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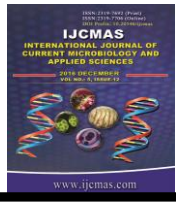

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Enhanced Production of β-glucosidase by New Strain *Aspergillus protuberus* on Solid State Fermentation in Rice Husk

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A B S T R A C T

The potential production of β-glucosidase by new strain *Aspergillus protuberus* under solid state fermentation was studied. Cultural and nutritional factors affecting β-glucosidase production were also investigated in order to optimize the fermentation conditions for the maximization of production. Different fermentation parameters such as different solid substrates, carbon and nitrogen sources, initial pH, moisture content, temperature, and optimized medium on β-glucosidase production were investigated. Rice husk served as the best solid support for maximal production of β-glucosidase. A maximum β-glucosidase production 26.06 U/g of rice husk was achieved under optimal conditions such as 1% glucose, 2% ammonium sulfate, pH 3, 40% moisture content, 30^{o}C temperature. An approximately 6-fold increase in β-glucosidase production was achieved in the optimized medium as compared with the nonoptimized medium. These results indicate that β-glucosidase production could be improved using these kind of processes. Also, taking into consideration locally available cheap substrates, economic side of the route is justified.

Introduction

Agricultural, forests and agro-industrial practices are among the causes of environmental pollution. Renewable lignocellulosic biomass, besides being cheap and abundant, has also the advantage that it does not compete with food production. Active efforts were being made to convert these lignocellulosic resources into either glucose or alcohol, and use this either as fuel or as valuable products (Kumakura *et al.*, 1988). Saccharification of polysaccharides to glucose by microbial hydrolytic enzymes which had attracted the attention of the researchers, as this was the first step of bioconversion of lignocellulosic material into valuable products such as sugar, fine chemicals and biofuels (Howard *et al.*, 2003). As the cost of cellulosic substrates play the central role in determining the economy of the saccharification process, lot of emphasis had been given to the usage of low price substrates and therefore screening of the agricultural wastes for release of sugars as organic wastes from renewable forest and agricultural residues (Heck *et al.*, 2002). The saccharification of different agro wastes had been reported by other workers employing enzymes from various organisms (Katzen and Fowler 1994; Van Wyk and

Leogale 2001; Baig *et al.,* 2004; Chandra *et al.,* 2007). ß-Glucosidases (ß-D-glucoside glucohydrolase, E.C. 3.2.1.21), under physiological conditions, catalyze the hydrolysis of ß-1,4-glycosidic bonds from the non-reducing termini presented in alkyl- and aryl-ß-D-glycosides, as well as different oligosaccharides (containing 2-6 monosaccharides). The deficiency in ß-glucosidase activity causes the accumulation of the disaccharide cellobiose, leading to the repression of enzyme biosynthesis and end-product inhibition of the upstream enzymes, which result in a limited hydrolysis yield (Zaldivar *et al.,* 2001). Therefore, commercially available cellulolytic preparations are often supplemented with ß-glucosidase to boost the overall activity, such as that prepared from *Trichoderma reesei* cellulases (Chauve *et al.,* 2010). ß-Glucosidase is the rate-limiting factor in the conversion of cellulose to glucose for the subsequent production of fuel ethanol.

ß-Glucosidases have been the subject of interest in recent research due to their potential for many biotechnological applications in food products, pulp and paper, jams, juices, biomass conversion and pollution control (Tolan and Foody, 1999). These enzymes can be recovered easily from SSF, making this system extremely appropriate for protein enrichment and cellulase production from lignocellulosics (Kim *et al.,* 1985). In general, the industrial applicability of an enzyme is closely related the cost of its production and physicochemical characteristics. The production costs can be reduced by screening hyper producer strains, associated with the cultivation process optimized in low-cost mediums (Leite *et al.,* 2008). The objective of this research was to evaluate the ß-glucosidase production by *A. protuberus* and to determine the influence of some parameters to maximize the production of this enzyme.

Materials and Methods

Culture and Preparation of Inoculum

Aspergillus protuberus isolated from the decaying Mahanandi forest litter soils was maintained on Czapek Dox medium and spore suspension was prepared from 7 days grown old slants by adding adequate amount of sterile distilled water with Tween-20 (0.2%,v/v). This fungal culture was maintained on Czapek Dox medium.

Lignocellulosic Substrates

Lignocellulosic substrates such as castor husk, sugarcane bagasse, sesame husk, groundnut fodder, rice husk, tea residue, sorghum husk and sawdust were chosen as solid matrices for use in solid state fermentation in this study because of their abundance in the local area at cheaper rates. Groundnut fodder, castor husk, cane bagasse and tea residue were collected from local farmers whereas rice husk and sawdust were obtained from rice mill and saw mill in Kadapa respectively. Sesame husk and sorghum husk were collected from farmers in Nandyal, Kurnool District. The substrates were individually sieved through a 2 mm screen, for uniform particle size.

Solid State Fermentation (SSF)

Solid state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks. Ten grams of different lignocellulosic substrates were dispensed. One liter of Czapek Dox liquid medium contained $NaNO_3$ - 2.0 g, K_2HPO_4 - 1.0 g, $MgSO_4$. $7H_2O$ - 0.5 g, KCl - 0.5 g, $FeSO_4$. $7H_2O$ - 0.01 g, Sucrose - 30.0 g and Cellulose - 5.0 g. The different lignocellulosic matrices require different volumes of water within a range of 10-15 ml for 50% moisturization of 10-gram samples. Each flask was covered with hydrophobic cotton and autoclaved at 121°C for 15 min.

Sterile solid culture medium in the flasks were inoculated with the spores of *A. protuberus* at density of 2×10^6 spores/flask and incubated at ambient temperature (30 ± 2^oC). At the regular intervals the samples were withdrawn for processing. Entire fermented bran in the flask was mixed with distilled water, the slurry was filtered through muslin cloth and the filtrate was centrifuged at 10,000 rpm for 20 min at 4^0C and the supernatant was used for determination of enzyme activity (Deswal *et al.*, 2011).

Effect of Carbon Source

To determine the appropriate carbon source for β-glucosidase production by *A. protuberus* normal Czapek Dox medium was supplemented with different carbon compounds (glucose, lactose, sucrose, fructose and maltose at concentration of 1% level. The supplemented medium was used to moisten the rice husk. Cultivation of *A. protuberus* was carried out in the same manner as specified above. The best carbon source was further amended at different concentrations to the normal Czapek Dox medium to find out optimal concentration for production of β-glucosidase.

Effect of Nitrogen Source

To determine the appropriate nitrogen source for β-glucosidase production by *A. protuberus* normal Czapek Dox medium was supplemented with six nitrogen compounds (sodium nitrate, urea, ammonium sulphate, peptone, beef extract and yeast extract at concentration of 1% level. The supplemented medium was used to moisten the rice husk. Cultivation of *A. protuberus* was carried out in the same manner as specified in SSF. The best nitrogen source was further amended at different concentrations to the normal Czapek Dox medium to find out optimal concentration for production of β-glucosidase.

Effect of pH

In order to determine the most suitable pH, the fermentation Czapek Dox medium was adjusted to different pH in the range of pH 3.0 – 6.0 using 0.1 M HCl and 0.1 M NaOH. Adjusted medium was used to moisten rice husk. *A. protuberus* was cultivated on rice husk in the manner as specified in SSF.

Effect of Moisture Content

To determine the optimum moisture content in the fermentation process for maximal production of β-glucosidase, only five milliliters of the normal Czapek Dox medium was added to the 10 g rice husk in the flasks at the beginning, and the remaining balance for achieving desired moisture level was provided to the respective matrix in the form of distilled water (Chandra *et al.*, 2008). Thus moisture content of the fermentation medium varied from 20 – 80%. *A. protuberus* on rice husk with different moisture levels was cultivated in the manner as specified in SSF.

Effect of Temperature

In order to determine the effective growth temperature for β-glucosidase production by *A. protuberus* the fermented medium was incubated at temperature in the range of 25 – 40^oC. Rice husk was incubated out after inoculation.

Formulation of Optimized Medium for Production of β-Glucosidase

Czapek Dox medium was amended with carbon, nitrogen sources and pH in the right proportion in accordance with the results of

earlier experiments for enhanced production of β-glucosidase. The medium contained (g/L) $NaNO_3$ 2.0, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.5, KCl 0.5, $FeSO_4.7H_2O$ 0.01, Glucose 10, Cellulose 5.0, $(NH_4)_2SO_4$ 20, Distilled water 1000 ml, pH 5.0. The optimized Czapek Dox medium was used to moisten the solid substrate-rice husk in subsequent experiments conducted.

β-Glucosidase Assay

β-Glucosidase activity was determined by reducing *P*-Nitro phenyl β-D-gluco pyranoside (PNPG) under acidic conditions (Herr 1979). For the determination of β-glucosidase activity in the assay mixture contained 0.2 ml of 5 mM *P*-Nitro phenyl β-D-gluco pyranoside (PNPG) in 1 ml of 0.05 M citrate buffer (pH4.8) and 0.5 ml of enzyme source was added and incubated at 50^OC for 30 mins. After incubation the reaction was stopped by adding 4 ml of 0.05 M NaOH Glycine buffer (pH 10.6) and the yellow color *P*-Nitrophenol liberated was determined at 420 nm by using Spectrophotometer (Shimadzu). One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1 µmole of *p*-nitro phenol per min under standard assay conditions.

Statistical Analysis

Data presented are the averages of replicates. Duncan's Multiple Range (DMR) test for all data was carried out (Megharaj *et al.,* 1999).

Results and Discussion

Selection of Substrate for β-Glucosidase Production

Selection of a suitable substrate for maximum production of enzyme in SSF is an important parameter. Among the tested solid substrates, the cultivation of *A. protuberus* in both rice husk and castor husk provided higher β-glucosidase production (3.46 U/g of substrate) and minimum activity (0.13 U/g of substrate) was recorded for sugarcane bagasse (fig 1). Both rice husk and castor showed maximum β-glucosidase production, but rice husk can be considered because of it's more abundance in our local area due to cultivation of rice in large area.

Higher β-glucosidase production can be achieved during the culturing of microorganisms in SSF, using rice husk as either the main substrate or as a substantial component of the mixture (Bhatti *et al.,* 2013). The titer of β-glucosidase production in the present study was higher when compared to the results in the study of Chandra *et al.,* 2007. According to this study, the titer of β-glucosidase activity (0.0169 U/g of substrate) was obtained from sawdust as solid support. Titer of β-glucosidase at peak production time interval in SSF were higher on rice husk than on other solid matrices in the present study. Thus, rice husk was selected for subsequent experiments in order to optimize the cultivation process for β-glucosidase production.

The association of cellulose with lignin and hemicelluloses in the lignocellulosic materials is an important factor limiting the hydrolys ability. Removal/degradation of hemicelluloses and lignin by pretreatments such as alkali or acid or H_2O_2 etc., open up the cell wall structure, thus increasing the accessibility of cellulose to cellulases (Zhang and Lynd, 2004). Pretreatment process may improve substrate utilization by the microbes and enhance enzyme yields. In the present study, only native lignocellulosic substrates without pretreatment were used. Use of pretreated lignocelluloses may

further increase yields of cellulolytic enzymes by microorganisms in SSF and needs to be further explored (Ortega *et al.*, 2000; Pandey *et al.*, 2000 and Pan *et al.*, 2006).

The effect of carbon sources on the induction of β-glucosidase production was investigated. Carbon sources tested for induction of β-glucosidase were glucose, lactose, sucrose, fructose and maltose at a concentration of 1% (w/v). As shown in fig. 2, glucose was found to be the best carbon source for maximum production of β-glucosidase. Sucrose was the second followed by maltose, fructose and lactose. This result is similar to that of *Trichosporon asahii* and *Fomitopsis pinicola* KCTC 6208 (Park *et al.*, 2015 and Wang *et al.*, 2015).

Different Concentrations of Glucose

Higher yields of β-glucosidase from fermentation of rice husk with glucose at 1% (w/v) used in the present study on 3rd day of incubation (fig. 3). Addition of glucose beyond 1 % level did not result in improvement of yields of β-glucosidase, in particular, on early incubation time (1st day) and caused decrease in activity of β-glucosidase

The presence of glucose in the fermentation medium was found to be the most effective for production of glucanase, as well as for production of cellulolytic enzymes by *T. viride*. On the other hand sucrose induced cellobiase better than glucose in the same organism. But, glucose induced FPase better than sucrose in other organism *P. chrysogenum* on lignocellulosics in SSF condition. Among the different concentrations of glucose tested in the present study, 1.0 % of glucose served as the best concentration followed by maltose, sucrose, fructose and lactose for β-glucosidase production.

Effect of Nitrogen Sources on β-Glucosidase Production

To optimize the nitrogen sources, various nitrogen sources including sodium nitrate, ammonium sulfate, urea, peptone, beef extract and yeast extract were tested in a same concentration (1% w/v). Fig 4 showed that maximum activity of β-glucosidase at ammonium sulfate as a nitrogen source. Sodium nitrate showed second highest for β-glucosidase activity followed by peptone, beef extract, yeast extract and urea.

Generally, microorganisms exhibit diversity in metabolic patterns in utilization of nitrogen sources. Badhan *et al.*, 2007 could achieve the optimal level (8 U/gds) of β-glucosidase activity by *Myceliophthara* sp. on combination of CH_3COONH_3 0.35%, $(NH_4)_2SO_4$ 0.7% and KH_2PO_4 0.4%. Introduction of ammonium sulphate at 2% N (w/w) enhanced extracellular soluble protein/g of substrate by *A. niger* in comparison to control by about 13.6% (Fadel 2000). This increase in soluble proteins was accompanied by increase to the extent of 21.3, 21.5 and 20% in the activity of FPase, CMCase and β-glucosidase respectively. Spiridonov and Wilson (1998) showed that NH_4 compounds were the most favorable nitrogen sources for production of protein and cellulase. Similarly, among nitrogen sources tested in the present study, ammonium sulphate served the best nitrogen source on the basis of yields of FPase, CMCase and β-glucosidase. The results of the present study were in conformity with observation made by other studies on the same or different organisms (Chahal *et al.*, 1996; Krishna 1999; Park *et al.*, 2002 and Badhan *et al.*, 2007).

The observations of Ilyas *et al.,* 2002 reported maximum cellulase activity with ammonium sulphate as nitrogen source which supports the result of the present study conducted. The efficiency of ammonium sulphate as nitrogen providing source may be due to its direct availability as nitrogen source for protein production (Mandals 1975).

Different Concentrations of Ammonium sulfate

Ammonium sulphate was found to be the best nitrogen source for production of β-glucosidase by *A. protuberus* according to the results of the previous experiment. $(NH_4)_2SO_4$ was tried only at 1% (w/v) concentration in that experiment. Dose response of ammonium sulphate supplementation within a range of 0.5 - 2.5% on production of β-glucosidase by *A. protuberus* was examined (fig 5). Maximum β-glucosidase activity with 26.34 U/g of rice husk was observed in the rice husk amended with 2.0% of ammonium sulphate on 5th day of incubation followed by 25.42 at 0.5% concentration of ammonium sulphate amended medium on 5th day of incubation.

Additional supply of nitrogen has been reported by various scientists to enhance the growth of the microbes and their physiology (Fan *et al.,* 1981; Yadav 1987 and Garg 1990). Ammonium salts in form of sulphate was found to facilitate higher production of cellulolytic enzymes by *Penicillium funiculosum, Myrothecium* sp., *A. terreus* (Harima *et al.,* 1980; Rao *et al.,* 1985 and Chahal *et al.,* 1996). Additional supply of ammonium sulphate to nitrogen deficient rice straw/wheat straw supported higher growth and high enzyme activity in *Aspergillus niger* as well as thermophilic fungal strains *Thermoascus aurantiacus* and *Myceliophthora* sp. (Kalogeris *et al.,* 1998; Park *et al.,* 2002 and Badhan *et al.,* 2007). Over all, on the basis of all together $(NH_4)_2SO_4$ at 2.0% was considered as optimal concentration of nitrogen source for higher production of β-glucosidase by *A. protuberus*.

Effect of pH

Maximum β-glucosidase activity (2.35 U/g of substrate) was observed at pH of 5 (fig 6). The β-glucosidase production by *A. protuberus* was affected when pH level was higher or lower than the optimum value of β-glucosidase production. The similar results were reported by Garcia (Garcia *et al.,* 2002). Most filamentous fungi show optimal growth in slightly acidic pH. The pH was not controlled during the cultivation process due to the heterogeneity of the process of SSF. According to Pandey *et al.,* 2000 the difficulty of monitoring and controlling fermentation parameters in SSF is perhaps, the main drawback of this process. Variations of pH during the fermentation process are due to the metabolic activity of the microorganisms, and may be increased or decreased according to the by-products released or the nutrients consumed during the process.

The fungus *A. niger* expressed high cellulase production on agro-residues at initial pH 4.5 – 5.5 in SSF (Wang *et al.,* 2012). Maximum production of β-glucosidase activity was observed at pH 5.0 by *Fusarium proliferatum* NRRL 26517 grown on *Ficus nitida* wastes (Fawzi 2003). The maximum activity of cellulolytic enzymes was obtained when the initial pH was adjusted to 7.0 for growth of *F. oxysporum* on corn stover (Panagiotou *et al.,* 2003). But in the present study of SSF, *A. protuberus* was cultivated on rice husk for generation of β-glucosidase enzyme at pH 5.0.

Effect of Temperature

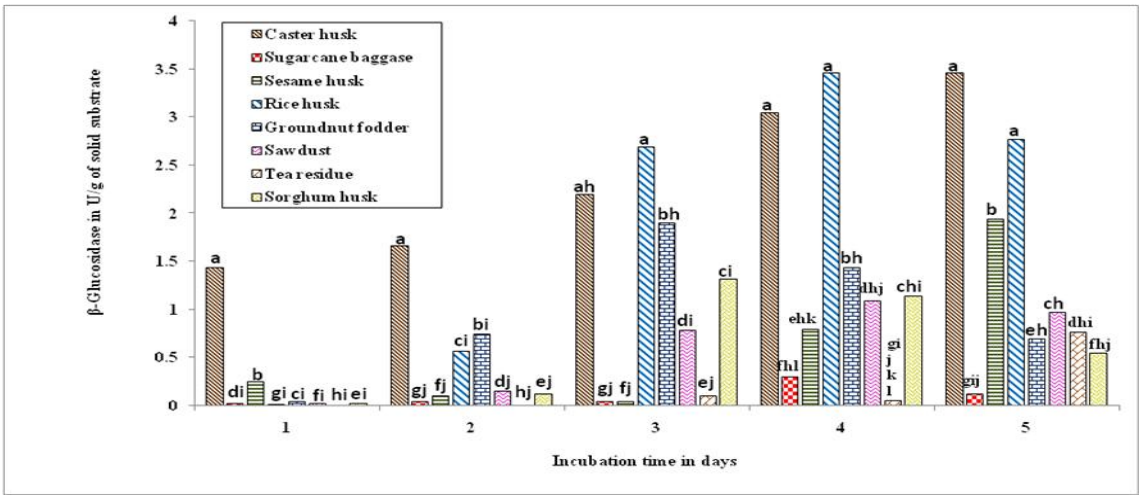
The incubation temperature of the fermentation medium is one of the ultimate factors influencing the production of enzymes. The ideal temperature for β-glucosidase production by *A. protuberus* was 30°C, about 3.67 U/g of substrate (Fig. 7).

The optimum temperature 32°C was recorded for the maximum production of cellulolytic enzymes by *A. niger* F-119 on radicle wastes (Fadel 2000). Badhan *et al.*, 2007 showed that the incubation temperature at 45°C was found to be optimum for production of cellulolytic enzymes by *Myceliophthora* sp. IMI 387099. The maximum cellulase yield was obtained at temperature of 28°C using *Trichoderma reesei* (Muthuvelayudham and Viruthagiri 2006). Krishna (1999) observed that the optimal growth and enzyme production were recorded at 35°C on banana wastes. Thus, occurrence of optimal production of enzyme at different temperatures may be related to the growth kinetics of the microorganism employed rather than the enzyme produced. In the present study, cultivation of *A. protuberus* in SSF on rice husk with temperature at 30°C generated higher production of β-glucosidase.

Effect of Moisture Content

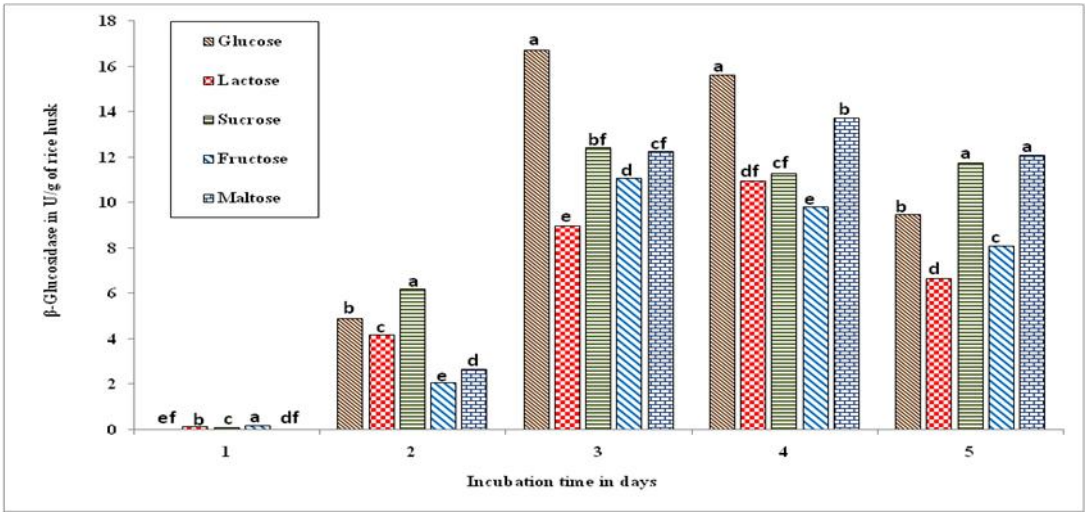
Among the moisture contents evaluated, the highest β-glucosidase production was obtained in rice husk with 40% of initial moisture on 5th day of incubation (fig 8). The production of β-glucosidase on rice husk at all moisture regimes was low on the 1st and 2nd day of incubation and increased significantly on 3rd day of the incubation onwards. The highest yield of β-glucosidase production was recovered (5.31 U/g) on 5th day of incubation with 40% moisture level.

Fig.1 Effect of different substrates on production of β-glucosidase by *A. protuberus* in SSF.



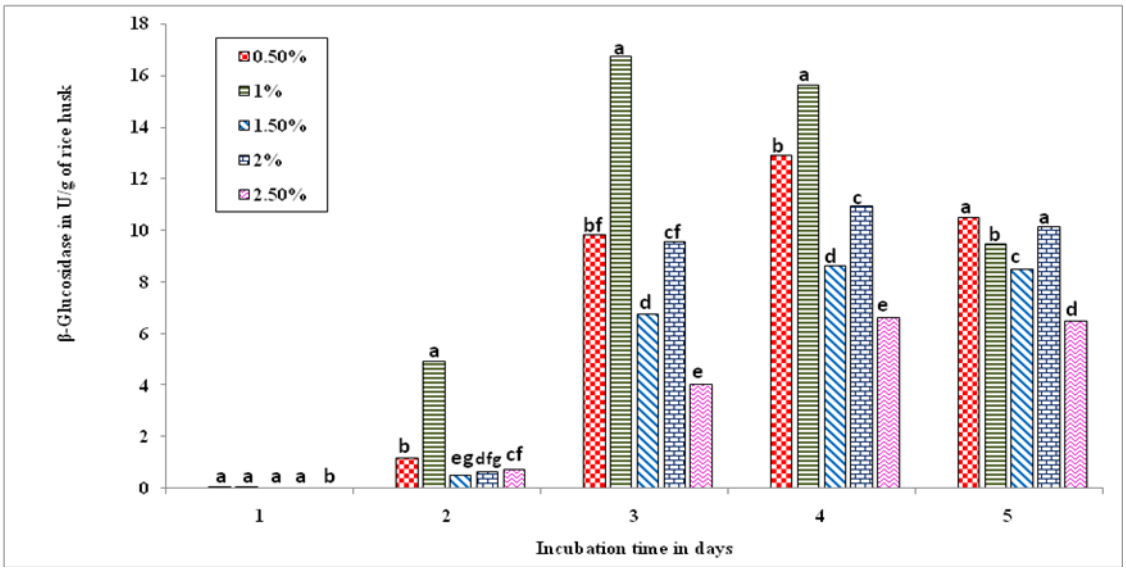
Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to DMR test.

Fig.2 Effect of supplementation of carbon sources on production of β-glucosidase in rice husk by *A. protuberus* in SSF.



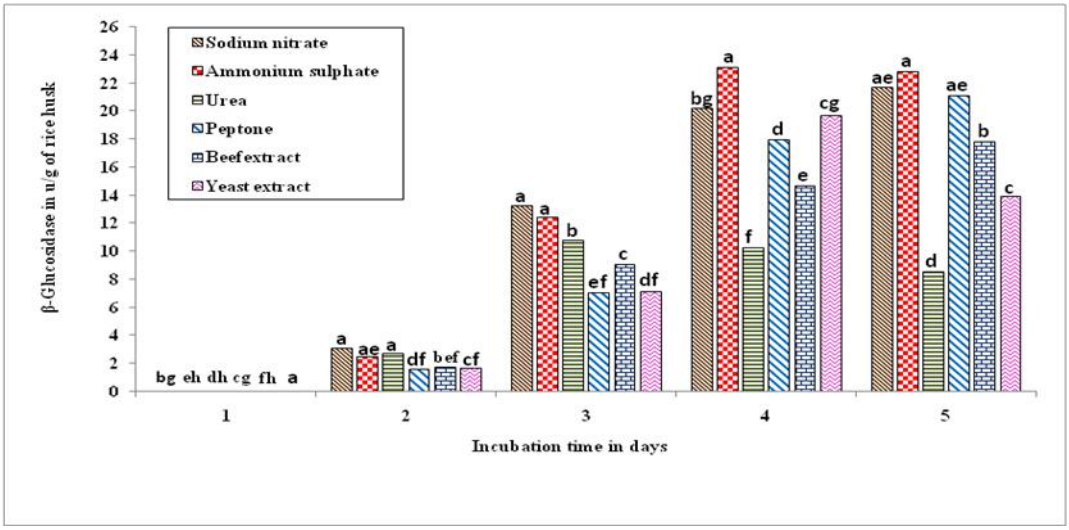
Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to DMR test.

Fig.3 Effect of supplementation glucose on production of β-glucosidase in rice husk by *A. protuberus* in SSF.



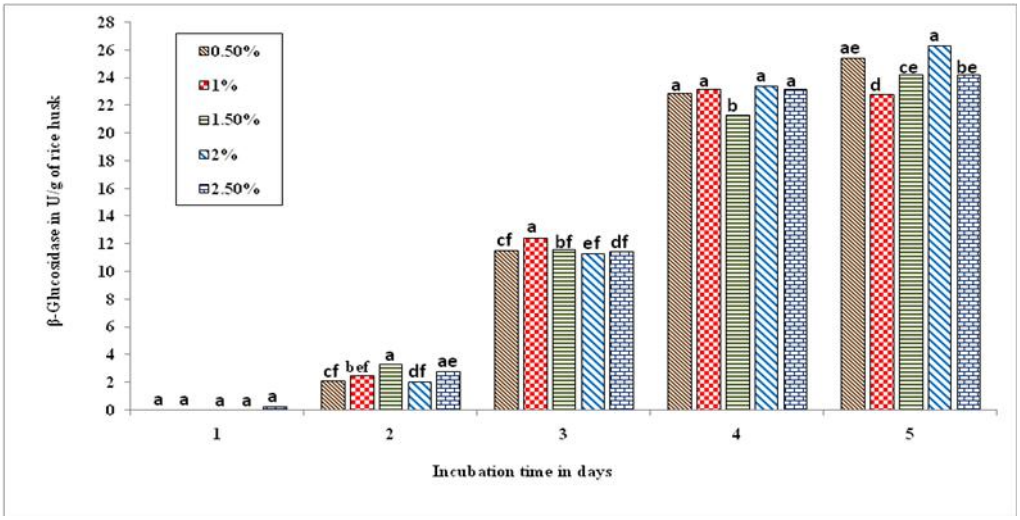
Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to DMR test.

Fig.4 Effect of supplementation of nitrogen sources on production of β-glucosidase in rice husk by *A.protuberus* in SSF.



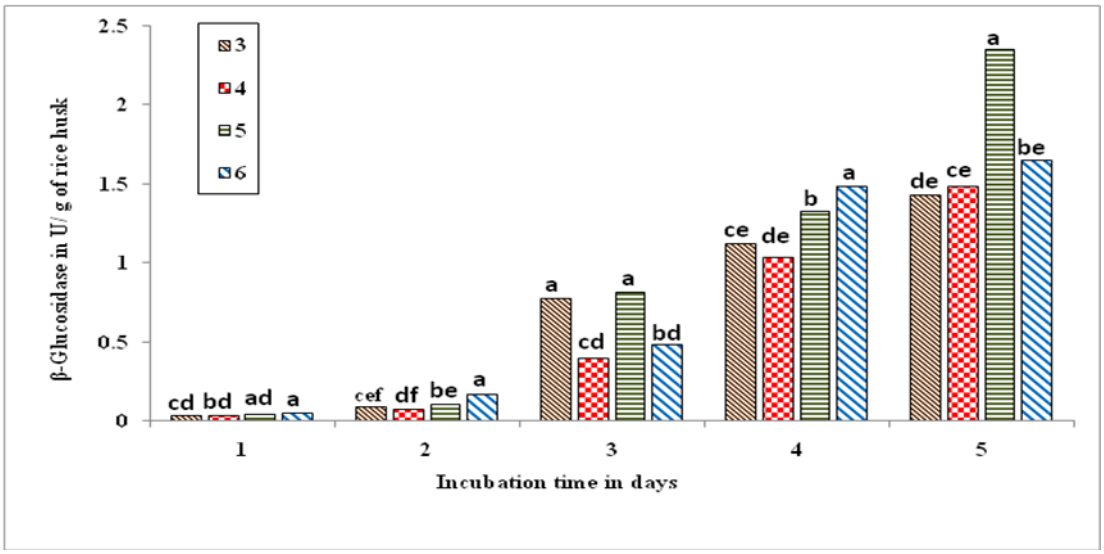
Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to DMR test.

Fig.5 Effect of supplementation of ammonium sulphate on production of β-glucosidase in rice husk by *A.protuberus* in SSF.



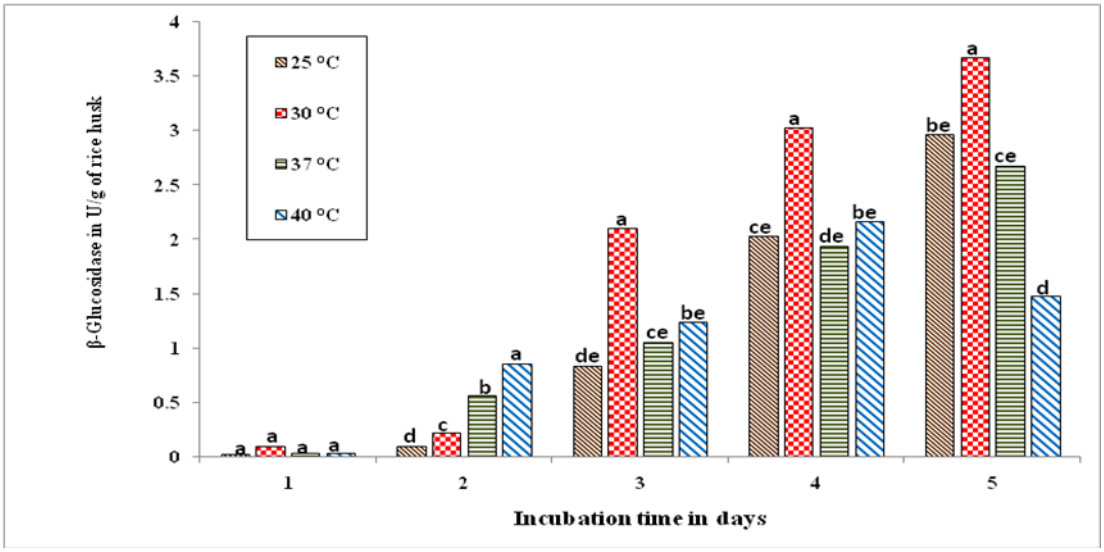
Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to DMR test.

Fig.6 Effect of initial pH on production of β-glucosidase in rice husk by *A. protuberus* in SSF.



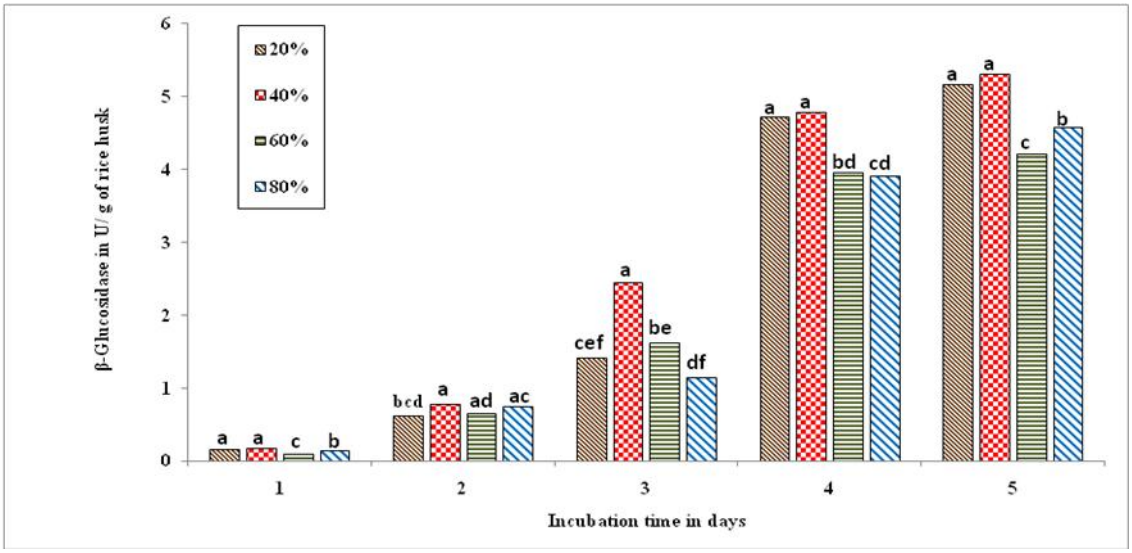
Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to DMR test.

Fig.7 Influence of growth temperature on production of β-glucosidase by *A.protuberus* in SSF.



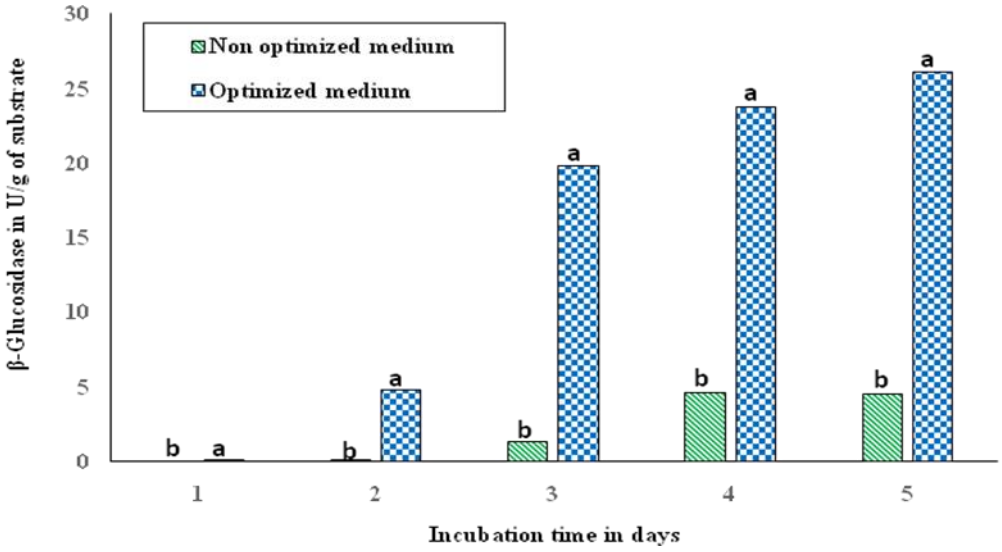
Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to DMR test.

Fig.8 Effect of moisture level on production of β-glucosidase in rice husk by 3 *A.protuberus* in SSF.



Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to DMR test.

Fig.9 Effect of optimized medium in the production of β-glucosidase in rice husk by *A.protuberus* in SSF.



Means, in each column, followed by the same letter are not significantly different ($p \leq 0.05$) from each other according to DMR test.

The moisture level demands in solid state fermentation differ according to enzyme to be produced, substrate, and microorganism as well as particle size of the substrate as

well as the configuration of the particles (Nandakumar *et al.*, 1994; Muniswaran *et al.*, 1994 and Krishna and Chandrasekharan 1996).

Effect of Optimized Medium

The higher β-glucosidase production 26.06 U/g rice husk was recorded at 5th day of incubation in optimized medium whereas in normal Czapek dox medium showed β-glucosidase production only 4.63 U/g rice husk at 4th day of incubation (fig 9). By using optimized medium β-glucosidase production was increased 6 times as compared with non-optimized medium normal Czapek Dox medium.

In conclusion, to the best of our knowledge, there has been no study on β-glucosidase production from *Aspergillus protuberus*. Therefore, an attempt was made in this study to maximize the β-glucosidase production using cheap locally available solid substrate- rice husk in SSF. Recent years, studies on ß-glucosidases are coming more frequent given the key role of this enzyme in ethanol production for biofuels. The cost-effective technologies are needed for the production of enzyme and SSF is a suitable technology for economical production of cellulases using lignocellulosic residues as substrate. Major parameters affecting the fermentation process for enzyme production were studied and optimal levels were identified. It is concluded from the findings that the strategy to produce β-glucosidase from rice husk was successful as it resulted in a considerably good amount of this enzyme produced (26.06 U/g of rice husk) by newly isolated strain *Aspergillus protuberus* under optimized conditions.

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