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Thermostable β-D-Glucosidase from Aspergillus flavus: Production, Purification and Characterization

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Abstract

The present study was focused on production, purification and characterization of thermostable β-D-glucosidase from the culture supernatant of *Aspergillus flavus* isolated from the wheat flour mill waste. Production of β-D-glucosidase was carried in shake flasks at 30^{o}C and pH 6.0. The results demonstrated that the β-D-glucosidase activity was high at day 6 of cultivation time, yielded 11.3 U/mL culture broth with a specific activity of 3 Units per milligram of total protein. The β-D-glucosidase purified by ammonium sulphate fractionation, followed by DEAE sepharose fast flow chromatography resulted ~2.07 fold with a final recovery of 75% and specific activity of 6 U/mg protein. The SDS-PAGE and zymogram analysis revealed monomeric protein with a molecular weight of 52.5 kDa. The enzyme was found to be stable up to temperature of 60^{o}C at pH 5, and retained its activity even after 30 minutes of incubation. The enzyme activity was found resistant (more than 80%) against wide range of surfactants (Tween-20, Tween-80, Triton X-100 and Sodium deoxycholate) at 0.5 and 1% concentration. Among metal ions tested at 5mM and 10 mM concentrations, Mg^{2+}, Ca^{2+} and Zn^{2+} were not affected, whereas Cu^{2+}, Hg^{2+} and Pb^{2+} were inhibitory to activity of β-D-glucosidase**.** Also, the β-D-glucosidase activity (80%) was resistant to product inhibition up to 200 mM glucose and further increase in its concentration decreased the enzyme activity. Kinetic analysis indicated that K_m and V_{max} of the enzyme were 0.124 mg/ml and 118 U/ml respectively.

Keywords: Aspergillus flavus, β-D-glucosidase, purification, characterization, surfactants, kinetics

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Introduction

Application of microbial sources for industrially important extracellular enzyme production has stimulated prominent renewed interest in food and biofuel processing industry. The industrial demand of highly active preparations of cellulolytic enzymes with appropriate specificity and stability to pH, temperature, metal ions and surfactants continues to stimulate the search for new enzyme sources. Isolation and screening of microorganisms from naturally occurring habitats is expected to provide new strains producing novel enzymes which are active and stable in desired conditions. Microbial degradation of cellulose involves the action of an enzymatic complex consisting of three types of enzymes where the first two enzymes act directly, depolymerizing

the cellulose to final products oligosaccharides of different sizes, and cellobiose and the third enzyme the β-D-glucosidase (EC3.2.1.21) hydrolyzes the last step, i.e., turning cellobiose into glucose [1, 2]. β-D-Glucosidases from microbial sources represent an important group of enzymes because of their potential use in various biotechnological processes, such as biomass degradation [3, 4], biofuel production [5- 7]. Surfactant active cellulolytic enzymes in laundry detergent formulation can boost cleaning performance and provide fabric care benefits by removing the hair, known as pills that occur on cotton clothes after repeated wearing and machine washing [8]. Surfactants as the typical auxiliaries are extensively used in the textile industry [9]. Surfactants are among known chemical protein denaturants, target site of these compounds on the protein structure is hydrophobic domains and/or charged groups of the proteins [10].

Recent studies have been reported on the effects of several surfactants on the enzymatic hydrolysis of cellulose [11-14]. Much attention has been given to β-D-glucosidase which is rate-limiting factor, and it is also very sensitive to glucose inhibition [2,15-18]. *Aspergillus* sp. produces and secretes variety of industrial enzymes such as cellulases, hemicellulases, pectinases, xylanases, α-amylases, glucoamylases and proteases [19 - 26]. It is interesting that *Trichoderma* species, seemingly the best source of cellulose solubilising activities, are poor producers of β-D-glucosidase [27]. On the other hand, *Aspergillus* species have been shown to be better producers of β-D-glucosidase [2, 28]. This enzyme is produced by a variety of fungi but the exclusive production of this enzyme in industry have been achieved mainly by *Aspergillus niger* [29], *Aspergillus oryzae* [30], *Aspergillus awamori* [31] and *Aspergillus terreus* [32] probably because of their ubiquitous nature and non-fastidious nutritional requirements of these organisms. *Aspergillus* can secrete copious amounts of homologous proteins into their culture medium. They also possess post-translational mechanisms capable of correctly processing proteins that are difficult to express in traditional host organisms such as *E. coli* or *S. cerevisiae***.** Since the 1980s, biotechnologists have been using a variety of *Aspergillus* strains to express heterologous proteins of commercial interest [2].

The use of *A. flavus* to produce β-D-glucosidase is well-documented [11, 22, 30, 33-35]. Several studies have shown that β-D-glucosidase isolated and produced by *A. flavus***,** assembles into functionally active β-D-glucosidase which is highly stable at different ionic concentration and, at a wide range of temperatures. An examination of the available literature reveals a wide variation in the reports by different groups, of temperature ranges from 50^{o}C to 70^{o}C with an ionic concentration, pH from 4 to 5 (Table 1). Most of the investigators have used either a naturally isolated strain or a genetically engineered host for production of enzymes. In this work, a detergent assisted production of β-D-glucosidase from a newly isolated strain with a simple traditional purification involving an ion-exchange chromatography and characterization of the

enzyme was presented. We demonstrate that our surfactant resistant β-D-glucosidase exhibited high enzyme activity at higher temperatures (50^{o}C with >90% activity for 60 minutes) and has a potential for application in food processing industries.

Materials and methods

Microorganism and culture conditions

Cellulolytic fungi were isolated from different environmental samples like wheat flour mill solid waste and saw mill solid waste around Tirupati, India. The ability to produce cellulolytic enzyme production and selection of promising cellulolytic fungi was screened by culturing on Czapek dox agar (supplemented with 1% carboxymethyl cellulose) medium at 30^{o}C and the Congo red staining plate assay method [36]. The spore suspension of 0.5 ml (~ 16 x10^{4}/ml) was inoculated to 50 ml production medium (yeast extract 0.05 g, sucrose 0.1 g, carboxymethyl cellulose 0.5 g, K_2HPO_4 0.05 g and $FeSO_4$ 0.001 g, pH adjusted to 6.0, distilled water 50 mL) containing 0.5 mL of the basal salt solution ($NaNO_3$ 10.0 g, KCl 2.5 g, $MgSO_4$ 2.5 g and distilled water 50 mL) in 250-mL Erlenmeyer flasks and the flasks were incubated at 30^{o}C with 120 rpm in an orbital shaking incubator. The culture medium was centrifuged at 10,000 rpm for 15 min at 4^{o}C and the centrifugal supernatant was used as crude enzyme source for β-D-glucosidase activity.

Assay of β-D-Glucosidase

Activity of β-D-glucosidase in the culture filtrate was quantified according to the method of Herr [37]. β-D-Glucosidase activity was measured using *p*-nitrophenyl β-D-glucopyranoside (pNPG) as a substrate. The yellow coloured *p*-nitrophenol liberated was determined by Spectrophotometry at 420 nm. One unit of β-D-glucosidase activity was defined as the amount of enzyme liberating 1 µmole of *p*-nitrophenol per min under standard assay conditions.

Purification of β-D-glucosidase

All the unit processes for enzyme purification were done at a temperature of 4^{o}C unless otherwise specified. The centrifugal culture supernatant was subjected to optimum ammonium sulphate fractionation (60-70%) overnight at 4^{o}C. The precipitate was collected by centrifugation at 15000 rpm for 25 min and the pellet was dissolved in small volume of 0.1 M acetate buffer (pH 5.0). Dialysis was carried out against 4 L acetate buffer (0.1 M, pH 5.0) wash for 10 h. The dialyzed sample pH was adjusted to pH 5.0 and dialyzed sample allowed to bind to the DEAE sepharose FF (Amersham Pharmacia Biotech, Sweden) pre-equilibrated with 0.1 M phosphate buffer (pH 5.0) using XK column (Amersham Pharmacia Biotech, Sweden). After sample binding, the column was washed with 50 mM phosphate buffer (pH 5.0) until the absorbance at 280 nm returned to baseline. Elution of bound enzyme was carried out with 50 mM phosphate buffer (Buffer A, pH 5.0) and 0.5 M NaCl in 50 mM phosphate buffer (Buffer B, pH 5.0) with linear gradient elution. Protein containing fractions (absorbance at 280 nm) were pooled and

checked for enzyme activity. The enzyme activity was measured as described above and the protein estimation was done by Folin's-Phenol method using BSA as standard [38].

SDS-PAGE analysis

SDS - PAGE was performed to analyze purified enzyme as described by Laemmli [33], using 12% resolving slab gel. For β-D-glucosidase detection, 4-methylumbelliferyl-β-D-glucuronide was added to a final concentration of 5 mM to the resolving gel mix [39]. After electrophoresis the enzyme protein band was visualized by staining with Coomassie brilliant blue R-250. The β-D-glucosidase activity in polyacrylamide gel was performed by renaturation of SDS-PAGE gel in 25% isopropanol solution for 2 h in shaking condition followed by distilled water and acetate buffer washes each of two times for 10 min at room temperature [40]. The protein after renaturation, associated with β-D-glucosidase activity was seen (clear zone formation) by incubating the gel at 50^{o}C for 30 min in 20 ml of acetate buffer (0.1 M, pH 5.0) and the image was captured under UVP gel doc system.

Optimization of assay conditions for β-D-glucosidase

Effect of temperature and pH on stability and activity

To find the optimum temperature for stability and activity of purified β-D-glucosidase, temperature ranging from 30–70^{o}C was tested. The temperature stability was tested by incubating the enzyme in acetate buffer without substrate from 30-60^{o}C for 5h and the remaining enzyme activity was measured. The effect of temperature was determined from 30–60^{o}C by varying the assay temperature under standard conditions as described. To find the optimum pH for stability and activity, different buffers with pH from 4 to 8 (pH 4 and 5 - 0.1 M acetate buffer and pH 6, 7 and 8 - 0.1 M potassium phosphate buffer) were used. The pH stability was measured by incubating the enzyme in selected buffers without substrate for 12 h and the residual enzyme activity was assayed as described earlier.

Effect of surfactants, heavy metal ions and EDTA

The effect of surfactants such as Tween-20, Tween-80, TritonX-100 and SDS, Chaps, Sodium deoxycholate at 0.5, and 1.0% were examined by incorporating them into the enzyme assay mix. To the optimized enzyme assay mix, the salt solution of metal ions such as Na, K, Zn, Mn, Mg and Cu and EDTA were made up to 5 and 10 mM, and the enzyme was assayed as described earlier.

Product inhibition of β-D-glucosidase activity

To determine the feedback inhibition of the purified β-D-glucosidase, increasing concentrations of product i.e., glucose ranging from 50-300 mM was incorporated into the enzyme assay

mixture. Under optimal enzyme assay conditions β-D-glucosidase activity was measured as described earlier.

Kinetics of β-D-glucosidase activity

To determine the kinetics of the purified β-D-glucosidase (K_m and V_{max}), pNPG was used as a substrate at concentrations ranging from 0.038 to 0.15 mg/ml under optimal conditions (pH 5, temperature 50^{o}C), and the enzyme activity was assayed as described earlier. The apparent Km and V_{max} values were calculated by using GraphPad Prism 5.0 software package.

Statistical analysis

Data presented are the averages of three replicates. Significance of the test variables were analyzed by the Experimental Design Module of Duncun's Multiple Range (DMR) test using the SPSS Statistical software package (SPSS 10.0 for windows).

Results and Discussion

Isolation and identification of the microorganism

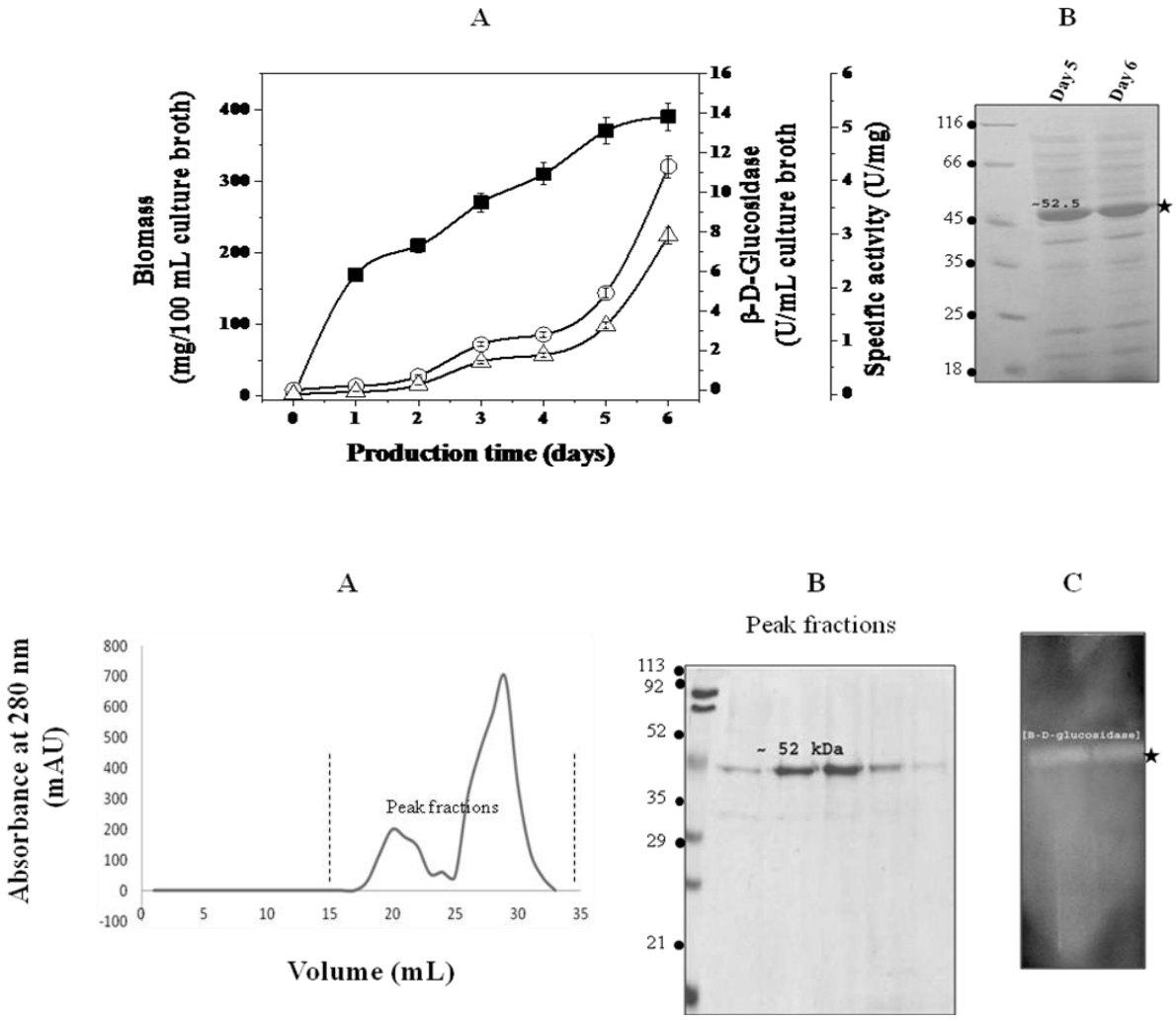
The strain used in this study was isolated from wheat flour mill solid waste. Clear halos of hydrolytic zone by Congo red staining around colonies on carboxymethyl cellulose agar gave an indication of cellulase (endoglucanase) producing strains. Individual colonies were purified through repeated streaking on fresh agar plates. Among more than 20 strains, isolated in the laboratory and screened for cellulase activity, *Aspergillus* sp. FME IX strain was selected (data not shown). Microbial type culture collection centre, Chandigarh, India identified the selected fungal isolate as *Aspergillus flavus*.

Purification, SDS-PAGE and zymogram analysis

An extracellular β-D-glucosidase was purified from the culture filtrates of *Aspergillus flavus* grown on optimized medium (Figure 1A & B, top). The purification was performed by salt precipitation followed by DEAE sepharose FF chromatography. The molecular weight determination of β-D-glucosidase was confirmed by SDS-PAGE separation of proteins followed by zymogram study in gel, it revealed with apparent molecular mass of β-D-glucosidase with 52.5 kDa (Figure 1A, B & C, bottom). The chromatogram and the SDS-PAGE of the present study was shown in Figure 1. Current protocol resulted in purification of enzyme by 2 folds with 75% of recovery. The result of purification of the present study was given in Table - 1. Zhang *et al.* [41] reported purification of β-D-glucosidase from *Aspergillus oryzae* using ammonium sulphate and ion exchange chromatography to 2.3 and 12.6 fold purification respectively. Peshin *et al.* [34] reported purification of β-D-glucosidase from *Aspergillus niger* with 6.0 fold purification. Reported on purification of β-D-glucosidase from *Cellulomonas flavigena* of wild and mutant PN-120 with 28.85, 15.63 folds respectively [42]. Gueguen *et al.* (2001) reported

purification of β-D-glucosidase from *Candida sake* with 35.5 fold purification [43]. The molecular weights of β-D-glucosidase from fungal and bacterial sources reported either monomers or dimers [18]. The polyacrylamide gel electrophoretic separation of microbial β-D-glucosidase showed wide range of molecular sizes from 47 to 340 kDa [44]. The molecular weight of β-D-glucosidase of present study compared with *Aspergillus niger* USDB 0827 and *A. niger* USDB 0828 [45], *Aspergillus oryzae* [41] showed in Table - 2.

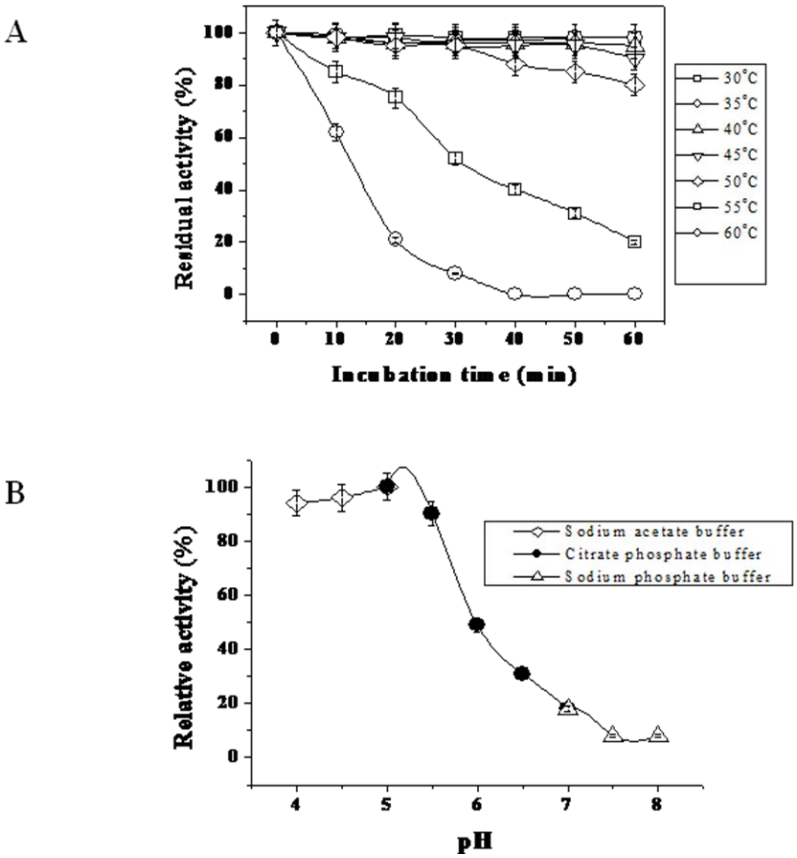
Figure 1: Time course production and optimization of β-D-glucosidase (A, B, top). SDS-PAGE analysis of purification and zymogram analysis of β-D-glucosidase. Protein standard marker DEAE-FF fractions and β-D-glucosidase activity in gel (A, B & C, bottom).



Temperature and pH dependence

The optimized assay conditions for purified β-D-glucosidase of the present investigation were as follows. The enzyme was stable up to 50°C and declined thereafter, and the optimum temperature for activity under the assay conditions was found at 50°C (Figure 2A). The pH stability of β-D-glucosidase from 5 - 7, and maximum enzyme activity was found at pH 5.0 (Figure 2B). There is great diversity for optimal temperature reported for β-D-glucosidase activity of different fungi from 35-75°C [46, 47]. Most fungal β-D-glucosidases reported optimal pH ranging from 5.0 to 6.5 [18]. Bastawde (1992) reported optimum temperature at 60°C and pH 4.8 for thermophilic *Aspergillus terreus* [48]. β-D-Glucosidase from *A. flavus* of the present study had no significant differences with other *Aspergillus* species in optimum pH, however optimum temperature found at 50°C instead of 60°C. The details of optimum temperature and pH for β-D-glucosidase activity of present study were compared with other reported *Aspergillus* species showed in Table - 2.

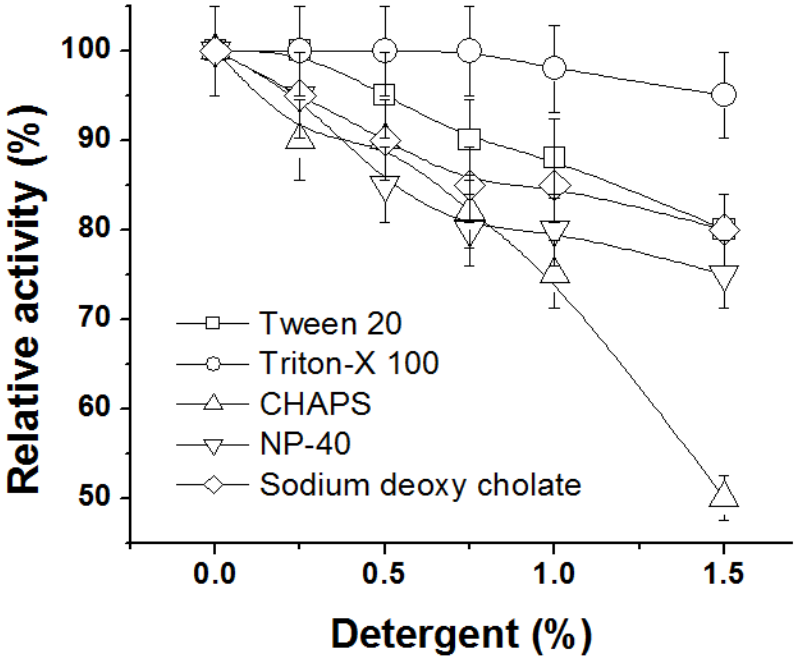
Figure 2: Temperature (A) and pH dependence (B) of β-D-glucosidase activity



Effect of surfactants, metal ions and EDTA

The extracellular β-D-glucosidase from *Aspergillus flavus* was active with various surfactants such as Tween-20, CHAPS, NP-40, TritonX-100 and SDS at concentrations of 0.5 and 1.5% (Figure 3). Similarly, higher activity of β-D-glucosidase of *A. niger* in the presence of detergents like Tween-80 and TritonX-100 was reported [49]. Several authors had proposed mechanisms to explain the saccharification of cellulose in the presence of surfactants [50, 51, 41]. The purified β-D-glucosidase was inhibited by detergents such as SDS, Tween 80 and Triton X-100 [52]. Among the tested metal ions, effect of Mg^{+}, Ca^{2+} and Zn^{2+} on β-D-glucosidase activity have no effect, Co^{2+} showed slight inhibition and Cu^{2+}, Hg^{2+} and Pb^{2+} showed significant inhibition. The metal chelating agent EDTA showed 40% inhibition of enzyme activity (Table 3). Similarly, Zhang *et al.* [41]reported the effect of metal ions Ca^{2+}, Mg^{2+} and Zn^{2+} have no significant effect on enzyme activity, but Cu^{2+} ion inhibited strongly enzyme from *Aspergillus oryzae*. Hoh *et al.* [46] reported β-D-glucosidase from *Aspergillus niger* USDB 0827 and *Aspergillus niger* USDB 0828 enzymes were relatively unaffected by Ca^{2+} , Cu^{2+} , Co^{2+} , Mg^{2+}, Mn^{2+} and Zn^{2+} [45]. Yeoh *et al.* reported β-D-glucosidase from *A. ornatus* inhibited by Ag^{2+} and Fe^{2+} [53]. The chelating agent EDTA did not inhibit the purified β-D-glucosidase from *Aspergillus oryzae*, but the activity was significantly inactivated by SDS [17]. The results of metal ions effect on β-D-Glucosidase of the present study are in agreement with those reported with other *Aspergillus* species.

Figure 3: Detergents stability on β-D-glucosidase activity



Enzyme kinetics and product (glucose) inhibition

The enzyme activity was increased with an increase in substrate (pNPG) concentration from 0.038 to 0.15 mg/ml, with further increase there was no significant increase in enzyme activity (Figure 4A). The K_m and V_{max} values of purified β-D-glucosidase were found to be 3.12 mM and 118 U/ml respectively (Figure 4B). K_m values ranging from 0.10 to 44mM PNPG have been reported for β-D-glucosidase from different fungal sources, including those from *Aspergillus* species [2, 54]. Such variations in K_m values could be attributed to different conditions of enzyme assay and substrate preferences. The purified extracellular β-D-glucosidase was found resistant to product (glucose) inhibition up to 50 mM glucose and further increase in its concentration decreased the activity. Glucose inhibition is a common characteristic of fungal β-D-glucosidases, that limits their use for the enzymatic hydrolysis of lignocellulosic substrates [2, 17]. Most microbial β-D-glucosidases have glucose inhibition ranging from 0.5 mM to 100 mM [17, 55, 56]. For example, some β-D-glucosidases from *Aspergillus* species exhibit glucose inhibition values ranging from 3 to 14 mM [57, 58]. The details of characteristic features of β-D-glucosidases of certain *Aspergillus* species, and their biochemical properties are given in Table - 2.

Figure 4: Feed-back (product) inhibition (A) and enzyme kinetics of β-D-glucosidase activity (B)

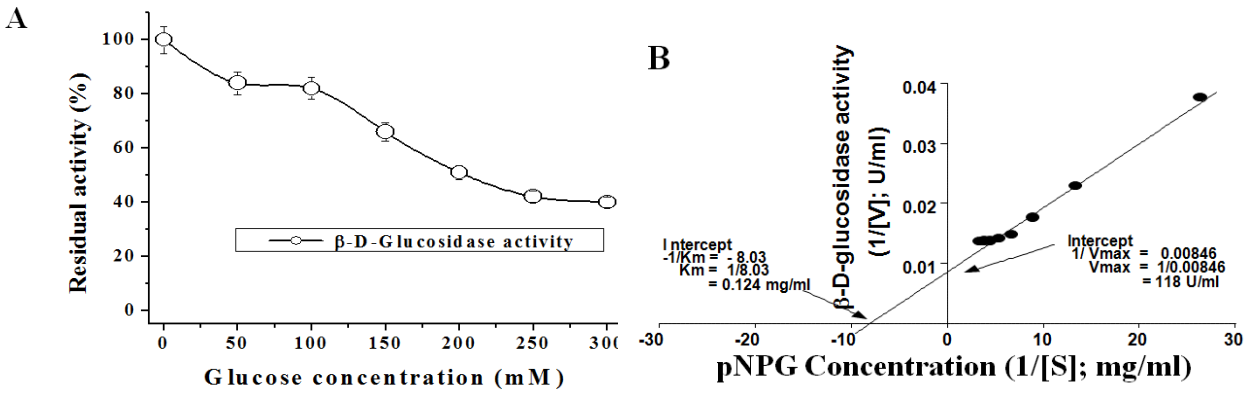


Table 1: Summary of purification of β-D-glucosidase from *A. flavus*

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Culture supernatant	216	645	2.99	100
Ammonium sulfate précipitation	167	580	3.47	89.8
DEAE-Cellulose	80	485	6	75

Table 2: Snap-shot of β-D-glucosidase features from *Aspergillus*-cell factory[a]

Host	Molecular mass (kDa)	Assay conditions		Kinetic analysis		Reference
		°C	pH	K_m (mM)	V_{max} (U)	
Aspergillus ornatus	nd	60	4.6	0.76	nd	[53]
Aspergillus niger	nd	55	4.5	0.9	nd	[11]
Aspergillus niger	nd	65	5.0	0.71	nd	[34]
Aspergillus niger	nd	70	4.6	1.11	nd	[31]
Aspergillus phoenicis	nd	60	4.5-5	0.58	14.2	[30]
Aspergillus oryzae	77	60	5	0.92	nd	[33]
Aspergillus usamii D5	128	55	4.5	-	-	[7]
Aspergillus oryzae	130,43	50	5	0.55	-	[17]
Aspergillus niger CCRC 31494	49	55	5	21.7	-	[58]
Aspergillus nidulans	125, 50	50, 60	5, 5.5	0.842, 0.465	1.4, 0.62	[57]
Aspergillus niger strain 322	64	50	5.5	100	-	[34]
Aspergillus niger	52.5	50	5	3.12	118	Present study

Table 3: Effect of metal ions, EDTA and detergents on β-D-glucosidase activity

Compound	Relative activity (%)	
	5 mM	10 mM
None	100	100
NaCl	100	100
KCl	98	96
$ZnCl_2$	100	89
$MgSO_4$	85	72
$MnSO_4$	92	84
$CuSO_4$	70	32
EDTA	80	74
Detergent	**Relative activity (%)**	
	0.50%	**1%**
Triton-X 100	100	100
Tween-20	100	93
Tween-80	92	85
Chaps	87	81
Sodium deoxycholate	88	83

Conclusion

In conclusion, the present investigation involves the purification and characterization of β-D-glucosidase from newly isolated *Aspergillus flavus*. The outstanding features of the enzyme to temperature and pH, absence of inhibition of its activity by certain metal ions, surfactants, product (glucose), makes this enzyme to be suitable for use as an effective additive to laundry detergents and saccharification of lignocellulosic materials.

Conflict of Interest

We declare that we have no conflict of interest

References

1. Lynd LR., Weimer PJ., van Zyl WH., Pretorius IS. (2002). Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol Mole Biol Rev 66 (3):506-577.
2. Woodward J., Wiseman A. (1982). Fungal and other β-D-glucosidases—their properties and applications. Enzyme Microbial Technol 4 (2):73-79.
3. Fawzi EM. (2003). Production and purification of β-glucosidase and protease by *Fusarium proliferatum* NRRL 26517 grown on Ficus nitida wastes. Annal Microbiol 53 (4):463-476.
4. Coughlan MP. (1985). The properties of fungal and bacterial cellulases with comment on their

production and application. Biotechnol Genetic Engin reviews 3:39-109.

5. Zhang L., Guo ZP., Hong JH., Ding ZY., Gao ZQ., He ZM., Shi GY. (2012). Expressing β-glucosidase from *Saccharomycopsis fibuligera* in industrial ethanol producing yeast and evaluation of the expressing sufficiency. Annal Microbiol 62 (2):539-544.
6. Bothast RJ., Saha BC. (1997). Ethanol production from agricultural biomass substrates. Advan appl Microbiol 44:261-286.
7. So JH., Do HJ., Rhee IK. (2010). Purification and characterization of β-glucosidase from *Aspergillus usamii* D5 capable of hydrolyzing isoflavone glycosides in soybean and astragali radix. J Korean Soc Appl Biol Chem 53 (5):626-633.
8. Kirk O., Borchert TV., Fuglsang CC. (2002). Industrial enzyme applications. Curr Opinion Biotechnol 13 (4):345-351.
9. Ueda M., Koo H., Wakida T., Yoshimura Y. (1994). Cellulase treatment of cotton fabrics. Part II: Inhibitory effect of surfactants on cellulase catalytic reaction. Textile Res J 64 (10):615-618.
10. Hemmatinejad N., Vahabzadeh F., Kordestani SS. (2002). Effect of surfactants on enzymatic hydrolysis of cellulosic fabric. Iranian Poly J 11:333-338.
11. Park JW., Takahata Y., Kajiuchi T., Akehata T. (1992). Effects of nonionic surfactant on enzymatic hydrolysis of used newspaper. Biotechnol Bioeng 39 (1):117-120.
12. Helle SS., Duff SJB., Cooper DG. (1993). Effect of surfactants on cellulose hydrolysis. Biotechnol Bioeng 42 (5):611-617.
13. Wu J., Ju LK. (1998). Enhancing enzymatic saccharification of waste newsprint by surfactant addition. Biotechnol Prog 14 (4):649-652.
14. Eriksson T., Börjesson J., Tjerneld F. (2002). Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microb Technol 31 (3):353-364.
15. Bisaria VS., Mishra S., Eveleigh DE. (1989). Regulatory aspects of cellulase biosynthesis and secretion. Critical Rev Biotechnol 9 (2):61-103.
16. Kubicek CP., Messner R., Gruber F., Mach RL., Kubicek-Pranz EM. (1993). The *Trichoderma* cellulase regulatory puzzle: From the interior life of a secretory fungus. Enzyme Microb Technol15 (2):90-99.
17. Riou C., Salmon JM., Vallier MJ., Günata Z., Barre P. (1998). Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae*. Appl Environ Microbiol 64 (10):3607-3614.
18. Bhatia Y., Mishra S., Bisaria VS. (2002). Microbial β-glucosidases: cloning, properties, and applications. Critical Rev Biotechnol 22 (4):375-407.
19. Tolan J., Foody B. (1999). Cellulase from submerged fermentation. Recent progress in bioconversion of lignocellulosics. 65:41-67.
20. Stewart JC., Heptinstall J. (1988). Cellulase and Hemicellulase from *Aspergillus fumigatus* Fresenius. Method Enzymol 160:264-274.

21. Heerd D., Yegin S., Tari C., Fernandez-Lahore M. (2012). Pectinase enzyme-complex production by *Aspergillus* spp. in solid-state fermentation: A comparative study. Food Bioproduct Proce 90:102-110.
22. Pal A., Khanum F. (2011). Efficacy of xylanase purified from *Aspergillus niger* DFR-5 alone and in combination with pectinase and cellulase to improve yield and clarity of pineapple juice. J Food Sci Technol 48 (5):560-568.
23. Ahmadi M. (2011). Solid-state fermentation applications in glucoamylase production by *Aspergillus niger*. Clinical Biochem 44 (13):S240-S241.
24. Negi S., Gupta S., Banerjee R. (2011). Extraction and purification of gucoamylase and protease produced by *Aspergillus awamori* in a single-stage fermentation. Food Technol Biotechnol 49 (3):310-315.
25. Vinetsky YP., Rozhkova AM., Sereda AS., Tsurikova NV., Nurtaeva AK., Semenova MV., Zorov IN., Sinitsyn AP. (2010). Increase in glucoamylase productivity of *Aspergillus awamori* strain by combination of radiation mutagenesis and plasmid transformation methods. Appl Biochem Microbiol 46 (6):633-640.
26. Koc O., Metin K. (2010). Purification and characterization of a thermostable glucoamylase produced by *Aspergillus flavus* HBF34. African J Biotechnol 9 (23):3414-3424.
27. Ryu DDY., Mandels M. (1980). Cellulases: biosynthesis and applications. Enzyme Microbial Technol 2 (2):91-102.
28. Kang S., Ko E., Lee J., Kim S. (1999). Over-production of β-glucosidase by *Aspergillus niger* mutant from lignocellulosic biomass. Biotechnol lett 21 (8):647-650.
29. Wang CL., Wu GH., Chen C., Chen SL. (2012). High Production of β-glucosidase by *Aspergillus niger* on corncob. Appl Biochem Biotechnol 168 (1):58-67.
30. Raza F., Raza NA., Hameed U., Ikram-ul-Haq., Mariam I. (2011). Solid state fermentation for the production of β-glucosidase by co-culture of *Aspergillus niger* and *A. oryzae*. Pak J Botany 43 (1):75-83.
31. Petrova SD., Andreev AA., Bakalova NG., Benadova RM., Kolev DN. (2002). Purification and biochemical characteristics of β-glucosidase from *Aspergillus awamori* K-1. Biotechnol Biotechnol Equip 16 (1):83-90.
32. Coughlan MP. (1985). The properties of fungal and bacterial cellulases with comment on their production and application. Biotechnol Genetic Eng Rev 3:39-109.
33. Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (5259):680-685.
34. Peshin A., Mathur JMS. (1999). Purification and characterization of β-glucosidase from *Aspergillus niger* strain 322. Letter Appl Microbiol 28 (5):401-404.
35. Petrova SD., Andreev AA., Bakalova NG., Benadova RM., Kolev DN. (2002). Purification and biochemical characteristics of β-glucosidase from *Aspergillus awamori* K-1. Biotechnol Biotechnol Equip 16 (1):83-90.

36. Farkaš V., Lišková M., Biely P. (1985). Novel media for detection of microbial producers of cellulase and xylanase. FEMS Microbiol lett 28 (2):137-140.
37. Herr D. (1979). Secretion of cellulase and β-glucosidase by *Trichoderma viride* ITCC-1433 in submerged culture on different substrates. Biotechnol Bioeng 21 (8):1361-1371.
38. Lowry OH., Rosebrough NJ., Farr AL., Randall RJ. (1951). Protein measurement with the Folin phenol reagent. J Biol Chem 193 (1):265-275.
39. Hosel W., Surholt E., Borgmann E. (1978). Characterization of β-glucosidase isoenzymes possibly involved in lignification from Chick Pea (*Cicer arietinum* L.) cell suspension cultures. Europ J Biochem 84 (2):487-492.
40. Blank A., Sugiyama R., Dekker CA. (1982). Activity staining of nucleolytic enzymes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis: use of aqueous isopropanol to remove detergent from gels. Analy Biochem 120 (2):267-275.
41. Zhang C., Li D., Yu H., Zhang B., Jin F. (2007). Purification and characterization of piceid-β-D-glucosidase from *Aspergillus oryzae*. Proc Biochem 42 (1):83-88.
42. Barrera-Islas GA., Ramos-Valdivia AC., Salgado LM., Ponce-Noyola T. (2007). Characterization of a β-glucosidase produced by a high-specific growth-rate mutant of Cellulomonas flavigena. Current Microbiol 54 (4):266-270.
43. Gueguen Y., Chemardin P., Arnaud A. (2001). Purification and characterization of an intracellular β-glucosidase from a Candida sake strain isolated from fruit juices. Appl Biochem Biotechnol 95 (3):151-162.
44. Shewale JG. (1982). β-Glucosidase: its role in cellulase synthesis and hydrolysis of cellulose. The International J Biochem 14 (6):435-443.
45. Hoh YK., Yeoh HH., Tan TK. (1992). Properties of β-glucosidase purified from *Aspergillus niger* mutants USDB 0827 and USDB 0828. Appl Microbiol Biotechnol 37 (5):590-593.
46. Mc Hale A., Coughlan MP. (1982). Properties of the β-glucosidases of *Talaromyces emersonii*. J Gen Microbiol 128 (10):2327-2331.
47. Macris BJ. (1984). Production and characterization of cellulase and β-glucosidase from a mutant of *Alternaria alternata*. Appl Environ Microbiol 47 (3):560-565.
48. Bastawde KB. (1992). Cellulolytic enzymes of a thermotolerant *Aspergillus terreus* strain and their action on cellulosic substrates. World J Microbiol Biotechnol 8 (1):45-49.
49. Narasimha G., Sridevi A., Ramanjaneyulu G., Rajasekhar Reddy B. (2015). Purification and charcterization of β-glucosidase from *Aspergillus niger*. International J Food Properties 19(3)652-661.
50. Castanon M., Wilke CR. (1981). Effects of the surfactant Tween 80 on enzymatic hydrolysis of newspaper. Biotechnol Bioeng 23 (6):1365-1372.
51. Ooshima H., Sakata M., Harano Y. (1986). Enhancement of enzymatic hydrolysis of cellulose by surfactant. Biotechnol Bioeng 28 (11):1727-1734.

52. Lucas R., Robles A., de Cienfuegos GA., Gálvez A. (2000). β-Glucosidase from chalara p aradoxa CH32: purification and properties. J Agri Food Chem 48 (8):3698-3703.
53. Yeoh H., Tan T., Koh S. (1986). Kinetic Properties of β-glucosidase from *Aspergillus ornatus*. Appl Microbiol Biotechnol 25:25-28.
54. Evans J. (1983). Rayonier produces animal feed from secondary sludge at sulfite mill.
55. Monedero V., Kuipers OP., Jamet E., Deutscher J. (2001). Regulatory functions of serine-46-phosphorylated HPr in *Lactococcus lactis*. J Bacteriol 183 (11):3391-3398.
56. Zúñiga M., Miralles MC., Pérez-Martínez G. (2002). The product of arcR, the sixth gene of the arc operon of *Lactobacillus sakei,* is essential for expression of the arginine deiminase pathway. Appl Environ Microbiol 68 (12):6051-6058.
57. Kwon KS., Gyoo Kang H., Chil Hah Y. (1992). Purification and characterization of two extracellular β-glucosidases from *Aspergillus nidulans*. FEMS Microbiol Lett 76 (1):149-153.
58. Yan TR., Lin CL. (1997). Purification and characterization of a glucose-tolerant β-glucosidase from *Aspergillus niger* CCRC 31494. Biosci Biotechnol Biochem 61 (6):965-970.

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