J Ind Microbiol Biotechnol
DOI 10.1007/s10295-013-1291-5

BIOENERGY/BIOFUELS/BIOCHEMICALS

# Highly glucose tolerant β-glucosidase from *Aspergillus unguis*: NII 08123 for enhanced hydrolysis of biomass

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Received: 4 March 2013 / Accepted: 10 May 2013


**Abstract** *Aspergillus unguis* NII-08123, a filamentous fungus isolated from soil, was found to produce β-glucosidase (BGL) activity with high glucose tolerance. Cultivation of the fungus in different carbon sources resulted in the secretion of different isoforms of the enzyme. A low molecular weight isoform, which retained ~60 % activity in the presence of 1.5 M glucose, was purified to homogeneity and the purified enzyme exhibited a temperature and pH optima of 60 °C and 6, respectively. The $K_m$ and $V_{max}$ of the enzyme were 4.85 mM and 2.95 U/mg, respectively, for 4-nitrophenyl β-D-glucopyranoside. The glucose inhibition constant of the enzyme was 0.8 M, indicating high glucose tolerance, and this is the second-highest glucose tolerance ever reported from the *Aspergillus nidulans* group. The glucose-tolerant BGL from *A. unguis*, when supplemented to cellulase preparation from *Penicillium*, could improve biomass hydrolysis efficiency by 20 % in 12 h compared to the enzyme without additional beta glucosidase supplementation. The beta glucosidase from *A. unguis* is proposed as a highly potent "blend-in" for biomass saccharifying enzyme preparations.

**Keywords** Beta glucosidase · Cellulase · Glucose tolerance · Biomass hydrolysis · Isoforms · *Aspergillus unguis*

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## Introduction

β-Glucosidases (EC 3.2.1.21; β-D-glucoside glucohydrolase) are key enzymes in cellulose hydrolysis, being the rate-limiting enzyme that is regulated by feedback inhibition from its own product—glucose. These enzymes are therefore of considerable interest as constituents of cellulose-degrading systems to be used for biomass conversion applications [19]. *Aspergilli* are known to be potent sources of β-glucosidase (BGL) and the most common industrial source of BGL is *Aspergillus niger* [3]. Inhibition by its product and substrate limits its use, especially in the context of biomass hydrolysis. β-glucosidase insensitive to glucose and cellobiose will significantly improve enzymatic conversion of cellulosic biomass to glucose for the subsequent production of fuel ethanol [16]. Glucose-tolerant β-glucosidases (GT-BGLs) have been reported from a few filamentous fungi, and differences in their properties were studied including molecular mass, isoelectric points, pH optima, and glucose inhibition constants [6, 9, 10, 15]. From the limited number of reports about GT-BGLs, it may be speculated that such enzymes are rather uncommon or understudied. However, the impact of the addition of GT-BGLs in the cellulase cocktails for biomass hydrolysis can be significant, and improvements in the yield and concentration of sugars may be achieved by using such blends.

As a part of our screening program, 200 fungal strains from soil samples were screened for the production of extracellular β-glucosidase and a significant number of them exhibited β-glucosidase activity (unpublished results). Further analyses proved that one of the isolates coded as BTC-F58 secreted a BGL that was active even in 1 M glucose. The fungal isolate was identified by morphological as well as molecular identification techniques to be of a strain of *Aspergillus unguis*. Detailed studies on the

Published online: 04 June 2013

# Highly glucose tolerant β-glucosidase from *Aspergillus unguis* - *NII 08123* for enhanced hydrolysis of biomass

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**Abstract**

*Aspergillus unguis* NII-08123, a filamentous fungus isolated from soil was found to produce β-glucosidase (BGL) activity with high glucose tolerance. Cultivation of the fungus in different carbon sources resulted in the secretion of different isoforms of the enzyme. A low molecular weight isoform which retained ~60% activity in the presence of 1.5M glucose was purified to homogeneity and the purified enzyme exhibited a temperature and pH optima of 60 °C and 6 respectively. The $K_m$ and $V_{max}$ of the enzyme were 4.85 mM and 2.95 U/mg respectively for 4-Nitrophenyl β-D-glucopyranoside. The glucose inhibition constant of the enzyme was 0.8M indicating high glucose tolerance, and this is the second highest glucose tolerance ever reported from *Aspergillus nidulans* group. The glucose tolerant BGL (GT-BGL) from *A. unguis* when supplemented to cellulase preparation from *Penicillium* could improve biomass hydrolysis efficiency by 20 % in 12h compared to the enzyme without additional beta glucosidase supplementation. The beta glucosidase from *A. unguis* is proposed as a highly potent '*blend –in*' for biomass saccharifying enzyme preparations.

Keywords*: beta glucosidase, cellulase, glucose tolerance, biomass hydrolysis, isoforms, Aspergillus unguis.*

## Introduction

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As a part of our screening programme, 200 fungal strains from soil samples were screened for the production of extracellular β-glucosidase and a significant number of them exhibited β-glucosidase activity (unpublished results). Further analyses proved that one of the isolates coded as BTC -F58 secreted a BGL that was active even in 1M glucose. The fungal isolate was identified by morphological as well as molecular identification techniques to be of a strain of *Aspergillus unguis*. Detailed studies on the GT-BGL production indicated that multiple isoforms of the enzyme were secreted by the fungus and the expression of different isoforms showed variation in response to the inducer/carbon source. SDS Polyacrylamide Gel Electrophoresis /Activity staining experiments showed that crude extract of *A. unguis* contained more than five isoforms of the enzyme. Zymogram analyses indicated that a low molecular weight isoform retained its catalytic activity even at very high glucose concentrations. This caused us to speculate that this isoform might be responsible for the glucose tolerance of the enzyme preparation from *A. unguis*. The low molecular weight isoform was isolated, purified and the properties were studied to characterize the suspected glucose tolerant isoform. Results showed that the enzyme of interest represented only minor fraction of the total β-glucosidase activity and the other

isoforms that formed the major fraction were highly sensitive to glucose inhibition. The enzyme when supplemented to *Penicillium janthinellum* cellulase could improve the hydrolysis performance of the enzyme cocktail.

## Materials and Methods

### Organisms and culture conditions

*Aspergillus unguis*, used in this study, was obtained from the soil samples and maintained on potato dextrose agar. For enzyme production, the culture was grown on minimal medium with the composition in g/L $(NH_4)_2SO_4$ -1.4, $KH_2PO_4$ -2.0, $CaCl_2.2H_2O$ -0.3, $MgSO_4.7H_2O$ -0.3, $FeSO_4.7H_2O$ -0.005, $MnSO_4.H_2O$ -0.0016, $ZnSO_4.7H_2O$ -0.002, Peptone -2.5, Yeast Extract -1.0, supplemented with an appropriate carbon source at a concentration of 1% (w/v). The initial pH of the medium was adjusted to 5.0. Erlenmeyer Flasks (250ml) containing 100 ml of the sterile liquid medium was inoculated with 1 x $10^6$ spores of the fungus suspended in 0.05% (v/v) Tween 80 solution. The culture was incubated at 30 ± 1 °C for 86h on a shaker incubator at 200 rpm and was then harvested by centrifugation (8000 rpm, 20 min at 4 ºC) followed by filtration through Whatman GF/A glass microfiber filters. These crude enzyme samples were assayed for BGL activity and were stored at 4 ºC for further studies.

### Identification of microorganism

The fungal isolate BTC-F58 was grown in potato dextrose agar (PDA) medium and the conidial characteristics were observed under a phase contrast microscope (Leica, Germany). Further, the conidia were coated with gold palladium particles using a JEOL JFC-1200 fine coater and their structures were observed using scanning electron microscope (SEM, JEOL Model JSM – 5600, Japan) at 15 kV and 500–5000X magnification. In addition to recording morphological characteristics of the fungal isolate, molecular systematic approach was also employed for identification using 18S rRNA gene sequence of the fungal isolate. A portion of the 18S rRNA gene was amplified from the genomic DNA by polymerase chain reaction (PCR) using the universal primers

NS1, NS4, NS3, NS8 [23]. Nucleotide sequences of the PCR amplicons were determined by dye terminator sequencing. Identity of the sequence assembly was established by BLAST analysis [1]. Later a homology search was performed and based on the results; a phylogenetic tree was constructed using the neighbour joining method implemented in PHYLIP [8].

**Differential Induction of BGL in response to Carbon source**

The carbon sources -cellulose, xylose, lactose, glucose, maltose, and sucrose were supplied in the medium at 1% w/v level and fermentation was carried out as outlined above. Culture filtrates were assayed for BGL activity and the samples were concentrated using vacuum centrifugation (Eppendorf, Germany). Secretion of BGL isoforms by the fungus in response to the inducer carbon source was studied by analyzing the Zymogram generated by perfroming native PAGE followed by activity staining of the gels.

**Native PAGE and Zymogram analysis**

Crude enzyme preparations were fractionated by Native PAGE using 12% Gel with 5% stacking Gel. Sample loadings were duplicated in same order in the same gel so as to perform the activity staining in presence and absence of glucose respectively. Activity staining of the gels was done by incubating them with a 10mM methyl umbelliferyl β-D-glucopyranoside (MUG) solution in citrate buffer (0.05 M, pH 4.8). While staining, identical conditions were maintained for both the sets except for presence of glucose (0.5-1.5M) in one of the sets. Activity bands were visualized as blue green fluorescence (methyl umbelliferone) by illumination with long wavelength UV trans-illumination and photographs were acquired using an imaging system (Syngene-GBox, UK). Both the sets of gels were pictured simultaneously to ensure even conditions of illumination and exposure. Band intensities were measured using ImageJ software [18]. The fluorescence intensities of the bands in presence and absence of glucose was compared to calculate the percentage activity retention as follows

% Activity Retention (Glucose Tolerance) = $T_g = (I_g/I_0) \times 100$

Where $I_g$ and $I_0$ are the fluorescence intensities in presence and absence of glucose respectively for each band.

**Enzyme assay**

β- Glucosidase activity was assayed using p-nitro phenyl-β-D glucopyranoside (pNPG) as substrate. The reaction mixture which consisted of 1 ml of citrate buffer (0.05 M, pH 4.8), 0.5 ml of enzyme sample and 0.5 ml of 10 mM pNPG was incubated at 40 °C for 15 min. Reaction was terminated by adding 2 ml of 0.2 M $Na_2CO_3$. The absorbance of p-nitro phenol (pNP) released was measured at 400nm. One unit enzyme activity was defined as the amount of enzyme required releasing 1 µM of pNP per minute and was expressed as IU/ml.

**Enzyme purification and characterization**

The culture broth was concentrated using acetone fractionation. Different isoforms were precipitated using varying concentrations of chilled acetone. Among the β-glucosidase active fractions, the fraction that showed glucose tolerance was further purified using chromatography. The samples were loaded on to Q Sepharose Fast Flow ion exchange column (Biorad, USA) pre-equilibrated with 0.05M Tris-HCl Buffer (pH-7). The column was eluted using a linear gradient of 0-1.0 M NaCl in 50mM Tris-HCl buffer (pH-7) at a flow rate of 0.75ml $min^{-1}$ when two activity peaks were detected. All fractions were analysed for β-glucosidase activity and fractions corresponding to the activity peaks were pooled and concentrated. Further gel elution was carried on Sephadex G75 column using 50mM, pH 4.8, Citrate buffer to separate the low molecular isoform, and the fractions corresponding to the peak was pooled and concentrated. Homogeneity of the purified enzyme was tested using SDS PAGE [12]. Enzyme kinetic studies were performed and the Michaelis Menten curve fitting and determination of $V_{max}$ and $K_m$ were performed using Graphpad Prism ® software (Graphpad Software Inc, USA). Inhibition kinetics was also studied using varying concentrations of inhibitors and glucose inhibition constant $K_i$ for BGL was determined using the software. Temperature optimum of the enzyme was determined by assaying the enzyme at different temperatures (40 -70 ˚C with 10 ˚C intervals) as outined above. The temperature at which maximum activity was obtained was considered as optimum. Similarly pH optimum for activity of the GT-BGL was determined by analyzing the activity of the enzyme at various pH (3-7) achieved by use of citrate and Tris buffers.

**Hydrolysis performance of enzyme cocktail containing *A.unguis* BGL**

The cellulase preparation used for the experiments was from *Penicillum janthinellum* and the substrate used was steam exploded bagasse (SEB). The *P. janthinellum* strain and SEB were kind gifts from Dr DV Gokhale and Dr AJ Verma respectively from the CSIR-National Chemical Laboratory, Pune, India. The *P.janthinellum* cellulase was produced following the protocol provided by Dr Gokhale. The hydrolysis was carried out in 100 ml screw capped conical flasks with a working volume of 30ml. The biomass loading was 10% w/w, and the cellulase and BGL loadings were 15 FPUs/g and 20 or 60 IU/g respectively. Incubation was performed at 50 °C for 60h with sampling at every 12h. Controls without BGL supplementation and with supplementation of 20 IU/g commercial BGL preparations were run in parallel. Samples were analyzed for glucose released by the NREL Laboratory Analytical Protocol for sugars using HPLC [13]

**Results**

**Identification of the fungus**

Fungal isolate BTC-F58 formed dark green colonies on PDA, and the microscopic features resembled that of *Aspergillus* sp. Morphological examination of the fungus under microscope revealed long roughened and thick walled spicular hyphae, hemispherical vesicles, biseriate phialides supported by metulae, and globose conidia dull green in color -the features characteristic of *Aspergillus unguis* [21] *(*Fig 1A-D*)*. PCR amplification of the 18S rRNA region yielded a 1678bp sequence which was submitted to Genbank with the accession JQ726491 (gi|387966750). The BLAST analyses of the partial DNA sequence showed 99% similarity with 18S rRNA sequence of *Aspergillus unguis* and progressively lesser similarity with those from other *Aspergilli* confirming that the isolate is a strain of *Aspergillus unguis*. The isolate was deposited in the NII culture collection with accession NII 08123. Pair wise alignments of the sequence with closest matches in the sequence depositories identified by BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) searches were performed, and the phylogenetic relationships were derived using neighbourhood joining methods and represented as a phylogenetic tree (Fig 2).

**Differential expression of BGL in response to C-sources and the existence of multiple BGL isoforms**

Since during the fermentation for BGL production by *A. unguis NII 08123*, there was a large amount of fluctuation in the glucose tolerance of BGL, it was speculated that there could be differences in the expression of enzyme depending on the immediate environment, especially the type of C source. To evaluate this, the isolate was cultured in the same basal medium under identical conditions but with different C sources, and the BGL activity and glucose tolerance was monitored. Effect of carbon source/inducer on β-glucosidase production by *A. unguis* and the expression of different isoforms are shown in Fig 3 and 4. Maximum glucose tolerance (32.75 % activity retained) was observed with lactose as C source, and the glucose tolerance and levels of BGL yield varied with C-sources (Fig 3).

Multiplicity of BGLs has been reported earlier by several authors [13, 6, 10, 16, 19]. Difference in expression of the BGL isoforms could be the reason for the differences in glucose tolerance of the crude enzyme preparation, since the actual amount of the glucose tolerant isoform could vary according to the carbon source. Existence of multiple isoforms of BGL in *A. unguis* was studied by growing the fungus in different C-sources and performing zymogram analyses. Since it was noted that the crude carbon source-wheat bran induced most of the BGL isoforms in *A. unguis*, BGL production was also conducted in media supplemented 1% w/v of either wheat bran or rice straw. Results shown in Fig 4 & 5 indicated the existence of multiple isoforms (5 detected here) of the enzyme. While wheat bran induced the production of 5 BGL isoforms (Fig 5, Lanes 1-3), rice straw and cellulose could induce only the high molecular weight and low molecular weight BGL isoforms. There was also a direct correlation between the signal intensity of the low molecular weight band and the glucose tolerance (as detected by pNPG assay) indicating that this could be the glucose tolerant isoform (GT-BGL)

This was further proved by fluorescence intensity analysis of the BGL activity bands observed at varying glucose concentrations. BGL produced using wheat bran as C source was run on Native PAGE and activity stains were performed in buffers containing varying concentrations of glucose (0.5-1.5M). Only the lower band was detected when assayed in presence of glucose and the intensity of this band was negatively correlated to the glucose concentration confirming that this band itself is the glucose tolerant isoform (Fig 5).

**Purification and characterization of β-glucosidase**

To study further the properties of the glucose tolerant BGL isoform, the low molecular weight BGL protein was purified to homogeneity from the culture filtrate of *A. unguis* grown on 1% w/v lactose as C source. Crude BGL sample (500ml) was precipitated using 2 volumes of chilled acetone and the precipitate was re-dissolved in 15ml of 0.05M, pH 4.8 citrate buffer. The concentrated sample contained 75% of the initial activity. Anion exchange chromatography on Q-Sepharose Fast Flow column resulted in the sample separation into two-a major and minor protein peaks (PI and PII respectively) both having BGL activity  Assay of the fractions corresponding to the peaks as well as SDS-PAGE analyses indicated that the GT-BGL protein has partitioned into the minor peak (Fig 6). Fractions corresponding to the two peaks were pooled separately and PII was run on Sephadex G75 column when one peak corresponding to the GT BGL was obtained.  The purification results are summarised in Table 1.

The purified GT-BGL was optimally active at 60 ºC and at pH 6.0. Increase in temperature beyond 60 °C (pH 6.00) resulted in a rapid decline in activity (Fig 7). At pH 7 the activity as almost the same as what was obtaiend at pH 6.00 (Fig 8).  The relative activity of GT-BGL was more than  60% of the activity at optimal pH over the entire range tested.

The standard assay mixture with pH 6.0 was used for the determination of reaction velocities of GT-BGL. $K_m$ and $V_{max}$ for the hydrolysis of pNPG by the enzyme was determined using 10-80 mM substrate. Michaelis-Menten kinetics was fitted and the determination of $K_m$ and $V_{max}$ was performed using the non linear regression implemented in Graphpad Prism. $K_m$ and $V_{max}$ were determined to be 4.85 mM and 2.95 U/mg, respectively for pNPG. End product inhibition was evaluated using pNPG as substrate and in presence of 0-1.0M Glucose as the inhibitor.GT-BGL was found to be highly glucose tolerant with a $K_i$ of 800mM.  Enzyme properties determined by the study are listed in Table 2.

**Hydrolysis performance of cellulase blends containing *A. unguis* BGL**

*P. janthinellum* cellulase preparation containing ~ 5.5 IU/ml BGL was blended with additional BGL from *A unguis* so that the final BGL concentrations were either 20 or 60 IU/g SEB to be hydrolyzed. Hydrolysis experiments showed that there was a considerable improvement in hydrolysis performance when *A. unguis* BGL was blended with the cellulase. With 60 IUs/g additional BGL loading, there was a 20 % improvement in glucose yield even within 12h of hydrolysis. There was a linear improvement in hydrolysis performance for blends containing higher BGL loading an efficiency of ~ 60 % of the theoretical maximum was achieved in 48h in blends containing 60 IU/g additional BGL loading (Table 3). Hydrolysis performance of blend containing 20 IU/g of commercial BGL was significantly better (71% efficiency attained in 48h). This could be due to the presence of cellulase and hemicellulase activities in the commercial BGL sample which we had detected. Hydrolysis study done with one of the best biomass hydrolyzing enzyme blends available in the market could yield >75% efficiency with this biomass (Data not shown)

**Discussion**

β-glucosidase plays a major role in the conversion of cellulosic biomass to ethanol, being the rate limiting enzyme that determines the action of all cellulase components. A large number of these enzymes from bacteria and plants have been purified and studied, but most microbial β-glucosidases are very sensitive to glucose inhibition [2,9]. There are only limited reports in publications on glucose tolerant BGLs and most of the reports on filamentous fungi describes *Aspergilli* as the producers of GT-BGLs. To the best of our knowledge, this is the first report on production of glucose tolerant β-glucosidase from *Aspergillus unguis* which belongs to the *Aspergillus nidulans* group. Glucose tolerant β-glucosidases have been reported previously in *A. oryzae* [16] and in *A. niger* [22]. However, the growth characteristics of *A. unguis* under submerged fermentation make it attractive for large scale production of the enzyme. Under our culture conditions the fungus grew as pellets in an even suspension and did not form large clumps/aggregates allowing better aeration and mixing. Other *Aspergilli* that we had used for BGL production (eg *A niger* [19]) either formed a mat or ball like structures in shake flasks

and larger non uniform aggregates in fermenter. This fact and the ability to use substrates like wheat bran for production makes *A unguis* a preferred choice for large scale BGL production for biomass hydrolysis.

The level of production of glucose tolerant isoforms was greatly influenced by the carbon source supplied. It is interesting to note that the highest tolerance (32.75%) was observed in cultures grown with lactose as carbon source. Lactose is a known inducer of cellulase and could have caused a selective induction of GT-BGL. From the zymogram analysis, we identified 5 active isoforms of β-glucosidase even though their expression level varies in the presence of different carbon source. Staining the Native as well as SDS PAGE gels using MUG in presence of different concentrations of glucose indicated that the low molecular weight isoform plays a key role in activity retention, even in presence of considerable amount of glucose. It has been reported previously that the glucose tolerant beta glucosidases are low molecular weight proteins expressed at lower levels compared to the highly expressed, high molecular weight glucose sensitive major BGLs in *Candida peltata* [17], *Aspergillus oryzae* [16], *A. foetidus* [6], *A. tubingensis* [7] and in *Aspergillus niger* [19]. BGL multiplicity can be attributed to the presence of multiple genes or due to differential post transcriptional modifications [5]. Differential expression of the various BGL proteins are reported in response to the carbon sources supplied in the medium or the conditions of culture [15, 24] and could be a probable adaptation of the fungi to respond to the changing immediate environments. This property however, could be exploited for selective expression of a desired isoform from a fungus by manipulating the culture conditions/carbon source carefully. This is apparently useful not only in context of producing BGL for biomass hydrolysis but also for flavour enhancement in wine and fruit juices, provided that the BGL in question has the suitable substrate specificity [22]

The high resistance of *A unguis* BGL to glucose inhibition and the advantages due to growth properties of the fungus underlines its importance in biomass hydrolysis. With few exceptions, most microbial β-glucosidases show competitive inhibition kinetics in presence of glucose [17]. Extracellular β-glucosidases are highly sensitive to glucose and normally have glucose inhibition constants ranging from 0.5-100mM. Noticeable exceptions like *A.oryzae* [16], *Candida peltata* [17] where the $K_i$ values have been reported to be 1.36 M and 1.4 M respectively, do exist though this is very rare. The $K_i$ (0.8M) obtained for *A.unguis* GT-BGL reported here, may be considered as the second highest tolerance ever reported from the *Aspergillus nidulans* group. The

inhibition kinetics graph was explained based on the Cornish-Bowden plot where the inhibition constant($K_i$) is greater than dissociation constant($K_i$). GT-BGL from *Aspergillus unguis* exhibited a type of mixed inhibition with a predominating un-competitive inhibition. Previously it has been reported that β-glucosidase from *Trichoderma viride* showed a type of mixed inhibition with competitive character [14].

Additional BGL supplementation in biomass hydrolyzing enzyme preparation is a common practice since typically the commercial cellulase producing microbial cultures contains very less BGL activity. In all the current commercial enzyme preparations for biomass hydrolysis, the BGL is also highly sensitive to glucose which limits their potential to achieve high sugar concentrations. In our preliminary studies conducted with steam exploded bagasse, supplementation of the GT-BGL containing BGL preparation from *A .unguis* could enhance the total sugar yield by ~20 % in 12h. Further optimizations of enzyme loadings and ratios are expected to enhance the sugar yield further. The results indicate the potential for further development of this enzyme as a potential "*blend-in*" for biomass hydrolyzing enzyme preparations. In conclusion, the short duration needed for production, possibility of modulation of the expression by varying carbon sources, as well as the high glucose tolerance of this enzyme are particularly favourable for the application of this BGL in the enzymatic hydrolysis of cellulose to glucose.

**Acknowledgements**

The work was supported by the research grant SR/FT/L-54/2006 to RKS from Department of Science and Technology, Govt. of India and the grants from TIFAC, Department of Science and Technology, Govt. of India. RKP is thankful for financial support from CSIR and MNRE, Govt of India. Authors are thankful to the Department of Forests, Kerala State for permission to carryout sampling in Silent Valley National Park. We thank Dr AJ Varma and Dr DV Gokhale, from CSIR-NCL for providing SEB and the *P. janthinellum* culture respectively. We acknowledge the Electron microscopy section of CSIR-NIIST for SEM analysis of the fungus and Dr Ramesh Kumar N, Biotechnology Division, CSIR-NIIST for DNA sequencing service.

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## Figure Captions and Legends

**Fig. 1** *Aspergillus unguis* strain morphology and BGL screening

*Fig.1: Legends*
*Aspergillus unguis* – A) colony on PDA agar, B) Conidiophore showing thick wall and biseriate phialides C) Screening of betaglucosidase production using MUG (Methyl umbelliferyl β-D glucopyranoside) soft agar method D) Electron micrograph of the conidiophores showing biseriate phialides with metuale

**Fig. 2 Phylogenetic tree of *Aspergillus unguis* NII 08123 (BTC-F58)**

*Fig.2: Legends*
Phylogenetic tree showing relationship between *Aspergillus unguis* NII 08123 (BTC-F58) and the other related species of *Aspergilli*

**Fig. 3 BGL production in response to carbon sources in the medium**

*Fig. 3: Legends*
BGL production in response to carbon sources in the medium(open bars). Hatched bars represent the BGL activity when asasyed in presence of 1.0 M Glucose and % activity retention/glucose tolerance ($T_g$) is represented by filled bars in the back ground

**Fig. 4 Differential expression of BGL in response to C source**

*Fig.4: Legends*
Differential expression of BGL in response to C source. Enzyme samples were subjected to native PAGE and incubated with MUG for detection of activity. Lane information -Carbon source followed by $T_g$ in parenthesis: 1) wheat bran (23%), rice straw (1.9%), cellulose (20.2%), glucose (0.0%). Image inverted for clarity

**Fig. 5 Evaluation of glucose tolerance by pixel density analysis**

*Fig. 5: Legends*
Glucose tolerance of the BGL proteins secreted by *A. unguis* when grown in 1% w/v wheat bran as C source. Enzyme samples were subjected to native PAGE and incubated with MUG for activity detection. Arrowheads indicate the isoforms. Lane information (Normalized fluorescence intensity and $T_g$ of GT-BGL in parentheses): lanes 1, 2, 3- BGL activity signals in gel incubated without glucose (0.148, 100%). Lanes 4&5 – BGL activity signals in presence of 0.5M glucose (0.118, 80%), Lanes 6&7 – BGL activity signals in presence of 1.0 M glucose (0.117, 79%), Lane 9 – BGL activity signal in presence of 1.5M glucose (0.086, 58%). Image inverted for clarity.

**Fig. 6 Activity staining of BGL fractions separated by chormatography**

*Fig. 6: Legends*

6A: Activity staining of the pooled fractions corresponding to the major and minor protein peaks obtained after ion exchange purification of BGL. Lane 1 & 2 – Peaks PI and PII respectively, run on native-PAGE and activity stained. Lanes 3 &4 - Peaks PI and PII respectively, run on native-PAGE and activity stained in presence of 1.0 M Glucose. The high molecular weight major isoform was not completely separated using the Q Sepharose ion exchange chromatography as can be gauged from the activity stain obtained for this protein (Lanes 2 & 4)

6B: SDS –PAGE analysis of he purified GT-BGL. Lane 1- moleculat weight marker, Lane 2- Purified GT-BGL – Arrowhead indicate the ~10 kDa GT-BGL protein band.

**Fig. 7 GT-BGL enzyme activity profile at different temperature**

**Fig. 8 GT-BGL enzyme activity profile at different pH**

Figure

**Fig. 1**

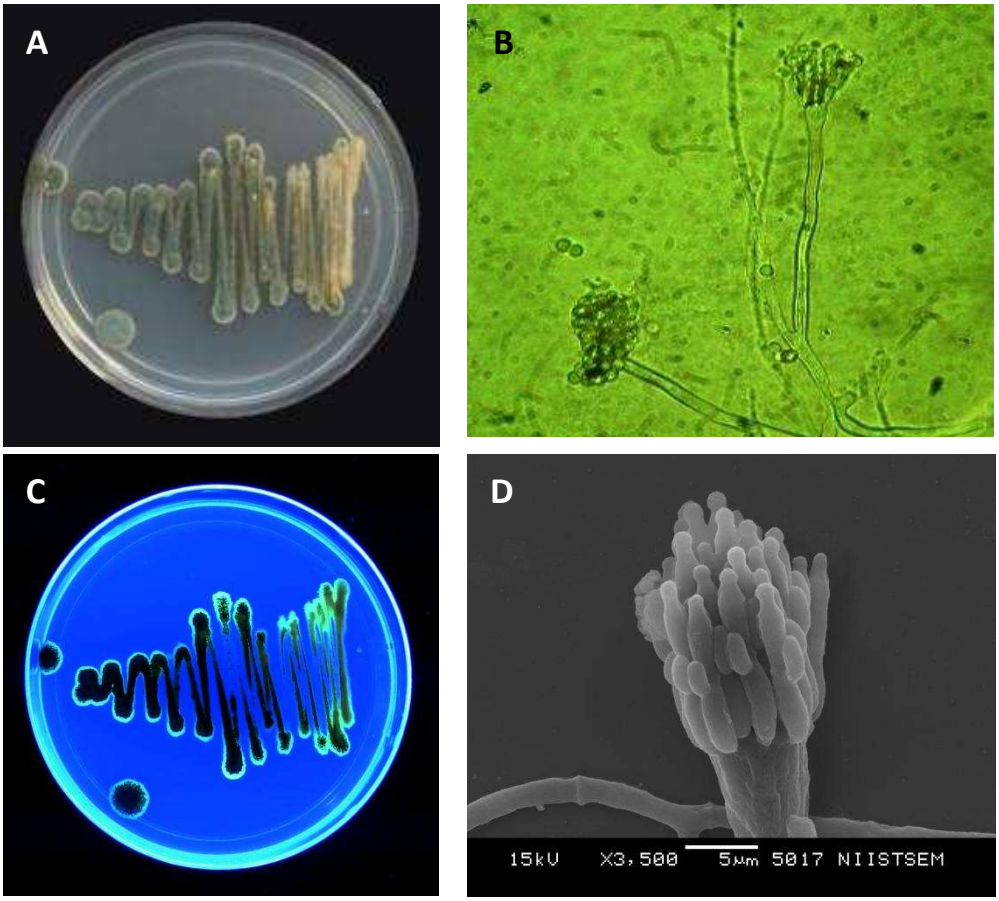


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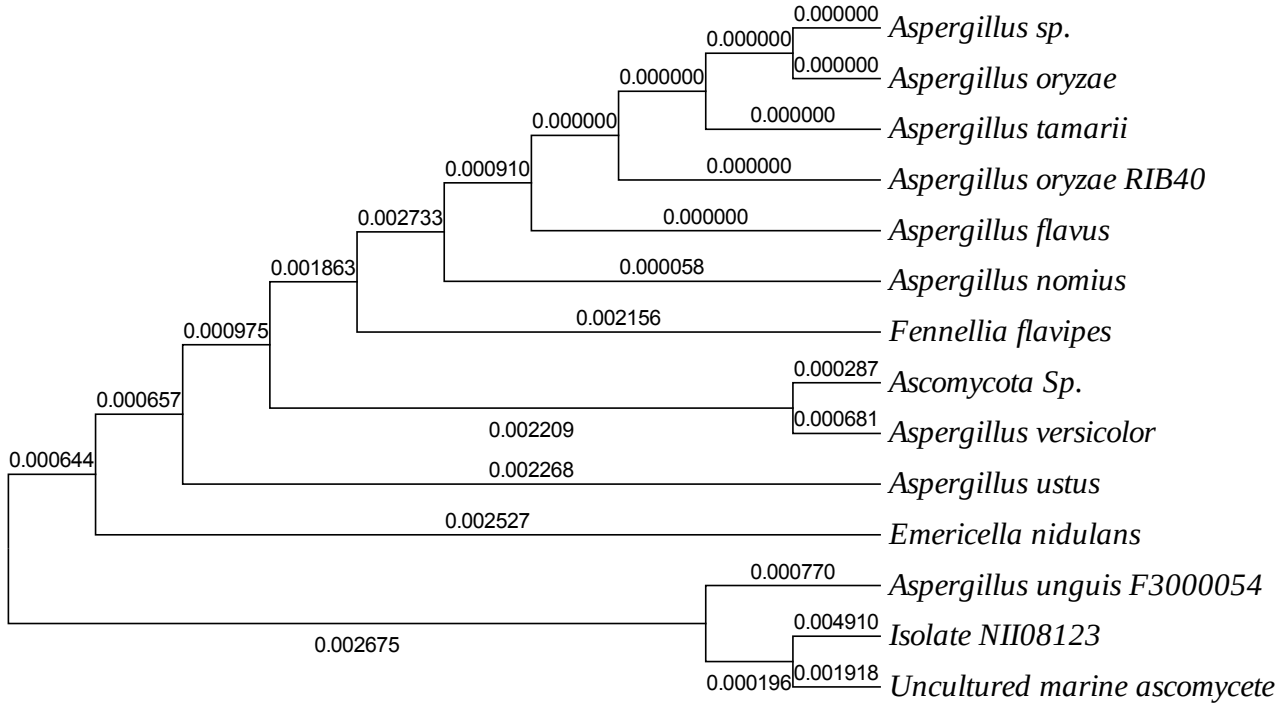
**Fig. 2**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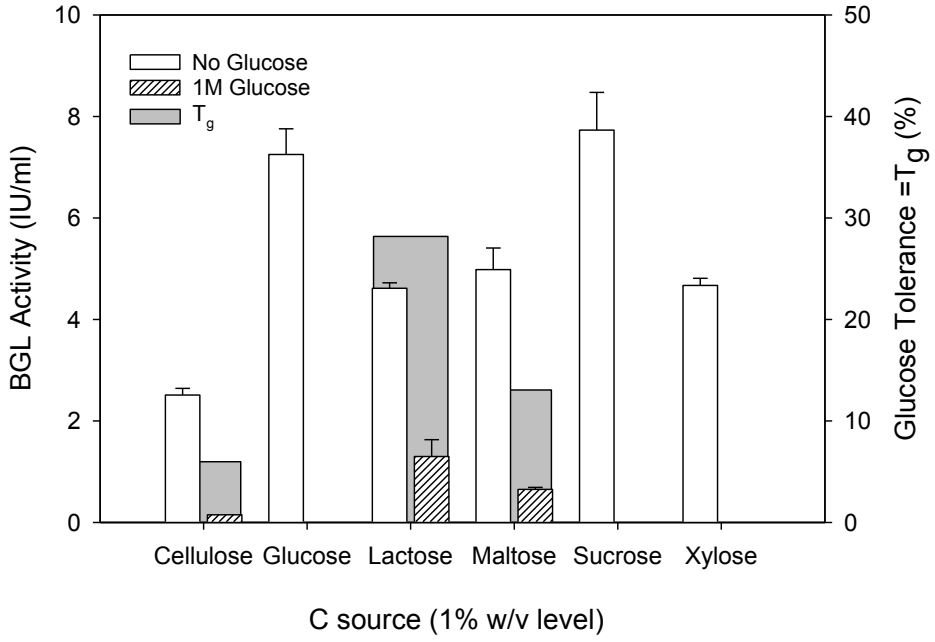
Aspergillus oryzae RIB40
Aspergillus flavus
Aspergillus nomius
Fennellia flavipes
Ascomycota Sp.
Aspergillus versicolor
Aspergillus ustus
Emericella nidulans
Aspergillus unguis F3000054
Isolate NII08123
Uncultured marine ascomycete

**Fig. 3**


No Glucose
1M Glucose
$T_g$
BGL Activity (IU/ml)
Glucose Tolerance =$T_g$ (%)
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2
4
6
8
10
0
10
20
30
40
50
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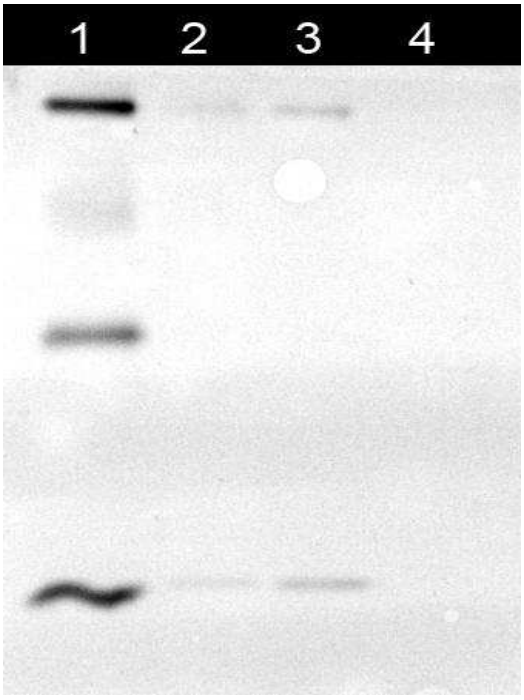
Glucose
Lactose
Maltose
Sucrose
Xylose
C source (1% w/v level)

**Fig. 4**



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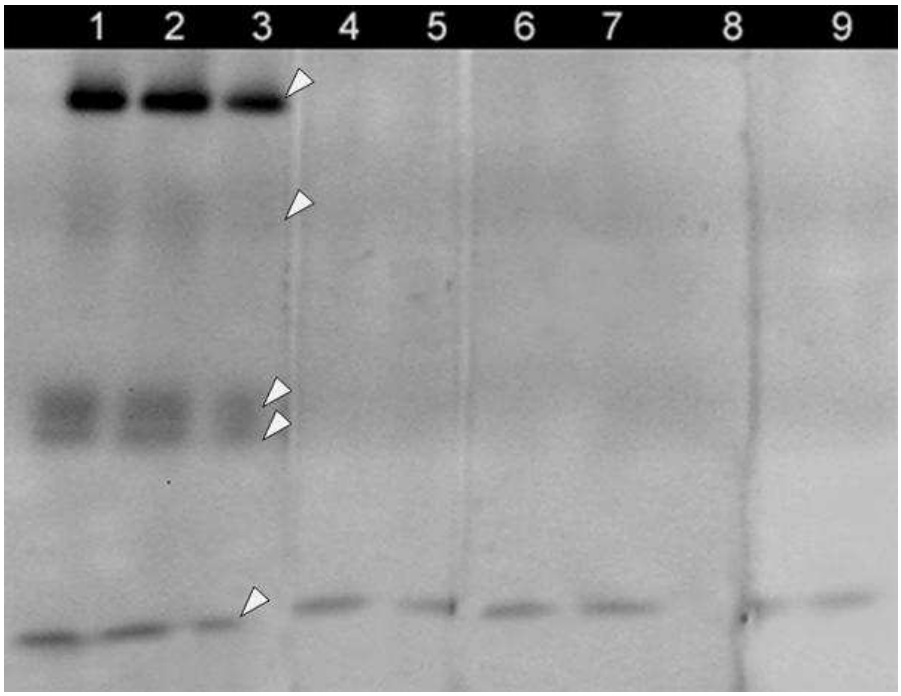


**Fig 6 A**

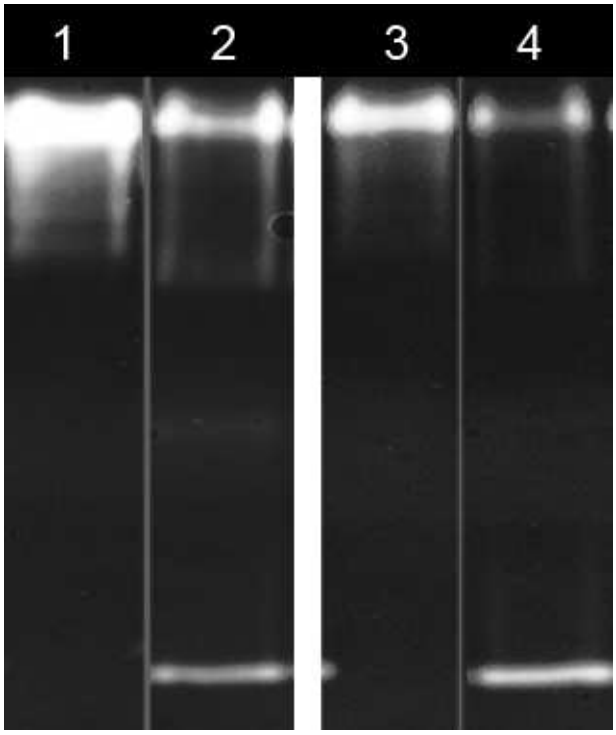



**Fig 6B**

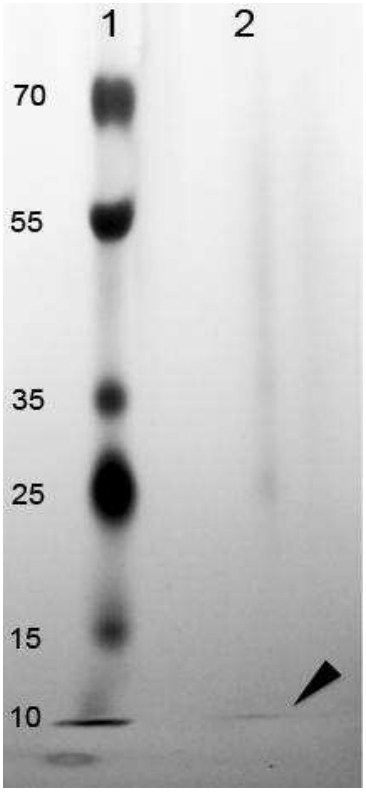


**Fig. 7**

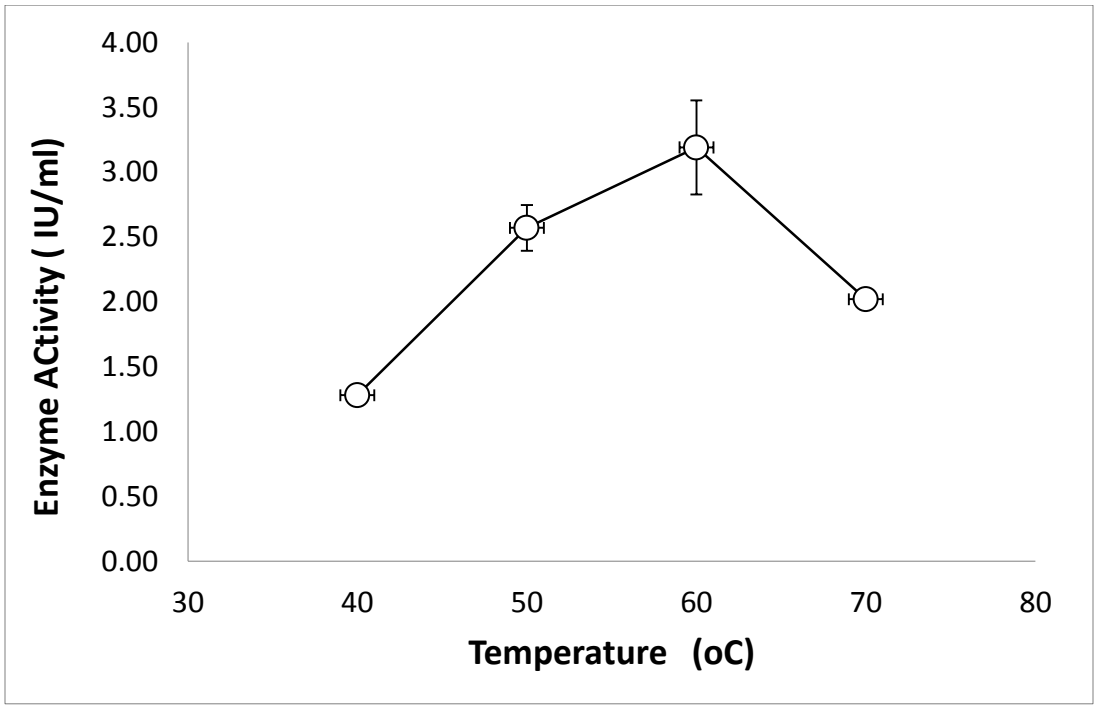
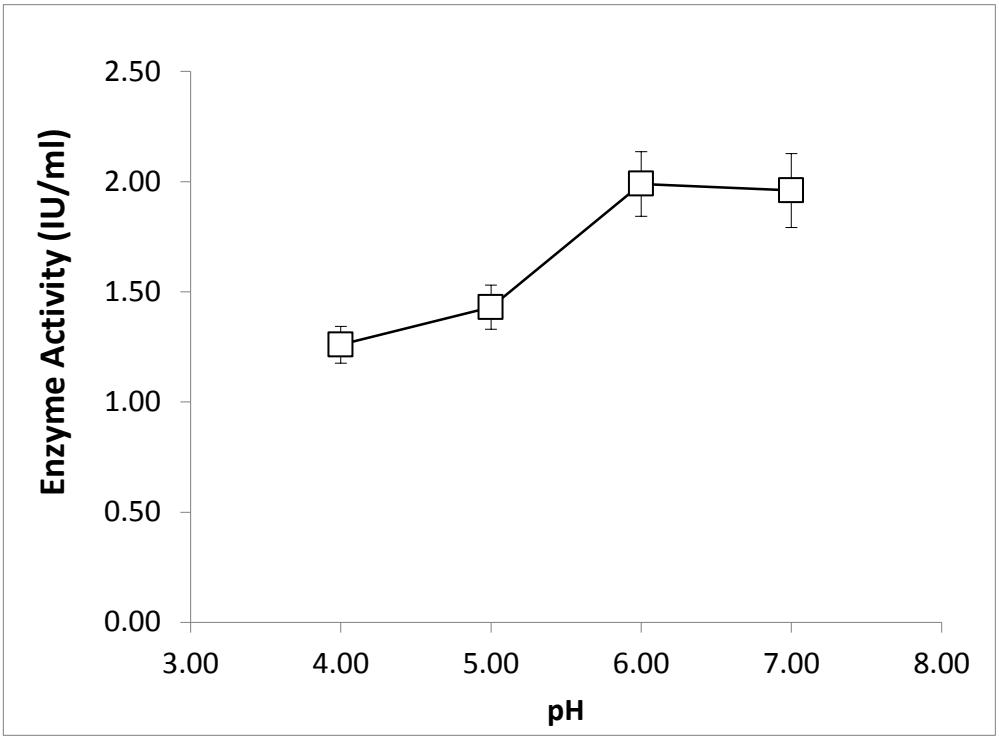

4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Enzyme ACtivity ( IU/ml)
30
40
50
60
70
80
Temperature (oC)

**Fig. 8**


Enzyme Activity (IU/ml)
2.50
2.00
1.50
1.00
0.50
0.00
3.00
4.00
5.00
6.00
7.00
8.00
pH

Table

**Table 1** Purification of GT-BGL from *A.unguis NII 08123*

| Purification stage | Total volume (ml) | Total protein (mg) | Total Activity (U) | Yield (%) | Specific Activity (U/mg) | Fold Purification |
|---|---|---|---|---|---|---|
| Crude Enzyme | 1400 | 482.7 | 715400 | 100 | 1482 | 1.00 |
| Acetone PPT | 15 | 3.9 | 12690 | 1.77 | 3275 | 2.21 |
| Q Sepharose-Peak II | 6 | 1.3 | 11652 | 1.63 | 9182 | 6.20 |
| Gel Elution of Peak II | 2 | 0.1 | 296 | 0.04 | 3131 | 2.11 |

**Table 2** Physicochemical and kinetic properties of *Aspergillus unguis* GT-BGL

| Feature | GT-BGL |
|---|---|
| Molecular weight (SDS-PAGE) | ~28 kDa |
| Optimal pH | 6 |
| Optimal Temperature (°C) | 50 |
| $K_m$ (mM) | 4.85 |
| $V_{max}$ (U/mg) | 2.95 |
| $K_i$ [glucose] (mM) | 800 |

**Table 3:** Hydrolysis of steam exploded bagasse using enzyme blends containing *A. unguis* BGL

| Time | Glucose Released (mg/g) | | | |
|---|---|---|---|---|
| (h) | 20 IU/g BGL | 60.0 IU/g BGL | No Addnl. BGL | Commercial BGL (20 IU/g) |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 12.0 | 179.7 | 195.8 | 149.5 | 135.5 |
| 24.0 | 228.5 | 251.5 | 229.1 | 358.4 |
| 36.0 | 306.0 | 322.0 | 292.0 | 404.0 |
| 48.0 | 308.0 | 353.4 | 325.9 | 425.4 |
| 60.0 | 320.0 | 354.4 | 315.2 | 396.9 |