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Short communication

Properties of a major β-glucosidase-BGL1 from *Aspergillus niger* NII-08121 expressed differentially in response to carbon sources

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ABSTRACT

Aspergillus niger NII-08121/MTCC 7956 exhibited differences in expression of β-glucosidase (BGL) in response to carbon sources provided in the medium. Activity staining with methyl umbelliferyl β-D-glucopyranoside (MUG) indicated that four different isoforms of BGL were expressed when *A. niger* was grown under submerged fermentation with either lactose or cellulose, whereas only two were expressed when wheat bran or rice straw was used as the carbon source. Among the four isoforms of BGL expressed during lactose supplementation, two were found to retain 92% and 82% activity respectively in presence of 250 mM glucose in the MUG assay. The major β-glucosidase (BGL1) was purified to homogeneity by electro elution from a Native PAGE gel. The purified 120 kDa protein was active at 50 °C and was stable for 48 h without any loss of activity. The optimum pH and temperature were 4.8 and 70 °C respectively.

1. Introduction

The enzymatic hydrolysis of cellulose is still considered as one of the major limiting steps of the biofuel production from lignocellulosic biomass. For efficient hydrolysis of cellulosic biomass, synergistic action of all the components of cellulase complex is required. In the cellulase complex, β-glucosidase is the rate limiting enzyme which is inhibited by its own end product (glucose) as well as substrate (cellobiose) [1]. The cellulase enzyme complex secreted by *Trichoderma reesei* has been intensively investigated. *T. reesei* strains produces low quantities of β-glucosidase and their enzymes are known to be highly glucose sensitive. Complete hydrolysis of cellulose often requires supplementation of *T. reesei* cellulases with a commercial β-glucosidase preparation such as that from *Aspergillus niger* [2]. The overall efficiency of saccharification may be improved by supplementation of cellulase with glucose tolerant β-glucosidase [3].

In this study, we have tried to address the problem of hydrolysis of biomass with homogenous cellulase by supplementing it with extra β-glucosidase with higher BGL activity and glucose tolerance. We hypothesized that it would be possible to express desirable glucose tolerant isoform of BGL by manipulating carbon source in the cultivation medium. Thus, the objective of this work was to study the properties of a major β-glucosidase-BGL1 from *A. niger* NII-08121 expressed differentially in response to carbon sources.

2. Materials and methods

A. niger NII 08121/MTCC 7956 was isolated from decaying wood at NIIST and maintained on PDA slants. Spores of the fungus were used as inoculum and were prepared by dislodging them from the surface of fully sporulated PDA slants into sterile 0.05% Tween 80 solution. Spore count was performed using a hemocytometer. All the chemicals used in the medium were reagent grade from Himedia (India) and Sigma (USA). Substrate for β-glucosidase, i.e. p-nitrophenyl β-D-glucopyranoside (pNPG) and methyl umbelliferyl β-D-glucopyranoside (MUG) used were from Sigma (USA).

2.1. Differential expression of β-glucosidase by A. niger in submerged fermentation in response to carbon source in the production medium

2.1.1. Medium and enzyme production

Mandel and Weber [4] medium (composition in g/l urea – 0.3, $(NH_4)_2SO_4$ – 1.4, KH_2PO_4 – 0.4, $MgSO_4{\cdot}7H_2O$ – 0.3, peptone – 0.75, yeast extract – 0.25, $FeSO_4{\cdot}7H_2O$ – 0.05, $MnSO_4{\cdot}7H_2O$ – 0.01, $ZnSO_4{\cdot}7H_2O$ – 0.01, $CoCl_2$ – 0.01) was used as the basal medium. This basal medium was supplemented with 1% wheat bran, rice straw, cellulose, lactose or glucose as carbon source. Initial pH of the medium was adjusted to 4.8. Medium was autoclaved and inoculated with 10^7 spores per 100 ml medium, and incubated at 30 °C for 96 h. Extracellular β-glucosidase was extracted by centrifugation of the culture broth at 8000 rpm for 15 min, and the supernatant was used as crude enzyme preparation.

2.1.2. Enzyme assay

Beta-glucosidase assay was done using pNPG as substrate (10 mM pNPG in 0.05 M citrate buffer, pH 4.8). Standard graph was prepared using varying concentrations of p-nitrophenol (5–50 μM) in same buffer. Half milliliter of appropriately

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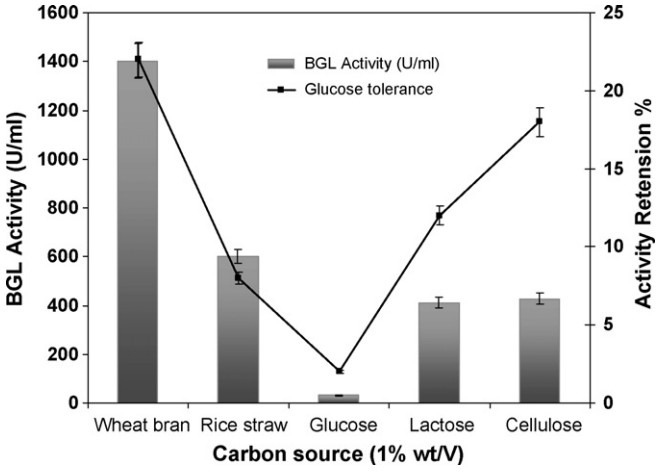

Fig. 1. BGL production and its activity retention in response to different carbon sources.

diluted enzyme sample was incubated with 0.5 ml of substrate solution at 40 °C for 15 min.The reaction was stopped by adding 2 ml of 0.2 M Na_2CO_3. The activity of β-glucosidase was estimated spectro-photometrically by reading the absorbance of the liberated p-nitrophenol at 400 nm. One unit of BGL activity was defined as the μM of p-nitrophenol released per milliliter of enzyme per minute under the standard assay conditions.

2.1.3. Zymogram

The crude enzyme preparation was concentrated by a centrifugal vacuum concentrator and Native PAGE (10%) was run with the samples normalized for total protein content. 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. Activities staining of the gels were done by incubating them with 10 mM 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 250 mM glucose in one set. Activity bands were visualized by illumination with long wavelength UV trans-illumination and photographs were acquired using an imaging system (Syngene-GBox, UK). Both the sets of gel were pictured simultaneously to ensure even conditions of illumination and exposure. Band intensities were measured using Scion Image (ScionCorp, MD, USA). The intensity of the band stained in presence of glucose gives the activity retention percentage of BGL in comparison to the band of the same sample stained in absence of glucose.

2.2. BGL purification and characterization

The protein bands showing BGL activity on Native PAGE were cut from the gel; size reduced and was transferred into a dialysis bag with 0.05 M citrate buffer (pH 4.8). This was subjected to 20 V DC current for 3 h in a submarine gel electrophoresis unit containing the same buffer. The dialysis bag was then inverted and run again for 5 min to detach the protein from the dialysis bag. The buffer containing eluted enzyme was aspirated using a micropipette and was used for further characterization. The sample was assayed for BGL activity, subjected to SDS PAGE for molecular weight determination. Activity staining using MUG was also done on the gels to confirm that the purified BGL is active. Optimal temperature and pH of the enzyme was determined by performing the assays at different temperatures (33, 40, 50, 60, 70 and 80 °C) and pH (3, 4, 4.8, 6, 7, 8, 9, and 10). To study the extended temperature stability of purified BGL, the samples were incubated in a water bath at 50 °C, and assays were performed at regular intervals till 72 h. The experiments were done in triplicates and the standard errors were calculated and were less than 5% which assures the reproducibility of the results.

3. Results and discussion

3.1. Differential expression of β-glucosidases

A. niger NII 08121 showed maximal BGL activity when wheat bran was used as carbon source in the medium (1400 U/ml). Supplementation of glucose resulted in a significant decrease in BGL activity while rice straw, cellulose and lactose supported lesser BGL activity compared to wheat bran (Fig. 1). Wheat bran is a rich source of nutrients that contain proteins, cellulose, starch and minerals and apparently can promote enhanced growth and enzyme production. High productivity in crude biomass could also be due to

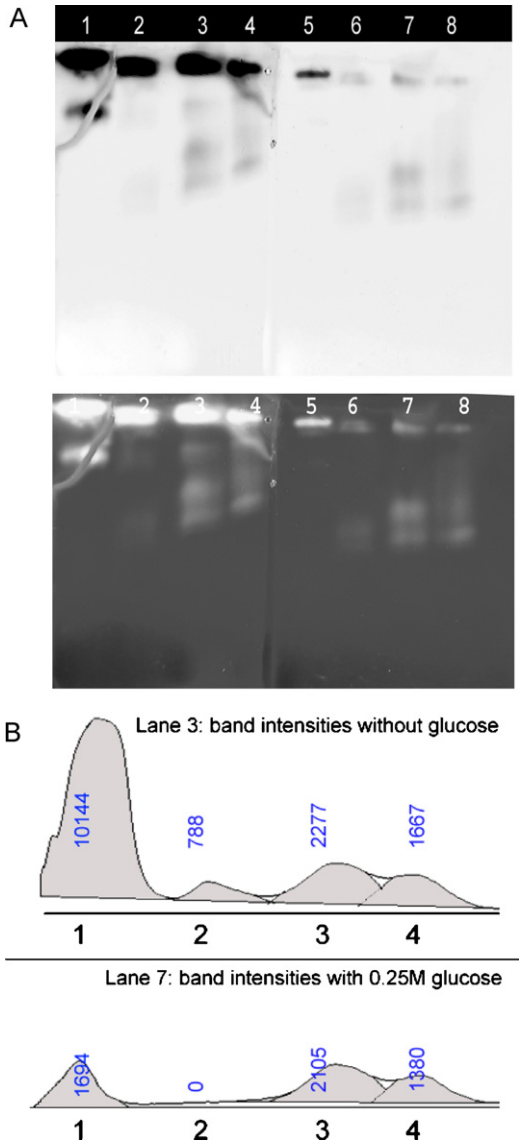

Fig. 2. Differential induction of BGL in response to different carbon sources. (A) Lanes 1 and 5: sample produced in WB as C source; lanes 2 and 6: sample produced in RS as C source; lanes 3 and 7: sample produced in lactose as C source; lanes 4 and 8: sample produced in cellulose as C source; lanes 1–4: staining done without glucose; lanes 5–8: staining done in presence of 0.25 M glucose. (B) Pixel density analysis of the activity bands obtained by MUG staining of BGL produced with lactose as carbon source, in presence (lane7, A) and absence (lane 3, A) of glucose. Area under each peak corresponds to the pixel densities (intensity of bands) and the values are indicated on the respective peaks.

the dual role of biomass as a nutrient source and a support matrix for fungal adherence. Lack of BGL production in presence of glucose may be because glucose is a catabolite repressor of many cellulase genes or due to the inhibitory effect of glucose on BGL activity [5]. The growth of fungi on mineral salt medium containing glucose generally represses cellulase activity and only after depletion of glucose, the fungus resumes production of the enzyme [6]. Differences in glucose tolerance of the enzyme were evident with change of carbon sources (Fig. 1). The difference in activity retention in presence of glucose for the enzymes prepared in different media could be due to the differences in expression of multiple BGL proteins since it is known that multiple isoforms of BGL is present in *Aspergilli* and few of them are reported to be glucose tolerant [7,8].

Zymogram analysis was done on the Native PAGE of samples to determine the presence of multiple isoforms of the enzyme.

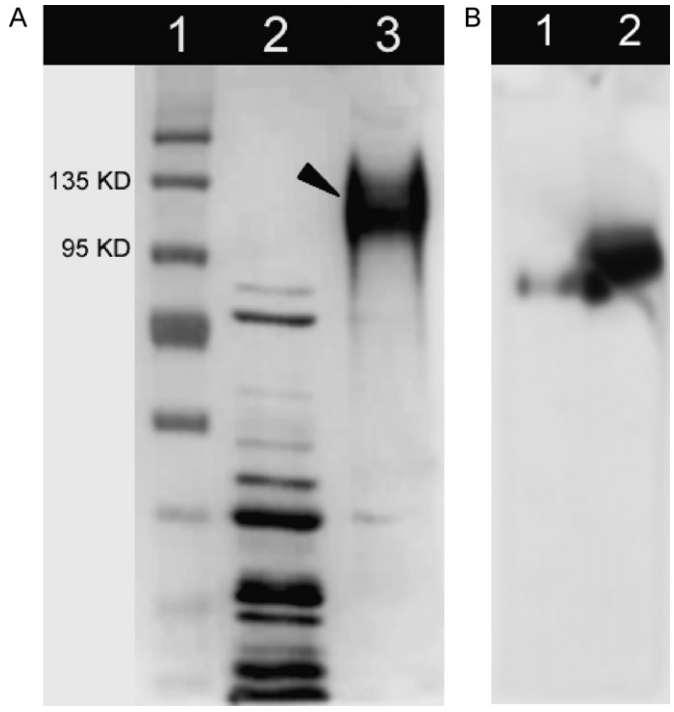

Fig. 3. Purification of major BGL1. (A) SDS PAGE of electroeluted major beta glucosidase (BGL1) protein showing single band at ~120 kDa. Lane 1: prestained marker; lane 3: electro-eluted major beta glucosidase protein. (B) Confirmation of BGL activity using MUG stained Zymogram. Lane 1: electro-eluted major beta glucosidase protein (low concentration); lane 2: electro-eluted major beta glucosidase protein (high concentration).

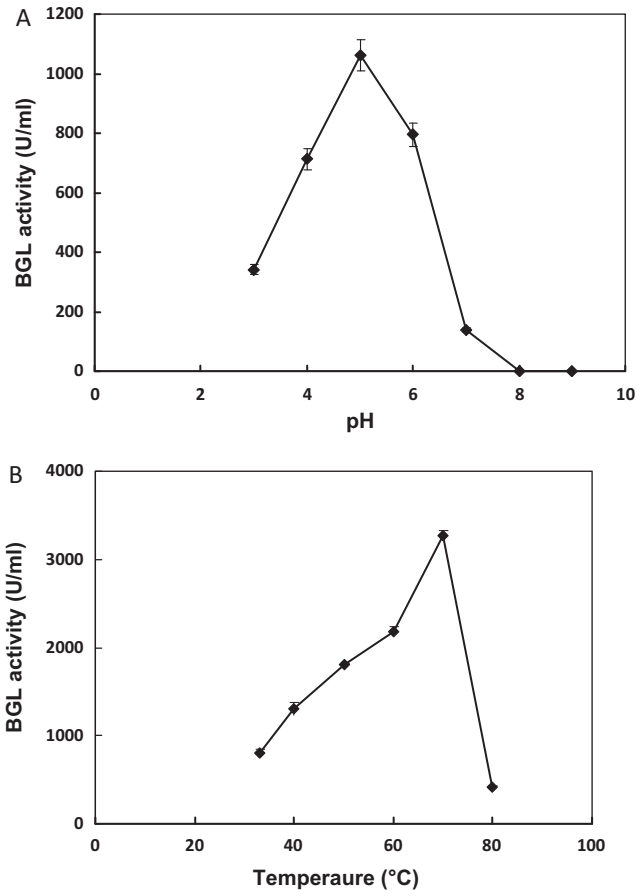

Fig. 4. (A) Determination of the optimal pH of major BGL1. Assay temperature: 40 °C, assay time: 15 min. (B) Determination of the optimal temperature of major BGL1. Assay temperature: varies from 37 °C to 80 °C, assay time: 15 min, pH: 4.8.

The analysis indicated the presence of at least 4 different activity bands which may be considered as different isoforms of the BGL protein. Proteins corresponding to the upper two activity bands were highly expressed while the lower two bands were expressed at comparatively lower levels (Fig. 2A). The lower two bands were more conspicuous in the case of samples where lactose or cellulose was the carbon source indicating the corresponding proteins are expressed more when these compounds were added in the medium (Fig. 2B). Simultaneous gel based activity assay performed in presence of glucose indicated that the proteins corresponding to the lower two bands are the ones which are glucose tolerant since the reduction in the intensity of these bands were very low compared to those corresponding to the high molecular weight BGL protein. The lower two BGL bands in case of lactose induced culture, showed activity retention of 92% and 82% respectively on the basis of pixel density analysis (Fig. 2B).

It may thus be assumed that the lower two bands are the ones which correspond to glucose tolerant BGLs and the expression of these are induced more by lactose compared to other carbon sources used in the medium. Lactose is known to induce BGL production in some fungi [9]. It supports the glucose tolerant BGL production and it is utilized in the commercial production of the enzyme owing to economic considerations [10]. The mechanism of lactose induction, however, is not fully understood [11].

It is generally believed that oligosaccharides play an important role in regulating the synthesis of wood-degrading enzymes which includes BGL [12,13], and oligosaccharides has been proved to be converted to inducer (such as sophorose and gentiobiose) by transglyosylation [14,15]. On this aspect, though results have not been shown here, *A. niger's* crude BGL preparation, incubated with sugar monomers when used as supplement in the medium, is found to increase the cellulase production which probably is due to generation of oligomers acting as inducers. Also, the effect of the growth environment manifests itself markedly in the type of carbohydrate metabolism of heterotrophic microorganism. Nature and quantity of the excreted metabolites is dependant on the nature of the growth limitations and carbon source [16].

A. niger, thus produced four different BGL proteins when induced with different carbon sources of which the high molecular weight activity band was the most highly expressed beta glucosidase. This beta glucosidase designated as BGL1 was the major BGL protein which was responsible for a very significant percentage of the total BGL activity of all samples. It shows about 16% of activity retension with 250 mM glucose level (Fig. 2B). BGLs can belong to different families of glycosyl hydrolases (e.g. GH1, GH3 and GH5) and the BGL isoforms from *A. niger* that we report here may also belong to different families since structural differences may be responsible for the differences in the level of glucose tolerance.

3.2. Protein purification and characterization

We had attempted to purify and characterize the BGL isoforms from *A. niger* samples grown in lactose containing medium. The attempts to purify the low molecular weight proteins were unsuccessful but the major BGL protein was recovered in active form by electro elution from the Native PAGE gel. Sample purity was confirmed by observing a single band on SDS PAGE (Fig. 3A). The molecular weight of the major BGL was approximately120 kDa by comparison with molecular weight markers. BGL activity was confirmed by staining a similarly run gel with MUG, when the bands fluoresced, signifying enzyme activity (Fig. 3B). Same sample lot

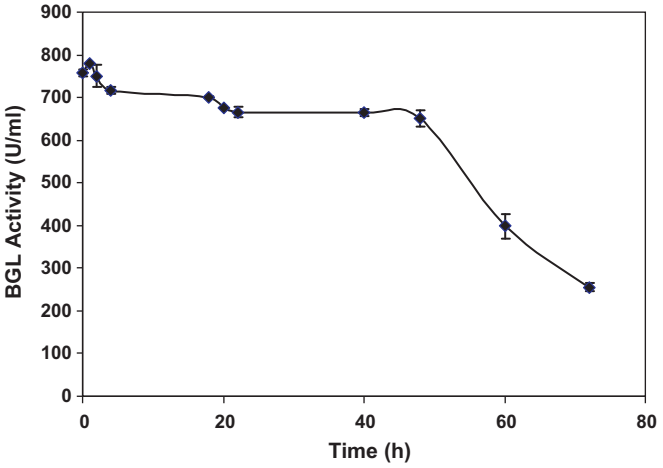

Fig. 5. Temperature stability of major BGL1 at 50 °C. Assay temperature: 40 °C, assay time: 15 min, pH: 4.8.

used for the gel analyses were used for further characterization of the properties of BGL1.

The optimal pH for BGL1 activity was 4.8 while the optimal temperature was 70 °C (Fig. 4A and B). Enzyme activity decreased with increase in pH after 4.8 indicating that the enzyme under study is acidic BGL. Interestingly, BGL1 activity increased with increase in assay temperature up to 70 °C and fall rapidly after that. Temperature stability of the protein was studied at 50 °C; as most of the enzymatic hydrolysis of biomass is operated at 50 °C [3,17] which is the target application of the enzyme. Enzyme assays, done at various time intervals showed no loss of activity till 48 h with a gradual decrease after that. Nevertheless, more than 50% activity was retained even after 72 h at 50 °C (Fig. 5). BGL 1 from *A. niger* NII 08121 is therefore can be considered as a very promising enzyme due its thermo-tolerance, acid activeness and extended thermo-stability at the temperatures normally employed in enzymatic biomass hydrolysis. Beta glucosidases with high thermo tolerance and stability are rarely reported in *A. niger* to our knowledge this is the first report of a major BGL enzyme from *A. niger* with optimal temperature of 70 °C and with an extended half life at 50 °C.

4. Conclusion

A. niger NII 08121 produced different BGL isoforms when grown on different carbon sources indicating a difference in the induction of these isoforms. Maximal production of moderately glucose tolerant isoforms was observed with lactose induction while a major BGL was produced in all carbon sources. The major beta glucosidase (BGL1) upon purification and characterization showed interesting properties including an optimum temperature of 70 °C, optimum pH of 4.8 and extended stability at 50 °C for more than 48 h. Normal enzymatic hydrolysis of biomass is performed at 50 °C over 24–72 h and loss of enzyme activity is a major limitation in such processes. BGL1 with its extended stability is a very potent candidate to be used in enzyme cocktails for biomass hydrolysis. The characterization of other BGL activities from *A. niger* would be interesting especially in light of the glucose tolerance exhibited by the low molecular weight BGLs observed in this study. We have been successful in cloning partial gene sequences of a GH family 3 BGL and is continuing the efforts to clone full length cDNAs of all the BGLs expressed by this fungus. These future studies are expected to provide more insights into differential expression of the BGL isoforms and also on the glucose tolerance of certain isoforms.

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