1}. All fractions were assayed for both glucoamylase activity and protein content.

Ion Exchange Chromatography:

The fractions with glucoamylase activity were pooled, concentrated and loaded into DEAE Sepharose CL-6B column chromatography (2.6 X 20 cm) which had been equilibrated with 0.2 M phosphate buffer (pH 6.0) containing 0.1 mM $CaCl_2$ and then eluted at a flow rate of 1 ml/min. The resulting active fractions were collected and used as the purified glucoamylase.

Determination Of The Molecular Weight Of The Purified Enzyme:

The molecular weight of the purified enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970).

Characterization of Purified JAN-25 Glucoamylase:

The following parameters were investigated and glucoamylase activity was assayed at the end of each treatment.

Optimum pH and temperature for JAN-25 Glucoamylase Activity:

The optimum pH was determined by measuring activity at 50 ºC using different buffers (0.1 M) with various pH values: citrate–phosphate (pH 4.0, 4.5, 5.0, 5.5 and 6.0); phosphate (pH 6.5 - 7.0); Tris-HCl (pH 8.0 - 9.0) and glycine-NaOH (pH 10.0). Optimum temperature was assayed by measuring activity at pH 5.0 with varying temperature (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C).

pH Stability and Thermostability for JAN-25 Glucoamylase Activity:

For studying pH stability, the enzyme solution was dispersed in 0.1 M buffer solutions (1:1), citrate–phosphate (pH 4.0, 4.5, 5.0, 5.5 and 6.0); phosphate (pH 6.5 - 7.0); Tris-HCl (pH 8.0 - 9.0) and glycine-NaOH (pH 10.0) and then incubated at 30 ºC for 24 h. An aliquot was used to measure the residual activity at pH 5.0 and 50 ºC. Heat stability of the glucoamylase enzyme was determined by incubating the purified enzyme

solution at various temperatures ranging from 25 to 80 ºC for 60 min then each treatment was terminated by exposing to ice-cold water and the residual activity was determined at optimum pH and temperature.

Effect of Substrate Concentrations:

Purified glucoamylase was incubated with soluble starch at different concentrations in the reaction mixture (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0%). The glucoamylase activity was determined under the optimized assay conditions.

Effect of Some Chemicals on Purified JAN-25 Glucoamylase Activity:

Purified glucoamylase was pre-incubated for 1 h at 30 ºC with different metal ions including Na^{+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Cd^{2+}, Ba^{2+}, Zn^{2+}, and Pb^{2+} at a final concentration of 5 mM. Also, the effect of different chemicals on the purified glucoamylase was studied by pre-incubating the enzyme for 30 min at 30 °C with several types of chemicals, including paramethyl sulfonyl fluoride (PMSF, at 5 and 10 mM), iodoacetamide (at 1.0 %), N-ethylmaleimide (at 5 and 10 mM), ethylenediaminetetraacetic acid (EDTA, at 10 and 50 mM), and ethylene glycol tetraacetic acid (EGTA, at 10 and 50 mM), separately. Residual activities in the presence of each chemical was then assayed and compared with the control activity obtained (hundred percent was assigned to the activity in the absence of these reagents).

RESULTS AND DISCUSSION

Selection and Identification of the Producing Strain:

Endophytic fungal cultures were isolated from marine soft coral *Dendronephtha hamprechii* as described before (Holler et al., 2000 and El-Bondkly and El-Gendy, 2010) on potato dextrose agar medium. All isolates were tested for amylolytic enzymes production by starch hydrolysis using starch agar medium, which inoculated with the isolated fungi and subsequently flooded with iodine solution, the zone of clearance around the microbial growth indicated the production of amylolytic enzymes (El-Safey and Ammar, 1994). On the basis of the area of clearance zone, one isolate under the isolation code JAN-25 was selected as the most promising producer of glucoamylase for further studies. The hyper amylolytic isolate JAN-25 was identified according to its morphologic and microscopic features according to the studies of Samson, (1979); Samson and Gams, (1984); Jernejc and Cimerman, (2001) and Watanab, (2002) as *Aspergillus* sp. JAN-25. Biotechnological advantages of laboratory solid-state fermentation of starchy materials with *Aspergillus* species were previously reported by many investigators (Hölker *et al.*, 2004; Zambare, 2010; Sidkey *et al.*, 2011 and Kumar *et al.*; 2011) and by endophytic fungi (Onofre *et al.*, 2011 and Marlida *et al.*, 2000a and b). Endophytic fungus *Colletotrichum gloeosporioides* has great capacity of producing amylolytic enzymes (α-amylase and glucoamylase) through fermentation in rice-based solid state (Onofre et al. 2011**).**

Optimization of Leaching Process of Glucoamylase Produced by Aspergillus sp. JAN-25 in SSF:

Among various leaching agents, 0.2 M citrate buffer (pH 4.0, 1:1, w/v) at 40 ºC and agitation state (150 rpm) leached maximum glucoamylase yield from the fermented rice bran (75.9 U gds^{-1}, Table 1).To evaluate the effect of solvent volume on the leaching process, the volume was varied from 1:1 to 1:10 (w/v). Citrate buffer volume of 1:6 (w/v) was capable of increasing extracting enzyme from the fermented rice bran from 75.9 to 135.1 $Ugds^{-1}$ (Table1). When glucoamylase was leached out and recovered at different contact time, a further increase of about 1.7-fold in leaching efficiency was observed when contact time was extended from 60 min to 120 min (Table1). The effect of pH on the extraction was also studied by incubating the fermented bran with citrate buffer of different pH ranging between 3.0 and 8.0. From Table 1, when pH of the extractant increased from 4.0 to 8.0, the leaching of enzyme from the fermented rice bran decreased and found maximum at pH 4.0 (229.8 U gds^{-1}). Moreover, data in Table 1 indicated that 45 ˚C was found to be the most effective temperature for the glucoamylase leaching out. When enzyme was recovered at 45 °C the quantum of recovery (264.5 $Ugds^{-1}$) was 125.11 % higher than at 30°C (117.5 $Ugds^{-1}$). Further increase or decrease in temperature resulted in decreases in the enzyme yield. To evaluate the effect of physical state on enzyme leaching from the fermented bran, the enzyme was extracted under agitation and static state, the leaching out of glucoamylase from fermented rice bran was much higher under agitation condition at 150 rpm (264.5 U gds^{-1}) than static condition (145.8 U gds^{-1}, Table 1). Recovery of the enzymes from the fermented matter is an important factor that affects the cost-effectiveness of the overall process. Negi and Banerjee (2009) tested various factors such as leaching agent selection, amount of leaching agent, soaking time, and temperature in order to determine optimum extraction conditions of glucoamylase produced by *Aspergillus awamari* nakazawa MTCC 6652 and optimum conditions were achieved in a 10% glycerol soaked for 2 h at 40 °C. Moreover, in a significant finding of Padmanabhan *et al.* (1992), the recovery of α-amylase from the solid fermented matter was depended on the temperature of extraction and when it was extracted at 50°C, the quantum of recovery was 2.2 fold higher than

at 30°C as well as they observed a further increase of about 19% in leaching efficiency when contact time was extended from 60 min to 120 min.

Optimization of Fermentation Conditions for the Production of Glucoamylase by Aspergillus sp. JAN-25 Screening Of Solid Substrates:

Aspergillus sp. JAN-25 was inoculated into Erlenmeyer flasks containing basal salt solution supplemented with 10 g of different solid substrates separately at 30 ºC, pH 5 with an initial moisture content of 60%, and inoculum level of 10^6 spore gds^{-1} for 1-5 days and then the enzyme was extracted and the productivity of the glucoamylase at different incubation periods in different solid substrates was recorded (Table 2). Among solid substrates screened for glucoamylase production in SSF, rice bran yielded substantially and consistently higher glucoamylase levels than wheat bran. The highest glucoamylase activity on rice bran was 264.53 U gds^{-1} compared with an activity of 225.16 U gds^{-1} obtained on wheat bran after 4 days of fermentation (Table 2). Moreover, potato peel followed by barely bran supported production of considerable amounts of enzyme (124.5 and 100.00 U gds^{-1}, respectively) while rice husk and corncob gave the lowest values of enzyme yields (43.61 U gds^{-1} and 57.14 U gds^{-1}, respectively). The best enzyme production on rice bran is probably due to the easy digestion of rice bran proteins as compared to that of the endosperm proteins present in wheat bran, as well as the higher content of starch in the former waste comparing to the later (Costa *et al.*, 1998). In a similar study, endophytic fungus *C. gloeosporioides* showed great capacity for producing amylolytic enzymes (α-amylase and glucoamylase) through fermentation in rice-based solid state without supplementation (Onofre *et al.*, 2011**).** The noticeable decreasing of glucoamylase productivity with rice bran after 4 days of fermentation (190.12 U gds^{-1} after 5 days of fermentation) may be due to the small flat particles of rice bran tended to pack together tightly to form a bed of low porosity. In contrast to rice bran, a bed of wheat bran had a more open structure. Therefore, wheat bran can improve rice bran properties for producing enzymes. Mixtures of rice bran and wheat bran were investigated as substrates for glucoamylase production (Table 2). A mixture of wheat bran and rice bran at a ratio of 1:2 by weight resulted in about 50.65 and 76.99 % increase in glucoamylase activity than using rice bran and wheat bran separately, respectively (after 4 days of fermentation). Therefore, in subsequent experiments this mixture was used as a substrate for glucoamylase production. In previous studies, production of very high levels of a hard starch-gel digesting amyloglucosidase under SSF using wheat bran, rice bran, other rice components and combinations of these has been reported (Singh and Soni, 2001). Moreover, rice bran and gingelly oil cake gave the highest and lowest specific activity of glucoamylase by *Aspergillus oryzae* (16.42 and 2.03 U/mg, respectively) but combination consisted of wheat bran, rice bran and ground oil coconut in the ratio of 1:2:2 showed significant increase in enzyme yield (Zambare, 2010). These data indicated that *Aspergillus* sp JAN-25 can be useful in bioprocessing application for saccharification of agro-residues. On the other hand, the highest yield of glucoamylase activity produced by *Aspergillus* sp. JAN-25 in short incubation period with mixed bran offers potential for inexpensive production of enzyme. Incubation period being an important parameter that has been controlled for optimum enzyme formation and it varies from organism to organism due to variation in the lag and log phases of growth (Bhatti *et al.*, 2007a and b). Regarding to incubation period our data are in line with those of glucoamylase produced by *Aspergillus niger* and *Fusarium moniliforme* in SSF, which reached their peak after 96 h of fermentation in SSF and then decreased (Bhatti *et al.*, 2007a and b).

Effect Of Substrate Mass To Flask Volume Ratio On Glucoamylase Production:

Effect of different amounts (5, 10, 15, 20, 25 and 30 g / flask) of the mixed bran (wheat bran: rice bran, 1:2) on glucoamylase production in 500 ml Erlenmeyer flasks was evaluated. Maximum enzyme activity (440.90 U gds^{-1}) was observed in the flasks containing 20 g of this mixture per 500 ml flask (2:50) (Table 3). Decreasing or increasing the level of substrate mass per flask gave relatively lower enzyme yields. In contrary to the present study, Ellaiah *et al.* (2002) and Bhatti *et al.* (2007b) reported that 1:50 and 1.5:50 of substrate mass to flask volume ratios were found optimum for glucoamylase production from *Aspergillus* sp. A3 and *Fusarium moniliforme* in SSF, respectively. The level of substrate per unit area of working volume of the flask influences the porosity and aeration of the substrate (Bhatti *et al.*, 2007 a,b) and hence affect the enzyme productivity.

Effect Of Initial Moisture Content On Glucoamylase Production:

The natural moisture (7–13%7) in bran is too low to support the metabolic activities of fungi; therefore the solid substrate needs to be moistened during preparation. Data in Table 3 clearly indicated that, 50 - 60% moisture content was found to be favorable for glucoamylase synthesis. Solid Substrate moistened at this level afforded a high glucoamylase activity value of about 440 U gds^{-1} at 96 h. Moisture levels higher than 60% reduced enzyme production as the substrate became waterlogged (Table 3). High moisture content is known to reduce porosity of substrate, causes particles to stick together and adversely impacts oxygen transfer to the mold. In contrast, a low moisture level reduces water activity to levels that are not conducive to supporting good fungal growth and metabolism (Norouzian *et al*., 2006). Maximum amylases yielded by *Penicillium* sp. X–1 was recorded when the initial level of moisture was 65%, which was 4.1-fold of that obtained at a moisture level

of 50% (Sun and Zhang, 2007). Moreover, the optimum initial moisture level for enzyme production by *Rhizopus* sp. A-11 and *Rhizopus* sp. MB46 were 48 and 45%, respectively (Morita *et al.*, 1998 and Morita and Fujio, 2000).

Effect Of Inoculum Size:

The impact of inoculum size on glucoamylase production was determined by varying the levels of inoculum (10^3 to 10^8 spore gds^{-1}) to 20 g of mixed bran at 60 % moisture after 96 h of incubation. Maximum enzyme activity (475.84 U gds^{-1}) was observed with 10^7 spore gds^{-1} (Table 3). A further increase in spore density leads to gradual decrease in glucoamylase formation. Inoculum density is an important consideration for SSF since higher inoculum levels are inhibitory factors for good growth and metabolites production while lower inoculum levels require more time for fermenting the substrates in SSF (Pandey *et al.*, 2005 and Bhatti *et al*., 2007b). On the other hand, not much difference in production was seen at different inoculum sizes compared to the production at optimum inoculum size (Anto *et al.*, 2006).

Effect Of Carbon Source Supplementation:

In mixed bran medium, better yield was achieved with the addition of starch followed by maltose and sucrose (491.5, 483.51 and 479.33 U gds^{-1}) (Table 3). Glucose and fructose supplementation resulted in the repression of enzyme production (195.24 and 210.43 U gds^{-1}, respectively). Data also clearly indicated that each of starch, maltose or sucrose lead to slight increase in enzyme productivity consequently *Aspergillus* sp. JAN-25 can produce high yield of glucoamylase without carbon supplementation. Similarly, Anto et al. (2006) reported that media supplementation with sucrose in wheat bran slightly increased enzyme production to 271 U gds^{-1}. On the other hand, productivity of raw starch degrading enzymes (RSDE) production is generally subjected to catabolite repression by glucose and other readily metabolizable substrates for example, catabolite repression of enzyme production by glucose was observed for *Aspergillus* sp. JGI 12 (Alva *et al.,* 2007). In contrary, Zambare (2010) reported maximum production of amylase when glucose was the carbon supplement and the solid substrate supplemented with glucose, sucrose or starch gives higher amylase yields than the un-supplemented SSF.

Effect of Nitrogen Source Supplementation:

The addition of inorganic nitrogen sources $(NH4)_2SO_4$, NH_4NO_3 and $NaNO_3$ along with the solid substrate show negligible increase in the yield of glucoamylase (0.28, 0.79 and 0.62 % respectively). In contrary, bran medium supplemented with organic nitrogen sources as soybean meal followed by the supplementation with malt extract, yeast extract, peptone and casein exhibited remarkably increase in the production of glucoamylase to 1.37, 1.25, 1.14, 1.12 and 1.05 -fold, respectively (Table 3). The supplementation of rice barn with nitrogen sources (organic or inorganic) to enhance amylolytic enzymes production by fungi was done with successful increasing of the yield of enzymes in SSF (Pandey, 2005). Whereas peptone and yeast extract are the common nitrogen sources for amylolytic enzymes production, soybean meal is a promising nitrogen source for amylases due to its low cost and availability (Pandey, 2005), Morita and Fujio (2000) reported that organic nitrogen sources negatively affected production of raw starch degrading enzymes by *Rhizopus* sp. MKU 40 since supplementation with organic nitrogen sources may induce the formation of protease, which resulted in the proteolysis of the enzyme degrading starchy materials.

Effect of Minerals Supplementation:

All minerals tested, showed enhanced glucoamylase yield. The best glucoamylase yield was achieved with $CaCl_2$ (700.25 U gds^{-1}) followed by NaCl (689.31 U gds^{-1}), $MgCl_2$ (682.5 U gds^{-1}) and KCl (679.04 U gds^{-1}), respectively (Table 3). Supplementation of salts of certain meneral ions influences the growth of microorganisms and thereby stimulates or inhibits enzyme production (Morita *et al*., 1998). For *Penicillium* sp. X-1, $MgSO_4$.$7H_2O$, $MnCl_2$, $ZnCl_2$ or $CaCl_2$ stimulated their production (Sun and Zhang, 2007). Therefore, the effect of metal ions on enzyme production varies from microorganism to another.

Effect of Some Detergents:

Whereas Tween-80 and Triton X-100, detergents stimulated glucoamylase activity by 32 and 25 %, SDS was negatively affected glucoamylase production by 9 % (Table 3). Some surfactants can influence the production and secretion of raw starch degrading enzymes through changing the permeability of cell membrane. These data are in line with that obtained for amylase production by *Penicillium* sp. X–1, which was doubled by Triton X-100 (Sun and zhang, 2007). On the other hand, Bhatti et al (2007b) reported a decrease in enzyme biosynthesis by *Fusarium solani* in SSF of wheat bran under optimum process conditions with the addition of surfactants. Therefore, surfactant effects depend on both its property and physiological responses of the fungal strain.

Effect of Incubation Temperature:

When the marine endophytic *Aspergillus* sp. JAN-25 was inoculated at different temperatures (30, 37, 40, 45, and 50 ºC) it showed maximum yield of glucoamylase (924.12 U gds^{-1}) at a range of 30 - 37 ºC in mixed bran medium. Then gradual decrease in the yield was observed as a response to increase in temperature (Table 3). However the optimum temperature for enzyme production was reported as 30ºC by Alva *et al.* (2007). According to Zambare (2010), higher temperature inactivation may be attributed to incorrect conformation of enzyme molecules due to hydrolysis of the peptide chain, destruction of amino acid or aggregation.

Effect of Initial pH:

As shown in Table 2, the initial pH of the substrates affected the peak of glucoamylase activity. The maximum yield of glucoamylase was found at pH 5.0. Glucoamylase production at this optimal initial pH was about 1.89 and 5.0-fold greater than at pH value of 7.0 and 9.0, respectively. This agrees with the knowledge that fungi and yeasts required acidic conditions (pH 4.0 - 6.5). According to Bhatti *et al*. (2007b) pH is one of the important factors that determine the enzyme secretion of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. The amylases yield by endophytic *Acremonium* sp. with the initial pH of 5.0 was 12.5-fold higher than that obtained on using initial pH 3.5 (Marlida *et al*., 2000 a, b). The present results showed a good coincidence with previous ones on fungal production of glucoamylase that have been reported acidic conditions for hyper glucoamylases production. It was pH 5.8 for glucoamylase production by *Aspergillus oryzae* (Zambare, 2010) and pH 5.4 at 96 h and 28°C for glucoamylase produced by endophytic fungus *C. gloeosporioides* (Onofre *et al*., 2011).

Purification of Glucoamylse from Aspergillus sp. JAN-25:

Crude glucoamylase preparation was purified by successive steps including, $(NH4)_2SO_4$ fractionation, gel filtration (sephadex G-200) and DEAE sepharose chromatography. The fraction attained at 70% saturation of ammonium sulphate resulted in specific activity of 47.64 U mg^{-1} proteins, 2.75 purification folds and recovery of 46.98% (Table 4). Following SephadexG-200 gel filtration, the purification of the glucoamylase was increased 14.64-fold with overall yields of 26.26% (Table 4). The purified enzyme obtained with the DEAE-Sepharose CL-6B column chromatography exhibited 23.34% of the total initial activity and there was a 35.41-fold increase in specific activity (613.30 U/mg) when compared with the crude enzyme. The purified glucoamylase appeared as a single protein band in SDS-PAGE with molecular weight of approximately 43 kDa (Fig. 1). Molecular weights of microbial amylolytic enzymes are usually ranged 40 - 60 kDa (Vihinen and Mantsala, 1989). Suntornsuk and Hang (1997) found that molecular weight of glucoamylase produced by *Rhizopus* oryzae mutant 4U2 was found to be 82 kDa but from other fungal species it varies between 58 and 100 kDa. With regards to the reported findings, the molecular weight of the amylolytic enzymes obtained from *Aspergillus flavus* F2Mbb, *A. flavus*, *A. niger* JGI 24, *Penicillium camemberti* PL21 and *A. niger* were approximately 56, 52.5 (+/- 2.5), 43, 60.5 and 118.17 kDa by SDS-PAGE, respectively (Sidkey *et al.*, 2011; Khoo *et al.*, 1994; Varalakshmi *et al.*, 2009; Nouadri *et al.*, 2010 and Slivinski *et al.*, 2006).

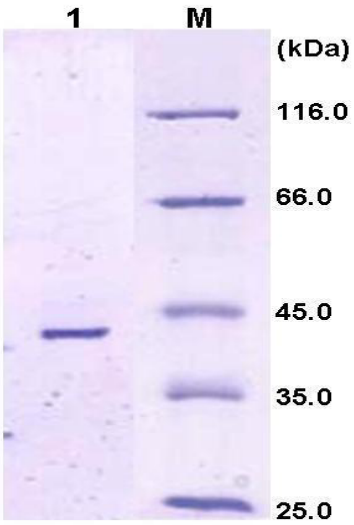

Fig. 1: SDS-PAGE of the purified glucoamylase from *Aspergillus* sp. JAN-25. Lane M, standard molecular mass markers (116.0, 66.0, 45.0, 35.0 and 25.0 kDa), lane 1, purified glucoamylase, proteins were stained with coomassie brilliant blue R-250.

Effect of Temperature on Purified Enzyme Activity and Stability:

In this study, fungal glucoamylase activity was maximal at 50 - 60 ˚C and above this temperature activity decreased. Moreover, enzyme being stable up to 65 ºC and above this temperature stability gradually decreased. It retained 97 and 83 % of its activity at 70 and 80˚C, respectively (Fig. 2). Optimum temperature of glucoamylase is generally in the range of 50 to 60 ºC; as reported by Anto *et al.* (2006). Our data are in line with that obtained by Kumar *et al*., (2011) they reported that the enzyme was optimally active at pH 5 and 50 °C with starch as substrate and it was almost stable at 60 °C even after 50 minutes of incubation. However, the optimum temperature of the purified amylases was found 30 °C for the enzyme produced by *A. flavus* F2Mbb (Sidkey *et al.*, 2011), *A. niger* JGI 24 (Varalakshmi *et al.*, 2009), *Penicillium camemberti* PL21 (Nouadri *et al.*, 2010) and *R. oryzae* (Suntornsuk and Hang, 1997) as well as 35 °C for that resulted from *A. flavus* var *columinaris* (El-Safey and Ammar, 2004). On the other hand, Khoo *et al.* (1994) reported optimum activity at 40 – 50 °C for the enzyme produced by *A. flavus*.

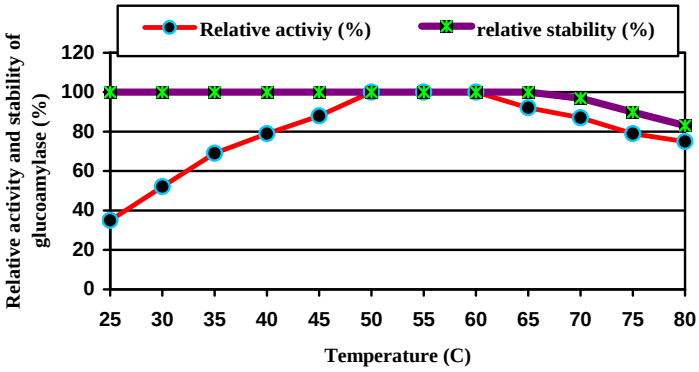

Fig. 2: Effect of temperature on activity and stability of glucoamylase produced by endophytic Aspergillus sp.JAN-25.

Effect of pH on Purified Enzyme Activity and Stability:

Glucoamylase exhibited maximum activity at pH values between 4.5 and 5.5 and then decreased with rising or lowering pH than the optimum one (Fig. 3) .It retained 90 and 54 % of its activity at pH 7.0 and 10.0, respectively. The study on pH stability of the glucoamylase enzyme revealed that the enzyme exhibited good stability over acidic pH range and retaining 100% activity at pH 4.0 - 6.5 (Fig. 3). It has been reported that fungal glucoamylase act well in lower pH (Kumar *et al*., 2011). In comparison with our result, maximum activity of amylolytic enzymes was obtained at pH 9.5 for glucoamylase of *A. niger* JGI 24, pH 6.4 for that of *A. flavus* F2Mbb, pH 6.2 for that of *A. flavus* var *columinaris* and 4.5-5.0 for that of *A. niger* (Varalakshmi *et al*., 2009; Sidkey *et al*., 2011; El-Safey and Ammar, 2004 and Slivinski *et al.*, 2006).

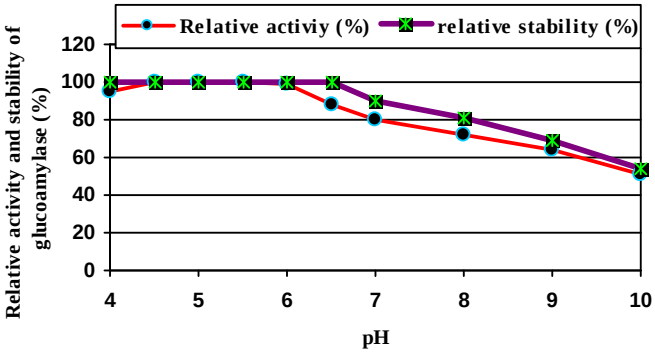

Fig. 3: Effect of pH on activity and stability of glucoamylase produced by endophytic Aspergillus sp. JAN-25.

Effect of Substrate Concentration on Purified Glucoamylase Activity:

Enzyme activity was evaluated with different concentrations (0.25 to 5%) of soluble starch and maximum activity was found with 2%. This activity remained unchangeable with further increase in starch concentration (Fig. 4). Sidkey *et al.* (2011) reported that maximum amylases activity from *A. flavus* F2Mbb was attained at the starch concentration 0.1%. Additionally, the optimum concentration of soluble starch for α-amylase activity was 0.2% for the enzyme attained from *A. flavus var columinaris* (El-Safey and Ammar, 2004) and 1% for that from *Penicillium camemberti* PL21 (Nouadri *et al*., 2010).

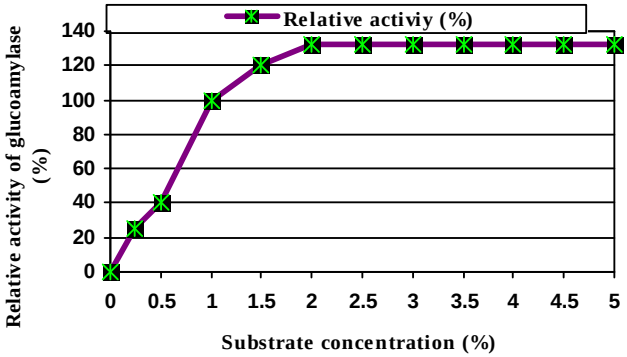

Fig. 4: Effect of substrate (starch) concentration on activity of glucoamylase produced by endophytic Aspergillus sp. JAN-25.

Effect of Different Chemicals on Purified Glucoamylase Activity:

Table (5) indicated that Cd^{2+}, Hg^{2+}, Pb^{2+}, Fe^{3+}, Zn^{2+}, Fe^{2+} and Cu^{2+} were negatively affected enzyme activity by 85, 80, 65, 43, 41, 35 and 30 %, respectively but Mn^{2+}, Ca^{2+}, Mg^{2+}, Na^{+} and Ba^{2+} markedly activate glucoamylase activity by 15, 12, 10, 9 and 5 % ,respectively. Co^{2+} exhibited no effect on glucoamylase activity. The inhibition observed with some metals may be due to the competition between the exogenous cation and the protein associated cation, resulting in reduce of metalloenzyme activity. N-ethylmaleimide had no inhibitory effect on the enzyme activity, hence it was concluded that, for this amylase no sulphydryl or carboxyl residues are essential for its catalytic activity. This result is in agreement with the amylase produced by *Penicillium janthinellum* NCIM 4960 (Sindhu *et al*., 2011). The inhibitors PMSF and iodoacetamide had no effects on glucoamylase activity suggest that serine and cysteine residues are not involved in the catalytic mechanisms as reported previously by El-Gendy (2010) for metallo-keratinase enzymes produced by endophytic *Penicillium* sp. Morsy1. On the other hand, the chelating agent EDTA and EGTA markedly decreased glucoamylase activity to 70 and 74 %, respectively at 10 mM and to 41 and 52 % at 50 mM, demonstrated the ion requirements of this glucoamylase activity. Most amylases are metallo enzymes containing at least one metal atom per molecule of enzyme, thus this type of inhibition is very common among amylolytic enzymes with ion requirements. Similarly, P. *janthinellum* NCIM 4960 amylases were inhibited by 32 % in the presence of EDTA and it was potent inhibited by Hg2+, Zn2+, Cu2+, Ag+ and Pb2+ as reported by Sindhu *et al*. (2011).

Table 1: Optimization of extraction process parameters of glucoamylase (U gds^{-1}) in SSF over different incubation periods.

Optimized parameters*	glucoamylase (U gds^{-1})
Screening of leaching agents (1:1, w/v with agitation)	
H_2O	31.4
Glycerol	47.0
Methanol	35.3
Ethanol	30.2
Acetone	56.9
Ethyl acetate	44.0
NaCl (1.0%)	31.5
KCl (1.0%)	31.0
$CaCl_2$ (1.0%)	35.7
Citrate buffer (pH 4.0, 0.2 M)	75.9
Phosphate buffer (pH 7.0, 0.2 M)	39.6
Glycine-NaOH buffer (pH 10.0, 0.2 M)	14.0
Tween 80 (0.1%)	60.0

Screening of extractant agent: fermented rice bran ratio (w /v)	
1:1	75.9
1:2	90.8
1:4	110.8
1:6	135.1
1:8	129.4
1:10	124.4
Screening of contact time between moldy bran and leaching agent (min)	
30	69. 5
60	135.1
90	152.3
120	229.8
150	170.7
180	122.0
Screening of pH of extraction process	
3	189.1
4	229.8
5	175.3
6	128.6
7	78.5
8	50.9
Screening of temperature of extraction process (ºC)	
30	117.5
40	199.4
45	264.5
50	200.9
60	157.0
70	100.6
Physical state	
Agitation condition (150 rpm)	264.5
Static condition	145.8

*Each parameter once optimized was fixed for subsequent studies.

Table 2: Screening and optimization of solid substrates for glucoamylase production (U gds^{-1}) in SSF over different incubation periods.

Solid substrates (10 g / flask)	**glucoamylase production(U gds^{-1}) during different fermentation period (day)**				
	1	**2**	**3**	**4**	**5**
Rice husk	8.74	14.30	20.55	39.7	43.61
Potato peel	33.5	110.24	118.12	124.5	124.00
Corncob	14.62	22.90	36.70	48.0	57.14
Rice bran	71.17	186.04	219.3	264.53	190.12
Wheat bran	42.15	90.56	170.25	225.16	225.00
Barely bran	35.02	62.10	80.16	100.00	100.00
Groundnut shell	27.04	39.68	55.92	75.7	81.36
Banana peel	10.96	25.78	49.06	76.8	80.00
wheat bran: rice bran mixtures					
1: 2	120.40	198.74	330.11	398.52	390.70
1: 1	105.38	161.0	290.50	328.90	318.04
2: 1	85.20	135.25	260.47	290.32	288.72

Table 3: Optimization of production conditions of glucoamylase (Ugds^{-1}) in SSF.

Optimized parameters*	**glucoamylase production (U gds^{-1})**
Substrate mass to flask volume ratio	
0.5 : 50	179.85
1.0 : 50	398.43
1.5 : 50	425.02
2.0 : 50	440.90
2.5 : 50	385.07
3.0 : 50	279.52
Initial moisture level (%)	
40	299.14
50	440.15
60	440.08
70	384.75
80	302.41
90	205.00
Spore concentration (spores gds^{-1})	
10^3	119.32
10^4	230.01

10^5	360.86
10^6	440.10
10^7	475.84
10^8	400.02
Effect of different carbon sources supplementation (0.04 g gds-1)	
Glucose	195.24
Fructose	210.43
Maltose	483.51
Lactose	423.0
Sucrose	479.33
Starch	491.50
Effect of different nitrogen sources supplementation (0.02 g gds $^{-1}$)	
Control	491.14
Yeast extract	560.90
Malt extract	612.82
Peptone	549.02
Soybean meal	674.60
Casein	514.54
$(NH4)_2SO_4$	492.50
NH4 NO_3	495.00
$NaNO_3$	494.18
Effect of minerals supplementation (1%)	
$CaCl_2$	700.25
NaCl	689.31
KCl	679.04
$MgCl_2$	682.50
Effect of detergents (1%)	
Triton X-100	875.49
Tween-80	924.00
SDS	637.23
Effect of incubation temperature (ºC)	
30	924.12
37	924.12
40	659.40
45	320.6
50	62.0
Effect of pH of medium	
4.0	611.72
4.5	852.90
5.0	920.27
5.5	847.25
6.0	724.15
6.5	593.48
7.0	487.74
8.0	290.00
9.0	184.05

*Each parameter once optimized was fixed for subsequent studies.

Table 4: Glucoamylase purification steps from *Aspergillus* sp. JAN-25.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude extract	359.65	6227.45	17.32	100	1
70% $(NH4)_2SO_4$ fraction	61.41	2925.54	47.64	46.98	2.75
Sephadex G-200	6.45	1635.55	253.57	26.26	14.64
DEAE Sepharose	2.37	1453.53	613.30	23.34	35.41

Table 5: Some factors affecting glucoamylase activity produced by *Aspergillus* sp. JAN-25.

Parameter	Concentration (mM)	Relative activity (%)*
Effect of different ions on glucoamylase activity		
None	0	100
Hg^{2+}	5	20
Zn^{2+}	5	59
Cu^{2+}	5	70
Cd^{2+}	5	15
Pb^{2+}	5	35
Fe^{2+}	5	65
Fe^{3+}	5	57
Mg^{2+}	5	110
Ba^{2+}	5	105
Mn^{2+}	5	115
Co^{2+}	5	100
Ca^{2+}	5	112
Na+	5	109

Effect of enzymatic inhibitors		
EDTA	10	70
	50	41
EGTA	10	74
	50	52
N-ethylmaleimide	5	100
	10	100
paramethyl sulfonyl fluoride (PMSF)	5	101
	10	100
Iodoacetamide	1%	100

*One hundred percent (%) was assigned to the activity in the absence of these reagents.

Conclusion:

Based on the above findings, it could be concluded that the endophytic fungus *Aspergillus* sp. JAN-25 demonstrated high capacity for the production of glucoamylase through agro/ agro industrial residues based solid state fermentation. Thus it can be industrially exploited for the synthesis of glucoamylase. As well as optimization and improvement of process parameters carried out in this study proved to be fruitful in enhancing programs for enzymes of biotechnological important.

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