substantial amount of glucoamylase in submerged (Berka *et al.* 1992) and solid-state fermentation (Alazard and Raimbault 1981).

Traditionally, glucoamylases have been produced by submerged fermentation (SmF). A recent focus of the research and development effort is the application of glucoamylases in the enzymatic degradation of carbohydrate rich polysaccharides for production of energy syrup. The development of microbial strains, media composition and process control all have contributed to the achievement of high levels of extracellular glucoamylases. However, the glucoamylase costs are still too high for the establishment of a cost effective production of energy syrup. One approach to overcome this obstacle is to employ solid state fermentation (SSF). The SSF process has the potential to significantly reduce the enzyme production costs because of lower energy requirements, increased productivity, smaller effluent volumes and simpler fermentation equipment (Ellaiah *et al.* 2002). In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and thus optimum moisture level of the substrate is the most important factor in enzyme production (Baysal *et al.* 2003).

Agro-industrial residues are generally considered best substrates for the SSF processes and enzyme production by SSF is not an exception to that (Ellaiah *et al.* 2002). Use of suitable low cost fermentation medium for production of α-amylase by using agricultural byproducts has been reported (Ikram-ul-Haq *et al.* 2003). The production of amylolytic enzymes, particularly glucoamylase on solid substrate is more advantageous for the fermentation industry [Joshi et al, 1999; Ghildyal *et al.* 1985). Cereal bran and flours, potato residue and other starchy waste materials have been utilized as fermentation substrate for glucoamylase production by filamentous fungi (Joshi *et al.* 1999; Biesebeke *et al.* 2005). Microbial degradation of these residues by GRAS (generally regarded as safe) strain may improve the substrate value as animal feed (Ramachandran *et al.* 2004). Glucoamylase production by *A. niger* was extensively studied using wheat bran in SmF and SSF by Kaur *et al.* 2003. Wheat bran, paddy husk, rice processing wastes or other such starch containing wastes have gained importance as supports for fungal growth during glucoamylase production (Arasarnam *et al.* 2001).

Since utility of *A. oryzae* has not been investigated for the commercial production of glucoamylase, the present study is undertaken to evaluate the use of agro-residues as solid substrate to cultivate *A. oryzae* for improved production of glucoamylase under SSF.

Materials and Methods

Microorganism and its maintenance

Aspergillus oryzae was propagated on Czapak Dox agar (CZA) medium (Difco, Germany). Slants were grown at 30^0C for 5 days and stored at 4^0C.

Inoculum preparation

A piece of culture from 6 days old slant was used to inoculate in seed flask containing 10 g wheat bran with 100% moisture and incubated for 6 days at 30^0C. After incubation, fermented dough was mixed aseptically followed by addition of 50 mL of saline containing 0.1% Tween-80. After 30 min mixture was filtered off through sterile glass wool to get spores. Spore count was determined by serial dilution and spread plating method.

Solid State fermentation

All agro residues rice husk (RH), wheat bran (WB), rice bran (RB), cotton seed powder (CSP), corn steep solid (CSS), bagasse powder (BP), coconut oil cake (COC), groundnut oil cake (GOC), corn steep liquor (CSL) and soybean meal were collected from local market and preserved at room temperature. Agro residues (10 g) were kept separately in a 250 mL Erlenmeyer flask and then moistened with 10 mL of water and sterilized at 121 ^{o}C for 30 min. The fermentation process was started by adding one mL of spore suspension (5 x 10^7 spores/mL) as prepared above. The whole content was mixed thoroughly and then incubated at 30 ^{o} C for 5 days in a stationary condition.

Enzyme extraction

To the fermented dough 50 mM citrate buffer (pH 5) (1:10) was added and homogenized for 2 h with a constant stirring at room temperature. This suspension was filtered through Whatman filter paper number 1 and the filtrate was again centrifuged at 6000 rpm for 15 min.
This solid-free supernatant was used as enzyme source for assaying glucoamylase activity.

Production optimization

Glucoamylase production was optimized with respect to various nutritional and environmental parameters such as 10g agro-residues (WB, RB, RH, CSP, CSS, BP, COCGOC); initial moisture content (50-110%, v/w); inoculum size (1-10%, v/w); nitrogen sources [inorganic (0.25% w/w) - ammonium sulphate, ammonium phosphate, ammonium nitrate, sodium nitrate, urea; organic (1%)-yeast extract, tryptone, beef extract, malt extract, peptone, CSL, soybean meal]; carbon inducer (1% w/w) [glucose, fructose, lactose, maltose, sucrose, starch, glycerol], pH (3-9) and temperature (20-40^0C). All the experiments were conducted independently in duplicate and the data presented here are mean value ± standard deviations (SD).

Enzyme 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. This reactive mixture was incubated at 50 ^{o} C for 20 min and then released reducing sugars were measured with 3, 5-dinitrosalicyclic acid (DNSA) reagent (Miller *et al.* 1959) using glucose as a standard. Glucoamylase activity unit (U) was expressed as the amount of enzyme releasing one µmole of glucose equivalent per minute under assay condition and enzyme activity is expressed in terms of units per gram dry fermented substrate (U/gdfs).

Biomass estimations

Biomass estimation was done by N-acetyl glucosamine released after acid hydrolysis of chitin present in fungal cell wall (Shivaramakrishnam *et al.* 2007). After acid treatment of biomass, liberated glucosamine from chitin was mixed with 1mL of acetyl acetone reagent and incubated in boiling water bath for 20 min. After cooling, 6 mL ethanol was added followed by addition of 1mL Ehrlich reagent and incubated at 65^0C for 10 min. After cooling optical density was read at 535nm against reagent blank and glucosamine as standard. Biomass is expressed in terms of mg of N-acetyl glucosamine released per gram of dry fermented substrate (mg/gdfs).

Time course of glucoamylase production

Glucoamylase was produced at optimum conditions of 10g, wheat bran, 1% (w/w) starch, 0.25%, (w/w) urea, pH 5, 5% inoculum and 30^0C. Samples were withdrawn aseptically after every 24 h and analyzed for glucoamylase activity and biomass by method as described above.

Results and Discussion

Total of 8 agro residues (Fig. 1) were screened. WB gave the highest enzyme production (1602U/gdfs) followed by RB (1271 U/gdfs). Wheat bran as the most promising substrate for glucoamylase production has been reported by several researchers (Kaur *et al.* 2003; Anto et al 2006; Pandey et al 1999). Production of very high levels of a hard starch-gel digesting amyloglucosidase under SSF using wheat bran, rice bran, other rice components and combination of these has been reported (Singh and Soni, 2001). Rice husk and cotton seed powder yielded almost same enzyme units (875 U/gdfs). Enzyme production was lower with BP, GOC, COC and CSS. Shivaramkrishnan *et al.* (2007) also studied agro residues for glucoamylase production from *A. oryzae var brunneus* and obtained maximum production with wheat bran and significantly good production with oil cakes.

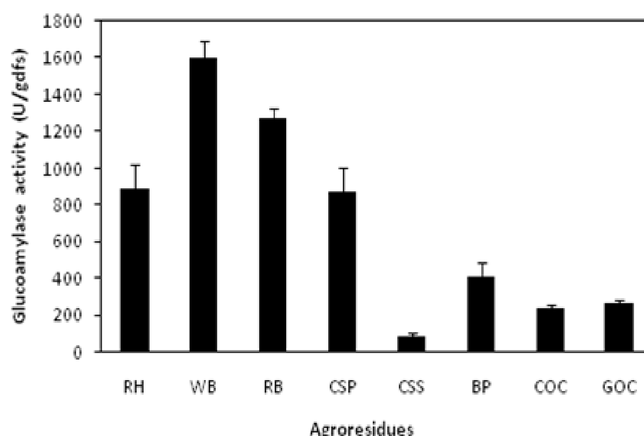

Figure 1. Effect of different agro-residues on glucoamylase by *A. oryzae* in SSF (pH7, 30^0C, 100% initial moisture, 1% inoculum, 120 hrs of incubation). Results represent the mean of duplicate analysis and bar indicates ± standard deviation.

Glucoamylase production increased with an increase in initial moisture content (Fig. 2) with optimum at initial moisture content 100% (v/w). Generally 40-70% of initial moisture content has been reported for fungal growth and substrate utilization. Fungal growth occurred at low moisture content but the enzyme yield was significantly low i.e. 21-54% residual activities from 50-80^{0}C. Kunamneni *et al.* (2005) reported maximum amylase production with 90% v/w initial moisture content. Reduction in enzyme activities was associated with early sporulation and also non - availability of nutrient due to low moisture or water activity. Low water activity affected the microbial activities because of limited nutrient solubilization and low degree of substrate swelling. Even high moisture content affected the microbial enzyme activities because of substrate stickiness, clumps, less porous nature of substrate and very limited oxygen transfer in vessel.

Highest enzyme production (1672-1693 U/gdfs) was observed at inoculum level of 5-8% (v/w) as shown in Fig 3. Kunamneni *et al.* (2005) reported maximum amylase production with 1% v/w inoculum. This indicated that high concentrations of spores are responsible for increased enzyme production because of higher substrate specificities and availability.

Except urea and ammonium sulphate all other organic and inorganic nitrogen sources used in our work had dimishing effect on glucoamylase production (Table 1). Urea (0.25%) was found to enhance enzyme activity by 10% as compare to basal media. The beneficial effects of the addition of urea nitrogen have been reported in submerged fermentations for glucoamylase production by *Aspergillus awamori* (Bertolin *et al.* 2003). Ammonium sulphate showed neutral effect on enzyme secretion while negative response was observed with ammonium phosphate, ammonium nitrate and sodium nitrate. Likewise, inorganic nitrogen sources were reported to negatively affect amylase production for *A. oryzae* (Jin *et al.* 1998) Organic nitrogen sources like yeast extract, tryptone, peptone and soybean meal reduced enzyme production by 30-35% while drastic reduction was observed with corn steep liquor, beef extract and malt extract. Previous findings have shown that peptone, sodium nitrate and casein hydrolysate are good nitrogen supplements for amylase production in *A. niger*, *Thermomyces lanuginosus* and *A. oryzae* (Ramachandran *et al.* 2004; Shivaramakrishnam *et al.* 2007; Kunamneni *et al.* 2005).

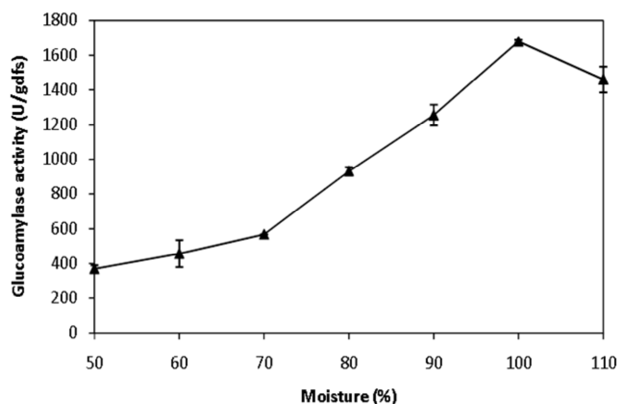

Figure 2. Effect of moisture on glucoamylase production by *A. oryzae* on wheat bran bed in SSF (pH7, 30^{0}C, 5% inoculum, 120 hrs of incubation). Results represent the mean of duplicate analysis and bar indicates ± standard deviation.

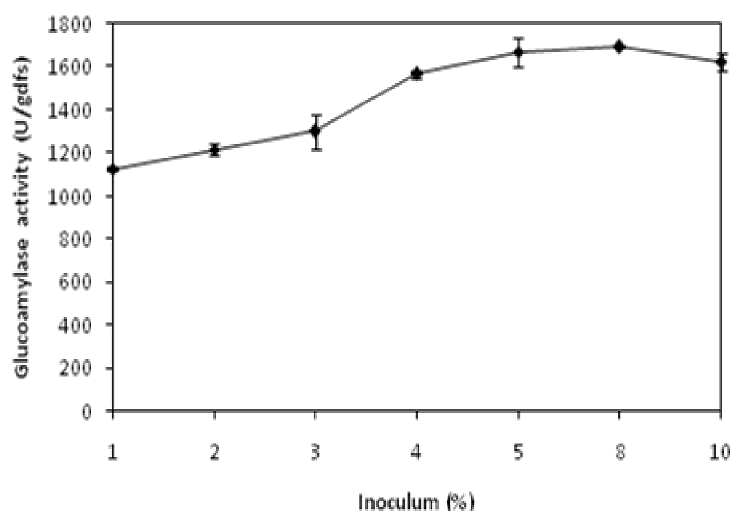

Figure 3. Effect of % inoculum size on glucoamylase production by *A. oryzae* on wheat bran bed in SSF (pH7, 30^0C, 100% initial moisture, 120 hrs of incubation). Results represent the mean of duplicate analysis and bar indicates ± standard deviation.

Table 1. Effect of nitrogen sources on glucoamylase production by *A. oryzae* under SSF (wheat bran bed, pH7, 30^0C, 5% inoculum, 100% initial moisture, 120 hrs of incubation)

Nitrogen sources	Glucoamylase activity (U/gfs) [a]
Inorganic nitrogen sources (0.25%, w/w)	
Ammonium sulphate	1674±21.2
Ammonium phosphate	677±2.82
Ammonium Chloride	772±4.94
Ammonium nitrate	873±24.74
Sodium nitrate	583±7.07
Urea	1870±27.57
Organic nitrogen sources (1%, w/w)	
Yeast extract	1289±2.1
Peptone	1115±28.2
Tryptone	979±16.2
Soybean meal	1050±6.36
CSL[b]	265±43.1
Beef extract	135±15.5
Malt extract	460±6.36
Control (without supplement)	1687±12.02

[a] Values are means of duplicate determinations with SD (±), [b] CSL, Corn steep liquor

A 1.16 fold induction in glucoamylase activity was observed with starch supplement at 1% concentration while other carbon supplements showed reduced effect (Table 2). Likewise starch as amylase inducer was reported by Shivaramakrishnan *et al.* (2007) and Cherry *et al.* (2004).

Table 2. Effect of carbon inducer on glucoamylase production by *A. oryzae* under SSF (wheat bran bed, 0.25% urea, pH7, 30^{0}C, 5% inoculum, 100% initial moisture, 120 hrs of incubation)

Carbon sources (1%)	Glucoamylase activity (U/gfs) [a]
Starch	1969±26.16
Sucrose	1092±21.9
Lactose	985±9.19
Maltose	1050±6.36
Glycerol	351±7.77
Glucose	550±9.1
Fructose	454±5.6
Control (without supplement)	1695±7.77

[a] Values are means of duplicate determinations with SD (±)

Glucoamylase was produced in pH range of 3-9 with optimum (1553 U/gdfs) at pH 5 (Fig. 4) indicating broad pH for enzyme secretions. Likewise, broad range of pH 3-9 was reported for synthesis of amylase from *A. oryzae* (Shivaramakrishnan *et al.* 2007).

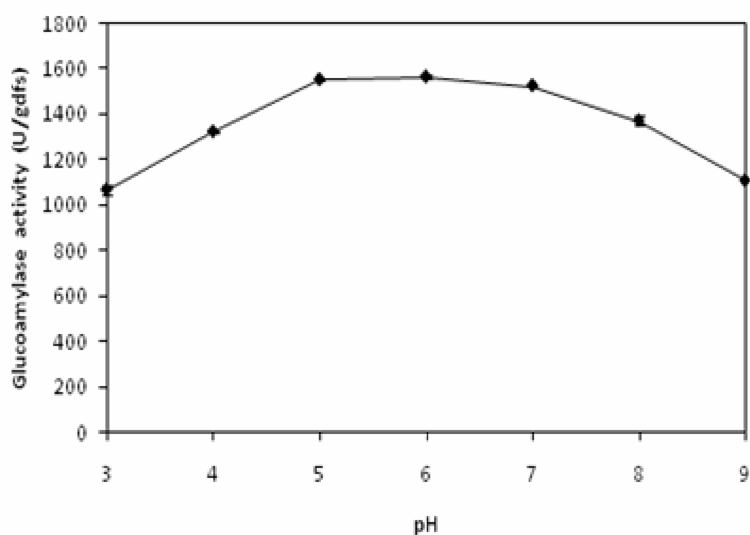

Figure 4. Effect of initial pH on glucoamylase production by *A. oryzae* on wheat bran bed supplemented with 1% starch, 0.25% urea, in SSF (30^{0}C, 5% inoculum, 100% initial moisture, 120 hrs of incubation). Results represent the mean of duplicate analysis and bar indicate ± standard deviation.

Glucoamylase synthesis occurred between 20-40^{0}C with optimum (1666 U/gdfs) at 30^{0}C (Fig. 5). A decrease in enzyme activities was observed in mesophilic temperature range. Similar results have been previously reported for amylase production by *A. oryzae (*Jin *et al.* (1998) and Francic *et al.* (2003).

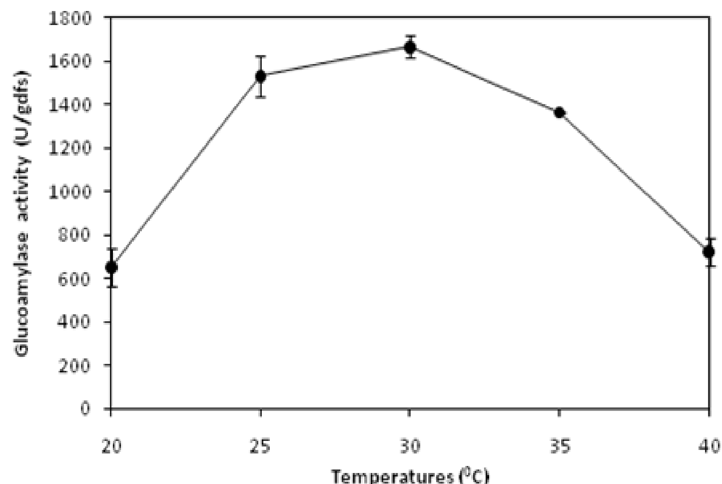

Figure 5. Effect of incubation temperature on glucoamylase production by *A. oryzae* on wheat bran bed supplemented with 1% starch, 0.25% urea in SSF (pH 5, 5% inoculum, 100% initial moisture, 120 hrs of incubation). Results represent the mean of analyses and bar indicate ± standard deviation.

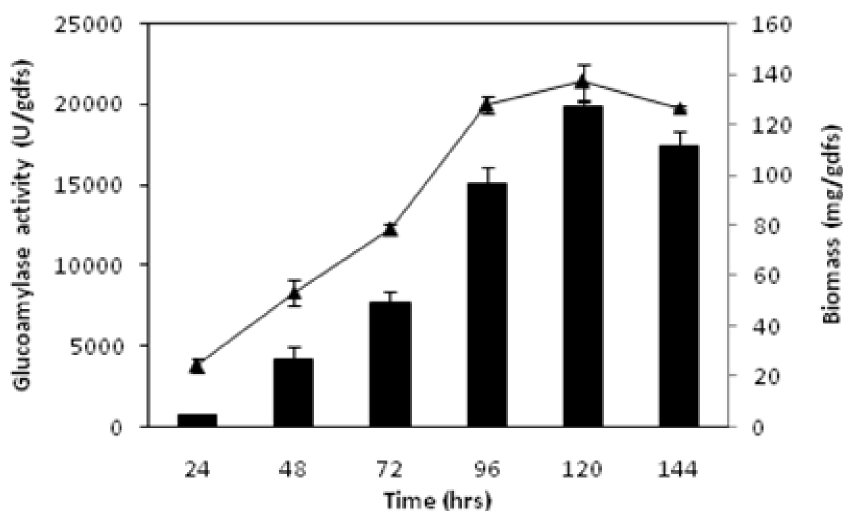

Figure 6. Time course of glucoamylase and biomass production by *A. oryzae* on wheat bran bed supplemented with 1% starch, 0.25% urea in SSF (pH5, 30^0C, 5% inoculum, 100% initial moisture). Results represent the mean of duplicate analysis and bars indicate ± standard deviation.

The maximum productivity of glucoamylase (1986 U/gdfs) was achieved in 120 h at 30^0C on a wheat bran substrate having an initial moisture content 100% at a pH of 5.0, an inoculum level of 5% (v/w), and soluble starch (1% w/w) and urea (0.25%) as supplements in a 250 mL flask per 10 g substrate. (Fig.6). Maximum glucoamylase production (726 U/g dry medium) from *A. niger* NCIM-548 was observed in 84 h on the solid surface of groundnut shell (0.5 mm particle size) supplemented with sucrose (1%, w/v) and yeast extract (0.5%, w/v) in *Aspergillus* minimal medium with 50% initial moisture content (Paulchamy 2008).

Conclusion

It has been demonstrated that *Aspergillus oryzae* has the potential to utilize agricultural waste for production of glucoamylase enzyme. The glucoamylase activity of 1986 U/gdfs can be obtained on inexpensive and easily available substrate-wheat bran by *A. oryzae* in SSF. Additional carbon (starch) and nitrogen (urea) supplements in wheat bran SSF increase glucoamylase yields. This can have important implications in enzymatic breakdown of polysaccharides by *A. oryzae* for the establishments of a robust and cost effective process in food and feed industry.

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