Journal of Molecular Catalysis B: Enzymatic 72 (2011) 107–115

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Purification and biochemical characterization of an atypical β-glucosidase from *Stachybotrys microspora*

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ARTICLE INFO

Article history:
Received 28 January 2011
Received in revised form 28 April 2011
Accepted 16 May 2011
Available online 26 May 2011

Keywords:
S. *microspora*
β-Glucosidase
Glucose
Ferrous ion
Coomassie blue
Retaining-enzyme

ABSTRACT

Stachybotrys microspora is a filamentous fungus secreting various β-glucosidases. The current work undertakes purification and biochemical characterization of the most particular one, named bglG, which is the only one to be highly produced on glucose and fairly on cellulose-based medium. Although produced on glucose, bglG activity continues to be highly inhibited by this sugar. After two chromatographic steps, bglG was purified to homogeneity and shown to be a monomeric protein with the molecular mass of 225 kDa. The highest bglG activity was obtained at pH 5 and a temperature range of 50–60 °C. This enzyme was shown to act through a retaining-enzyme mechanism. The N-terminal sequence analysis did not reveal any homology with all available sequences in the database. BglG is somehow atypical for multiple reasons: (1) BglG is insensitive to the conventional Coomassie staining protocol and $CuCl_2$ method was applied to reveal the protein; (2) the bglG activity is strongly enhanced by ferrous ion (Fe^{2+}) to 161% at 5 and 10 mM of Fe^{2+}. Flame spectrometry analysis showed that iron was stoechiometrically and strongly bound to bglG; (3) besides cellobiose, BglG is active on sucrose (114%); a rarely described property among β-glucosidases and (4) bglG is significantly stimulated by xylose. BglG could be considered as very original, since all known β-glucosidases, did not share these properties.

1. Introduction

Cellulose is the major component of plant cell wall and the most abundant renewable biological resource in the biosphere [1]. The biological depolymerisation of cellulose requires a multi enzymatic system composed of three enzymes functioning in synergy [2,3]: endoglucanases (EC3.2.1.9), cellobiohydrolases (EC3.2.1.4) and β-glucosidases (EC3.2.1.21) [4,5]. Indeed, endoglucanases attack the amorphous regions of cellulose fibres, liberating long oligosaccharides, which are readily taken by the cellobiohydrolases, processive enzymes which sequentially split cellobiose units from their extremities [3]. Finally, the β-glucosidases terminate the cellulolysis by hydrolysing cellobiose and other cellooligosaccharides into glucose monomers.

β-Glucosidase enzymes are important for cellulolytic fungi as by hydrolysing cellobiose, which is a strong cellobiohydrolase inhibitor, they minimize such end-product inhibition of cellulases [6]; they also convert cellobiose into sophorose, which is a very potent inducer of cellulolytic genes [7]. β-Glucosidases are also important for insects and animals as they cleave various glycosilated biomolecules into deglycosilated forms more or less actives [8,9]. Other studies proved that *Bacillus thuringiensis* δ-endotoxin exerts its bio-insecticide effect through β-glucosidase activity [10]. Industrially, β-glucosidases provide glucose stream for their use as carbon and energy sources such as in bio-conversion to ethanol [11–13] and other industrially important molecules by fermentative microorganisms [14]. Commercially, available cellulase preparations are often supplemented with β-glucosidase to boost the overall cellulolytic activity on biomass [15].

Among cellulases, only β-glucosidases possess the capacity not only to hydrolyse cellobiose and other cellooligosaccharides but also to synthesize oligosaccharides [8,16–18]. Thus, they contribute efficiently to the synthesis of valuables molecules in medical, pharmaceutical and industrial fields [19].

The catalytic mechanism mediated by β-glucosidase requires the presence of two acidic amino-acid residues in the catalytic site. The reaction occurs in one or two displacement steps depending on the space between the two acidic residues, 9.5 or 5.5 Å respectively, leading to an inverting or retaining mechanism according to the anomeric carbon configuration of the liberated glycone molecule [20]. β-Glucosidases are classified into three GH families: GH1, GH3 and GH9. GH1 and GH3 are families with a retaining mechanism while GH9 presented an inverting mechanism and mostly contains endoglucanases. Retaining enzymes often display transglycosylation abilities [21].

Glucose is the most known catabolic repressor of several genes, which are therefore controlled negatively by glucose at the transcriptional level. Being the end-product of the reaction

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doi:10.1016/j.molcatb.2011.05.007

catalyzed by the cellulolytic complex, glucose is also a very potent inhibitor of the cellulolytic enzymes and particularly the β-glucosidases. Nonetheless, exceptionally, some β-glucosidase genes are expressed/induced in presence of glucose even though they continue to be inhibited by the same substrate [8,22].

Stachybotrys microspora secretes a large number of β-glucosidases. The production of the last ones was directed differentially by the use of various carbon sources [23], two of these β-glucosidases were already purified and characterized [8,22]. In the current work, we undertake the purification and biochemical characterization of the most particular one, named (bglG). This β-glucosidase is not only produced in presence of glucose as unique carbon source, but also characterized by some peculiar properties such as its activation by xylose and ferrous ion or its capacity to efficiently hydrolyze sucrose, in addition to its insensitivity to ordinary protein staining methods. BglG thermo-activity, thermo-stability and refolding recovery were improved by wheat dehydrin, a somehow chaperone-like molecule, as we have already proven in our previous work [24].

2. Materials and methods

2.1. Biological strain

The biological stain used in this work is a filamentous fungus A19 that was isolated in our laboratory and belongs to *S. microspora* [22].

2.2. Production and purification of bglG

A19 strain was grown on potato dextrose agar medium at 30 °C for 4 days. The spores were harvested in 0.1% Tween 80 solution and used to inoculate Mandels medium [25] that was modified as follows, per litre: 2 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 1 g yeast extract, 0.69 g urea, 0.3 g $CaCl_2{\cdot}2H_2O$, 0.3 g $MgSO_4{\cdot}7H_2O$, 1 mL Tween 80 and 1 mL trace element solution composed of 1.6 g/L $MnSO_4$, 2 g/L $ZnSO_4$, 0.5 g/L $CuSO_4$, 0.5 g/L $CoSO_4$. Glucose at the concentration of 1% was used as a unique carbon source, during the pre-culture and the culture of the fungus. 10 mL of pre-culture inoculated 100 mL of modified Mandels medium in 500 mL erlenmmeyer.

S. microspora was grown at 30 °C for 5 days and the supernatant was used for the purification and characterization of the bglG. The culture medium was centrifuged for 15 min at 4000 × *g*. The supernatant was concentrated using twice volumes of cold acetone, incubated for 2 h at −20 °C and centrifuged for 30 min at 10,000 × *g* at 4 °C. The pellet was resuspended in buffer A (20 mM Tris–HCl buffer pH 8), dialysed against the same buffer and applied to an anionic exchange column (Q sepharose big beads, 1.5 cm × 20 cm) equilibrated with the same buffer. Proteins were eluted with a linear gradient of NaCl from 0 to 1 M in buffer A (Buffer B). The active fractions were pooled, concentrated with cold acetone as described previously, resuspended in 50 mM sodium acetate buffer pH 5.6 and applied onto a Sephacryl S-200 gel filtration column (1 cm × 90 cm) pre-equilibrated in the same acetate buffer. The active fractions were pooled and analyzed by gel filtration-HPLC system (Schodex 300 mm × 8 mm) and SDS-PAGE analysis in order to judge the protein purity and to determine its molecular mass.

2.3. Enzyme assays

The β-glucosidase activity was monitored using para-nitrophenyl-β-D-glucopuranoside (pNPG) as substrate. 0.2 mL of 1 mM pNPG (in 0.1 M sodium acetate buffer pH 5) was incubated with bglG at the appropriate dilution at 50 °C for 15 min. The reaction was stopped by adding 0.6 mL of 0.4 M glycine–NaOH buffer pH 10.8; the amount of the liberated *p*-nitrophenol (pNP) was determined by measuring the optical density at 400 nm. The molecular extinction coefficient of the pNP is 18,000 L/mol cm. One unit of enzymatic activity was determined as the amount of enzyme required to release 1 μmol of pNP per min under the assay conditions.

Various substrates were used to measure the hydrolytic capacity and specificity of bglG. The substrates used were chromogenic substrates like pNPG, oNPG (ortho-nitro-phenyl-β-D-glucopuranoside), pNPX (para-nitro-phenyl-β-D-xyloside), pNPA (para-nitro-phenyl-β-D-arabinofuranoside) and others such as salicine, amygdaline, arbutine, esculine, cellobiose, lactose, sucrose, avicel cellulose, CM-cellulose, starch, xylan, pectin and casein. For the chromogenic substrates, we used the same assay as with pNPG. For the remaining substrates, the activity was assessed by determining the liberated reducing sugars using the method of 3,5-dinitrosalicilic acid (DNS) [26]. Alternatively, the liberated glucose was also determined via the glucose oxidase/peroxidase kit (GOD kit was purchased from Biomaghreb company) as follows: 10 μL of the sample was added to 1 mL of GOD-kit, incubated at 37 °C for 10 min and the optical density was determined at 505 nm against standard curve prepared with glucose solution.

2.4. Protein assay, electrophoresis and staining

The protein content of the samples was determined using the Biorad-Bradford assay with optical density reading at 595 nm and BSA used as standard [27]. After electrophoresis, proteins were stained using conventional Coomassie blue staining solution.

Alternatively, $CuCl_2$ staining method was applied. Such method is 2–3 times more sensitive than Coomassie blue staining, much more easier and faster and the gels can be stored at 4 °C for several months without fading [28–30]. Practically, the gel was immersed in $CuCl_2$ solution at the concentration of 0.2 M for 5 min, washed with water and observed in dark phase. Using this method, the gel can be subsequently used in zymogram analysis, just after washing the gel with water, which eliminates the copper ion. This staining method depends partly on the tyrosin and tryptophan content [31].

The protein band was excised from the gel and transferred onto PVDF membrane as described in [31,32]. The purified enzyme was then subjected to N-terminal sequence analysis using an automated protein sequencer (Procise 492 cLc, Applied Biosystem).

2.5. Zymogram analysis

Proteins were mixed with the same loading buffer as in SDS-PAGE but they were not heated before loading on SDS-gel [33]. After electrophoresis, the gel was incubated during 2 h in 20 mM Tris–HCl pH 8 to get rid of SDS, allowing the renaturation of proteins. After 15 min of equilibration in 0.1 M sodium acetate buffer pH 5, the gel was superposed against 1% agar gel containing 100 μL of 4-methylumbelliferyl-β-D-glucoside (MUG) at 25 μg/mL. Following a suitable period of incubation, the system was observed under UV light, with excitation at 366 nm and emission at 445 nm. The zone of activity was indicated by the fluorescence emitted by methylumbelliferol (MU) released during enzyme action. The zymogram analysis can also be conducted using pNPG as substrate. Indeed, after the renaturation step, the gel was immersed in sodium acetate buffer 0.1 M pH 5 containing 10 mM pNPG and incubated for up to 15 min at 50 °C. The gel was briefly washed in distilled water and then immersed in 0.4 M glycine–NaOH buffer pH 10.8. The yellow color characteristic of pNP develops thereafter at the site of enzyme activity.

2.6. Determination of the molecular mass of proteins

The purified enzyme was applied onto a Sephacryl S-200 (93 cm × 1.5 cm) gel filtration chromatography and eluted with

50 mM sodium acetate buffer (pH 5.6) at a flow rate of 0.8 mL/min. The column was calibrated with gel filtration standard markers (dextrin blue 20×10^3 kDa, catalase (230 kDa), BSA (67 kDa), ovalbumin (43 kDa) and vitamin B_{12} 1.350 kDa). The plot representing the function: Log (MW) = *F* (Ve/V0) permitted the determination of the molecular weight of bglG. The SDS-PAGE [33] with 8% separating gel and 5% stacking gel was also used to determine the purity, the molecular weight and to confirm the monomeric state of bglG. The colored molecular weight marker is composed of: blue (225 kDa), red (150 kDa), green (102 kDa), yellow (76 kDa), purple (52 kDa), blue (38 kDa), orange (31 kDa), green (24 kDa), blue (17 kDa) and red (12 kDa).

2.7. Determination of the glycosylation state of bglG

The absence/presence of glycosylation on bglG was monitored as follows: the purified enzyme was digested with Endoglycosidase H (Endo H) according to the manufacturer's instructions (New England Biolabs, Beverly, MA, France). 100 μL of bglG was mixed with 100 μL sodium acetate buffer (pH 5), 0.2 M, SDS 0.1%. The final volume was divided into two aliquots E1 and E2. One microlitre of Endo H was added to E1 and the two aliquots (E1 and E2) were incubated for 16 h at 37 °C. E1 and E2 were analysed by zymogram analysis using MUG as substrate. Alternatively, the concanavalin A-sepharose affinity chromatography (ConA-Sepharose (10 cm × 1.2 cm) from Pharmacia) was used to bind the glycosilated enzyme. The fractions were eluted using N-acetylglucosamine (from 0 to 1 M) and assayed for the β-glucosidase activity, using pNPG as substrate [34].

2.8. Effect of ionic strength (I) on bglG activity

BglG activity was determined in the presence of sodium acetate buffer, NaCl and KCl solutions at different concentrations varying from 0.05 to 1 M. The ionic force was determined using the relative equation: $I = (1/2) \sum C_i z_i^2$; with C_i and z_i are the concentration and the ion charge, respectively. "*I*" was expressed in mol/L [35].

2.9. Configuration of the anomeric carbon of the released glucose

The method described in [20] was adapted to identify the anomeric configuration of the glucose liberated following the action of the β-glucosidase. BglG was incubated with 1 mM pNPG for 15 min at 50 °C. The reaction was stopped by the addition of δ-gluconolactone (100 mM final concentration) which completely and specifically inhibits the β-glucosidase activity. Two equivalent samples were removed, the first before and the second after boiling the reaction tube for 5 min. Heating insures the displacement of the equilibrium between the two anomeric forms of glucose towards the α form. The GOD kit was used to measure the liberated glucose using two controls consisting in the fresh and boiled solution of glucose. The method is based on the fact that glucose oxidase reacts exclusively with β-glucose form [20].

2.10. Effect of pH on bglG activity and stability

The optimal pH was examined by measuring bglG activity at various pH values ranging from 3 to 11 using different buffers at 0.1 M (pH 3–5: sodium–citrate buffer; pH 6–7: phosphate (KH_2PO_4/K_2HPO_4) buffer; pH 8: Tris–HCl buffer; pH 9–11: glycine–NaOH buffer). The pH stability of bglG was studied by incubating the purified bglG in various buffers with pH ranging from 3 to 10 during 24 h at 4 °C, followed by the determination of bglG residual activity.

2.11. Effect of temperature on bglG activity and stability

The bglG activity was measured at various temperatures ranging from 0 to 70 °C in 0.1 M sodium acetate buffer pH 5. The thermal stability was assessed by incubating the purified enzyme for 30 min at the desired temperature, followed by measuring the residual activity. Incubating the bglG at 50 °C in presence or absence of reactants (cationic solutions) at 5 mM was used to determine their effect on the bglG thermo-activity and/or thermo-stability, during activity assay. The half-life time (t1/2) of bglG was also determined in presence of cations.

2.12. Effect of reactants (salts, chelators, sugars, polyols and reducing agents) on bglG activity

The bglG activity was measured in presence of various effectors and the residual activity was determined. The stock solutions used in this study were prepared in ultra pure water at 1 M. $BaCl_2$, $CoSO_4$, $CoCl_2$, $MnSO_4$, $CuSO_4$, $CuCl_2$, $HgSO_4$, $CaCl_2$, $ZnSO_4$, $ZnCl_2$, $FeCl_2$, $FeSO_4$, $FeCl_3$, EDTA, glucose, xylose, xylitol, mannose, β-mercaptoethanol, urea, Cys and GSH were used as additives. All these reactants were added individually to the reaction mixture containing the pNPG, used as substrate. The *Ki* of glucose and mannose were determined using Lineweaver–Burk representation [36].

2.13. Metal ions analysis by flame atomic absorption spectrometry

A Perkin-Elmer Analyst 200 atomic absorption spectrophotometer (Norwalk, CT), equipped with a deuterium lamp background correction system, was used for metal ion quantification. The manganese hollow cathode lamps (wavelength, 279.5 nm; slit, 0.2 nm; Perkin-Elmer) were used as the primary radiation source. Analytical measurements were based on the average absorbance under the recommended conditions. The purified enzyme was dialyzed against ultra-pure water, treated with an equal volume of absolute HNO_3 and absolute HCl during 2 h at 200 °C, then, ultra-pure water and absolute HNO_3 (V/V) were added, followed by incubation at 100 °C until desiccation. Finally, 25 mL of ultra-pure water were added and used for flame atomic absorption analysis.

2.14. Kinetic parameters of bglG for pNPG

Kinetic parameters (K_m and V_{max}) of the purified bglG were determined for pNPG as substrate, using a specific enzymology program (Hyper 32, exe program, version 1.0.0, 2003; free ware on the web). BglG was incubated with pNPG at various concentrations (0.25, 0.5, 1, 2, 3, 4 and 5 mM) under optimal experimental conditions. The initial velocities were determined for the indicated substrate concentrations and used to determine K_m and V_{max} following the Lineweaver–Burk plot [36]. The same strategy was adopted to determine the K_m and V_{max} when cellobiose was used as substrate at various concentrations ranging from 0.25% to 2%.

2.15. The capacity of bglG towards cellobiose and cellotetraose

BglG (0.4 U/mL) was added to 5 g/L of cellobiose or cellotetraose solution (prepared in 0.1 M sodium acetate buffer pH 5), at the final volume of 5 mL. The mixture was incubated at 50 °C. Aliquots (50 μL) were taken during various time intervals and analyzed by HPLC (Aminex Fast carbohydrate 100 mm × 7.8 mm, 0.5 mL/min); the peak areas were determined and converted into percentages of glucose versus cellobiose or cellotetraose. The results were confirmed by the GOD method [37].

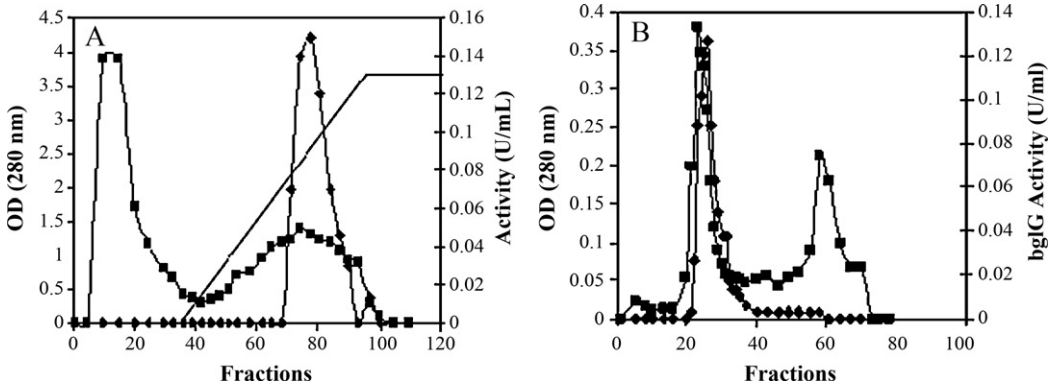

Fig. 1. Purification profiles of bglG. (A) Chromatographic profile of the precipitated crude extract on Q sepharose (big beads) exchange anionic column; (B) S-200 gel filtration chromatographic profile of the active fraction pooled from the first step on column. (♦) Symbolizes bglG activity expressed on U mL^{-1}, (■) optical density at 280 nm.

3. Results and discussion

3.1. Production and purification of bglG

Two steps were conducted at 4 °C for the purification of bglG; the third β-glucosidase to be purified from *S. microspora* strain. This fungus was grown for 5 days in liquid modified Mandels medium supplemented with 1% glucose as sole carbon and energy sources. The medium was centrifuged, the recuperated supernatant was precipitated using two volumes of acetone to concentrate sample, dialyzed against buffer A and injected onto anionic exchange column (Q Sepharose big beads) (Fig. 1A). A single peak of β-glucosidase activity was eluted with buffer B. The increase of the percentage of recovery observed during Q Sepharose chromatography (Table 1) might be due to the elimination of an inhibitor such as an ion or a metabolite, which had an activating effect on bglG activity. The active fractions were pooled and injected on gel filtration column (Sephacryl S-200) giving rise to the elution profile shown on Fig. 1B.

3.2. BglG is insensitive to Coomassie blue staining method

To check the purity and the molecular weight of the novel β-glucosidase, we performed SDS-PAGE and zymogram analysis. We were surprised to discover the complete absence of any protein band in the lane of the purified enzyme, after staining by the conventional Coomassie blue method (Fig. 2A(1)). We checked that the molecular weight marker bands were clearly revealed on the same stained gel (Fig. 2A(M)). Inversely, a positive response was observed on the bglG corresponding lanes of the zymogram, which was very intense since it appeared just after few minutes of overlay and exposure to UV (with MUG used as substrate) (Fig. 2B(3)). The same result was observed after immersing gel in pNPG as described in zymogram analysis section of Section 2 (Fig. 2B(4)).

Since we were absolutely sure that we have loaded enough amounts of purified enzyme on the gel, we hypothesized that probably the G 250 Coomassie blue did not stain it in our conditions. We therefore tested another staining method, using $CuCl_2$ [28–30]. The copper produces a "negative image" of colorless protein bands against a semiopaque background. It reacts partly, with tyrosin residues, leading to opacity but leaving the proteins as uncolored zones (Fig. 2A(2)). Consequently, this method allowed us to reveal the bglG enzyme on gel and to ascertain its purity.

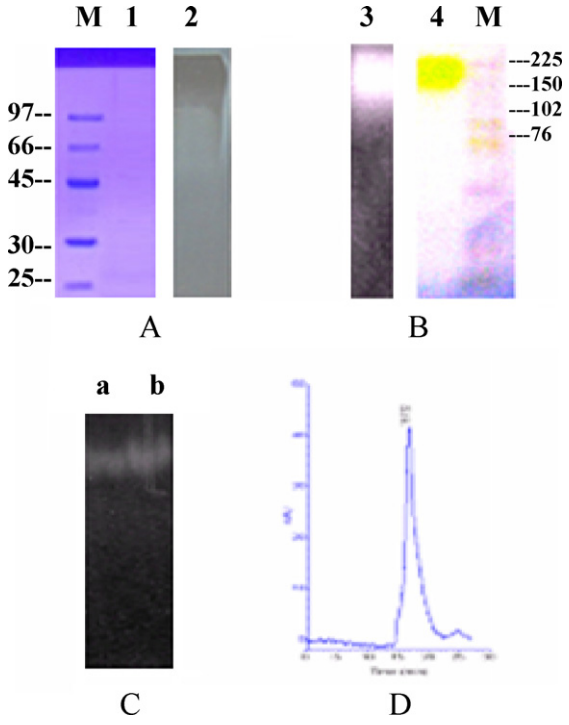

Fig. 2. Biochemical analysis of purified bglG enzyme. (A) Staining of BglG: on the right: G250 Coomassie blue staining of 8% SDS-PAGE with (M) molecular weight markers. (1) Purified bglG. (2) $CuCl_2$ staining of the purified bglG migrated. (B) Zymogram profiles of bglG migrated on SDS-PAGE using MUG as substrate (in 3) and pNPG (4) (M) colored molecular weight marker. The colored marker used is composed of: blue (225 kDa), red (150 kDa), green (102 kDa), yellow (76 kDa), purple (52 kDa), blue (38 kDa), orange (31 kDa), green (24 kDa), blue (17 kDa) and red (12 kDa). (C) Endo H test on bglG, revealed by zymogram using MUG; (a) EndoH treated bglG and (b) untreated bglG. (D) HPLC chromatographic profile of the purified bglG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

We hypothesized that hyper-glycosylation could preclude the interaction between glycoprotein and Coomassie blue staining

Table 1
Summary of purification steps of bglG. Enzyme activities were determined using pNPG as a substrate at the concentration of 1 mM.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	120	150	1.25	100	1
Q-sepharose	32	160	5	106	4
Sephacryl S-200	6	120	20	75	16

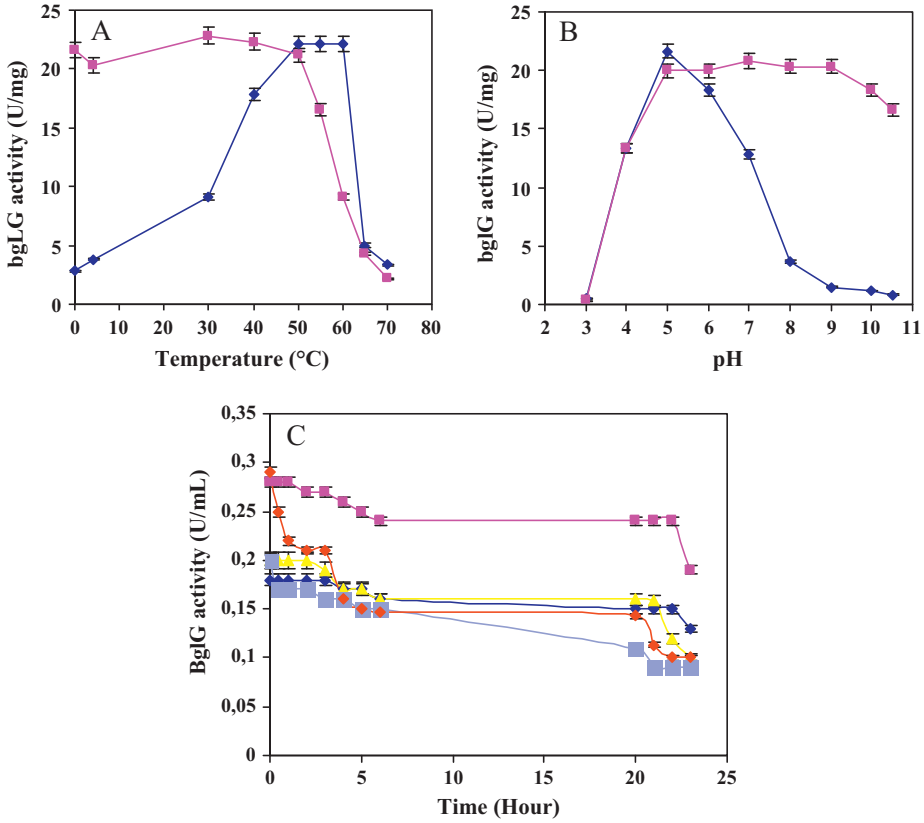

Fig. 3. (A) Thermo-activity and thermo-stability of the purified bglG. Residual activity was determined under optimal conditions. The non-heated enzyme was considered as control (100%): (♦) thermo-activity (■) thermo-stability. (B) Effect of pH on the activity and the stability of the purified bglG. (♦) Effect of pH on bglG activity: the maximum activity obtained at pH 5 was considered as 100%. (■) Effect of pH on stability of bglG incubated in different buffers for 24 h and the residual activity was measured at pH 5 and 50 °C. The enzyme activity before incubation was taken as 100%. (C) Thermo-stability of bglG without (♦) and with cations: ferrous ion (■), calcium (▲), barium (♦), manganese (■). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

solution. In order to verify such hypothesis, bglG was treated by Endo H glycosidase and migrated on gel and revealed by zymogram analysis; the results showed that bglG is not glycosilated (Fig. 2C). The use of concanavalin A confirmed this result. Indeed, after gentle mixing with concanavalin A and centrifugation, bglG was present on the supernatant.

Table 1 summarizes all purification steps of bglG. The purified enzyme, named bglG, from the last step was considered as homogeneous, as shown by SDS-PAGE and HPLC gel filtration profile (Fig. 2D) and was used to determine its biochemical and kinetic properties.

3.3. Molecular weight and NH_2-terminal sequence determination

BglG molecular weight was estimated, using gel filtration calibrated markers, to be about 225 kDa. The analysis on 8% SDS-PAGE indicated that bglG behaved as a monomer and confirmed its determined molecular mass. The NH_2-terminal sequence is NH_2-YYMFVMPEE as determined by the automated Edman method. Blasted to protein databases, this sequence did not show any homology with known proteins.

3.4. The substrate concentration affects bglG activity

The effect of the substrate concentration on bglG activity was studied in order to optimize the molarity that should be used in kinetic studies. The pNPG concentration was varied from 0.125 to 5 mM. This study shows the gradual increase of the amount of pNP with the increase in substrate concentration till 1 mM, and then it continues constant (data not shown). Therefore, 1 mM was chosen for further assays of bglG activity. Perfect proportional relationship was observed between bglG activity and bglG concentration (data not shown), which suggests firmly the Michaelian behaviour of the purified bglG. In addition, we also note that bglG resists to a high ionic strength, as high as 1 M of sodium acetate, KCl and NaCl (data not shown). Such result is very important for biotechnological applications in which stability and efficiency of the enzyme against ionic potential are required.

3.5. Substrate specificity of S. microspora bglG

The substrate specificity of bglG activity was investigated using two substrate categories; synthetic and natural ones. Table 2 shows that bglG presented a higher specificity for the hydrolysis of pNPG than that for oNPG. Hence, the position of NO_2 radical is crucial for the affinity between the enzyme and its substrate. Indeed, bglG was able to cleave oNPG with only 17% relative to pNPG (Table 2). In the same context the specificity of bglG is exemplified by the absence of activity on pNPA (Table 2).

The second interesting result concerned the activity of bglG on sucrose. BglG is, not only active on cellobiose and esculine (classical substrates of β-glucosidases) but also on sucrose. It is less active on salicine, amygdaline, arbutine and finally was completely inactive on lactose, cellulose, CM-cellulose, xylan, starch, pectin and casein (Table 2). While several β-glucosidases are known to split esculine, their ability to act on sucrose is very unusual. We even note that bglG cleaves sucrose more efficiently than that cellobiose and esculine, as shown in Table 2 (114% against 100%). We advanced the following hypothesis to explain such behaviour: (1)

Table 2
BglG specificity and stereospecificity on various substrates.

Substrate	Concentration	Relative activity (%)
p-NP-glucose	1 mM	100
o-NP-glucose	1 mM	17
p-NP-xylose	1 mM	8
p-NP-arabinose	1 mM	0
Cellobiose	2%	100
Lactose	2%	8
Sucrose	2%	114
Esculin	2%	100
Amygdalin	2%	16
Arbutin	2%	19
Salicin	2%	10
CMC	1%	0
Cellulose avicel	1%	0
Xylan	1%	0
Starch	1%	0
Pectin	1%	0
Casein	1%	0

bglG has a double function (β-glucosidase and invertase activities) and (2) bglG was co-purified with an invertase enzyme. To disclose between these two hypotheses, the hydrolysis of sucrose was tested in presence of δ-gluconolactone, a specific and strong inhibitor of β-glucosidases. This resulted in the annihilation of both activities and confirmed the first hypothesis: bglG possesses a double function: β-glucosidase (cellobiase) and invertase capacities. It should be noted here that bglG shares this property with almond β-glucosidase, which cleaves sucrose with 77% of its relative activity on glucose [10]. The invertase activity of bglG could be exploited to produce glucose from molasses (highly rich in sucrose), which could be fermented into bio-ethanol [10].

3.6. Kinetic and physico-chemical properties of bglG

The K_m and V_{max} kinetic parameters were determined for bglG towards the pNPG substrate using the Hyper 32 program. The values of 0.9 mM and 27.77 U/mg for the K_m and V_{max} respectively, were in the range of other known β-glucosidases [38,39]. Regarding cellobiose, the K_m and V_{max} are 1.9 mM and 21.14 U/mg respectively. Therefore, the bglG behaved much more as aryl β-glucosidase as it exhibited higher velocity and affinity towards pNPG, compared to cellobiose. BglG possess more affinity to pNPG than F2 [8] and less affinity than M9 [22].

BglG presented an optimum of temperature ranging from 50 to 60 °C (Fig. 3A). BglG was also quite stable at temperatures ranging from 4 to 50 °C, such stability decreased at 60 °C as it conserved only 20% of its activity (Fig. 3A).

BglG presented an optimal pH around 5 and could be considered as quite stable since it conserved 100% of its activity between pH 5 and pH 9 (Fig. 3B).

3.7. Effects of ions and other additives on the bglG activity

The effect of several divalent cations was assessed on the pNPG hydrolysing capacity of bglG. Interestingly, ferrous ion upgrades bglG activity to 161% at 5–10 mM (Fig. 4) while ferric ion inhibited strongly the purified enzyme, suggesting that Fe^{2+} is specifically recognised by some residues of bglG.

Other ions improved the activity to moderate level, such as manganese (140%) and barium and calcium (both 120%). However, mercury decreases the activity to 15.79% (Fig. 4). Cobalt had no effect, neither did EDTA. We checked that the anionic form (sulphate or chloride) did not influence these effects as no differences were observed between both forms.

Thereafter, the bglG thermo-stability was tested in presence of Fe^{2+} and other activator ions at 50 °C. Fig. 3C indicates that

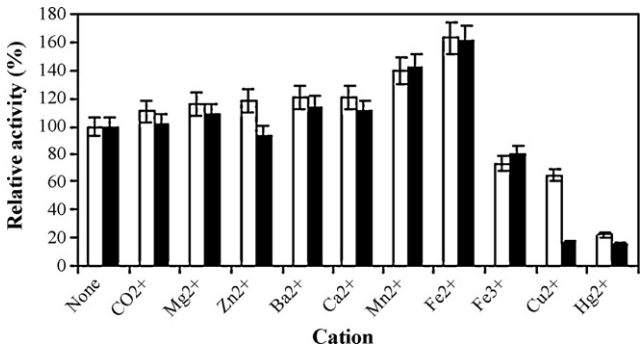

Fig. 4. Divalent cations effect on bglG at two ionic concentrations (5 mM (□) and 10 mM (■)).

bglG thermo-stability was clearly improved in presence of iron while it remained unchanged in presence of calcium and barium and slightly decreased in presence of manganese. Table 3 indicates the progress in bglG thermo-stability through its half-life time (t1/2) in presence of some cations. The half-life time of bglG alone is more than 23 h. It continues to be constant in presence of Fe^{2+} and Ca^{2+} (both 23 h). The Mn^{2+} t1/2 is 20 h and Ba^{2+} is less than 6 h. Thus, Fe^{2+} plays a double enhancing role on both thermo-activity and thermo-stability of bglG. Ca^{2+} and Ba^{2+} are considered as two thermo-activator effectors but not thermo-stabilizator ones. However, the remained cations (Hg^{2+}, Cu^{2+} and CO^{2+}) affected negatively bglG thermo-activity and thermo-stability. Mn^{2+} affected positively bglG thermo-activity (140%) and negatively bglG thermo-stability (t1/2 = 20 h). The half-life time is determined as the time required to detect the loss of 50% of the bglG activity in presence or absence of the effector.

The thermostability of bglG in presence of the activator ions was tested to assess their exact role: thermo activation or stabilisation or both. In fact, not only iron upgrades bglG thermoactivity by 161% but we proved that it maintains an elevated level of activation during thermo stability studies (more than 23 h). Hence, it is true that the half-life time of bglG is conserved with or without iron but it is upgraded. Therefore, we can conclude that this ion might play a dual role on the enzyme in terms of thermoactivation and thermostabilisation.

3.8. Detection of iron in bglG structure by flame atomic absorption spectrometry

The enhancing effect of Fe^{2+} ion prompted us to seek for the presence of such ion in the bglG structure. The purified bglG was treated with 10 mM EDTA and dialysed against ultra-pure water. The measurement of bglG activity, before and after this treatment, showed no significant difference. The analysis by flame atomic absorption spectrometry indicated the presence of one iron atom per protein molecule (226 ppm of iron was detected on the analysed

Table 3
Illustration of the half-life time of bglG in presence of cationic solutions. The half-life time (t1/2) was determined in presence of Ba^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+}, Ca^{2+} and Fe^{2+}, at the concentration of 10 mM, against bglG used alone.

	Half-life time (t1/2) (h)
BglG	>23
BglG + Fe^{2+}	>23
BglG + Ca^{2+}	23
BglG + Mn^{2+}	20
BglG + Ba^{2+}	6
BglG + Cu^{2+}	1.5
BglG + CO^{2+}	0.75
BglG + Hg^{2+}	0

Table 4
Effect of inhibitors and reducing agents on bglG activity, using pNPG as substrate at the concentration of 1 mM. Effect of carbohydrates on the bglG activity, using pNPG as substrate at the concentration of 1 mM.

	Concentration (mM)	Relative activity (%)
None	–	100
Iodoacetamide	5	104
NEM	5	102
Cystein	5	63
GSH	5	87
2-ME	1	157
DTNB	1	171
Urea	10	105
Xylose	10	120
Xylitol	10	108
δ-gluconolactone	1	7
Glucose	10	33
Mannose	10	50
N- acetylglucosamine	10	110

sample (5 mg/mL were used in this study) by flame spectrometry). This finding strongly suggests that iron is closely bound to bglG, in such away that the EDTA treatment cannot remove it. Such behaviour has been already reported for many proteases, amylases, isomerases and cellulases in which the calcium, chloride, cobalt ions were strongly bound to the enzyme [40–43].

3.9. BglG activity is stimulated by xylose and inhibited by glucose

Table 3 shows that bglG activity was enhanced, to 120% by xylose, a C5 sugar, as well as its alcohol derivative, the xylitol, to 108% (Table 4). Such stimulation was observed with either 5 or 10 mM of both molecules. It has been suggested that these additives can act as alternatives to a water molecule in accepting the β-glucosyl moiety from the β-glucosyl-enzyme complex formed during bglG catalysis [39]. Another obvious explanation is that the xylose enhancing effect could be due to a trans-glycosylation reaction as suggested in [44]. Indeed, pNPG could play the role of glycone donor and xylose would then play the role of acceptor of the released glycone [44–46]. Hence, xylose attracts the released glucose and increased the frequency of meeting between the enzyme and the substrate. It should be also recalled here that glucose constitutes a strong inhibitor of bglG.

Inversely to xylose, glucose inhibited bglG as only 33% of residual activity was obtained in presence of 10 mM glucose (Table 4). As this sugar is the end-product of β-glucosidase catalyzed reaction, feed-back inhibition is evoked to explain such result. The mannose, an epimer of glucose, also inhibited bglG to 50% at the same concentration of 10 mM (Table 4). The *Ki* value of glucose and mannose are 2.11 and 3.3 mM, respectively. According to these findings, we conclude that glucose possess more affinity to bglG than mannose. This fact explains the difference on the level of inhibition observed in presence of glucose. We deduce also that the level of inhibition is governed by the inhibition severity observed with glucose and mannose.

3.10. Effect of iodoacetamide and NEM on bglG activity

The negative effect of mercury on bglG activity, would suggest the involvement of a free cystein residue in the vicinity of the catalytic site as it was suggested for other enzymes affected by mercury [39]. In this context, we tested agents acting on free cystein. Except the DTNB, which activated bglG, some other compounds have no effect, such as those known as thiol alkylating agents as iodoacetamide and NEM. DTNB (or Ellman's reagent) is known to react, generally rapidly and selectively, with the free sulfhydryl side chain of cystein to form an S–S bond between the protein and a thionitrobenzoic acid (TNB) residue [47]. It is possible that the 1-cystein residues, reacting with DTNB, are not the same that react with NEM or iodoacetamide; 2- those residues should be maintained at reduced or alkylated state to give a more active open-structure to BglG. Usually, DTNB inhibits the enzymes by covalently linking cystein engaged in the active site as in the glutamate dehydrogenase [48] but in certain cases, it exerts an activating effect was already reported in the fructose diphosphatase by [49].

In this context, it is also interesting to note that reducing agents which are able to prevent the oxidation of free sulfhydryl residues (cysteines) and to reduce disulfide bonds, showed also controversial effect. Indeed, while mercaptoethanol (used at the concentration of 1.66%) efficiently increased the bglG activity to 2.5 fold, other reducing agents showed slight inhibitory effect such as cystein and GSH with 63 and 87% of residual activity, respectively (Table 4).

In this context, we shall recall that the oxidation of free cystein residues into disulfide bonds can lead to non-specific aggregation of the enzyme or its inactivity and denaturation. It is also known that reducing agents can bind metals and trace metal compounds (such the ferrous ion in bglG), inactivating by the way the reducing agent and the metal. This could explain the action difference of the mercaptoethanol and other reducing agents.

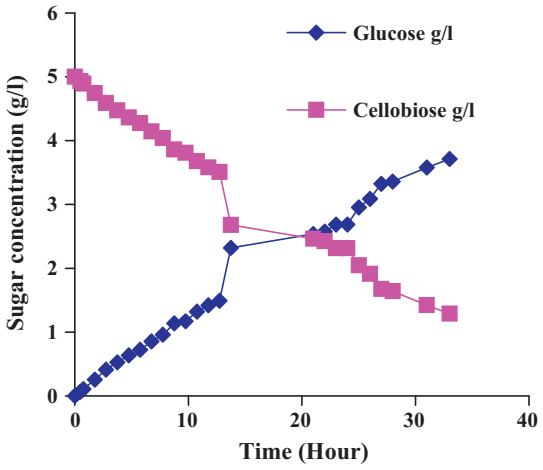

Fig. 5. BglG hydrolysis of cellobiose, with (■) concentration of cellobiose (◆) concentration of glucose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.11. Cellobiase and cellotetraose bglG hydrolysis capability

β-Glucosidases may be divided into three groups on the basis of substrate specificity. (1) Aryl β-glucosidases exclusively hydrolysing or showing a great preference towards aryl β-glucosides; (2) cellobiases hydrolysing cellobiose and small oligosaccharides and finally (3) the members of the third group, termed as broad-specificity β-glucosidases, that act on both substrates (aryl-β-glucosides, cellobiose and cellooligosaccharides) and are the most commonly observed group in cellulolytic microbes [39].

The bglG hydrolysing capacity was tested on cellobiose (5 g/L) in a small bioreactor. Aliquots were taken at various reaction times and their glucose contents were determined by HPLC. The profile in Fig. 5 shows that the conversion of cellobiose into glucose reached 74% of recovery. Hence, bglG rejoins the two other β-glucosidases previously purified from *S. microspora* and specified as cellobiases. However, kinetic conversions and hydrolytic recoveries are different for each enzyme. BglG is more efficient than the two other β-glucosidases purified from *S. microspora* [8,22].

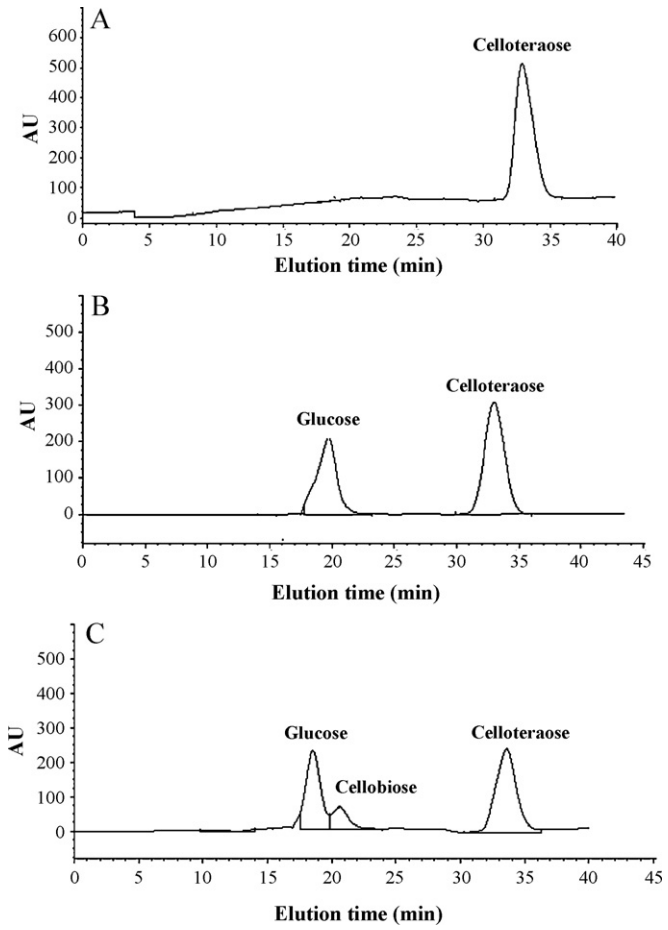

Fig. 6. The capacity of bglG to hydrolysis cellotetraose. Chromatographic profile illustrating the cleavage of cellotetraose by the purified bglG under the experimental conditions described in Section 2. (A) Represents the time zero; (B) after 1 h and (C) after 24 h.

The capacity of bglG to hydrolysis cellotetraose was also assessed under the same experimental conditions described for cellobiose conversion reaction towards glucose monomer. Chromatographic profiles shown in Fig. 6 prove that bglG have the ability to hydrolyze cellotetraose into glucose units. Indeed, after 1 h of the conversion reaction, only glucose appeared but not cellotriose or cellobiose. This fact could be explained by the fact that such saccharids are probably immediately cleaved into glucose units. After 24 h, the hydrolysis efficiency decreased giving rise to the appearance of the intermediates (cellobiose and cellotriose). In comparison to cellobiose, bglG seems to be more efficient in hydrolysing celloteraose since after only 1 h, bglG shows a degradation yield of about 63.34% while it reached 74% with cellobiose after 24 h.

Based on the above results and those in Table 2, bglG belongs to the third group of β-glucosidases described in [50], because of the capacity of bglG to hydrolyze cellobiose, cellotetraose and also aryl- and alkyl-glucosides.

3.12. BglG is a retaining-enzyme

According to the protocol described in Section 2.9, adapted from [20], the configuration of the anomeric carbon of glucose (end-product of pNPG hydrolysis) was determined. At the end of the reaction, the liberated glucose can have β or α form depending on the action mechanism of the enzyme, qualified respectively as a retaining or inverting mode. It is already known that (1) the β-glucose might be converted into α form by a simple heating at 100 °C for 10 min; (2) the glucose oxidase can act only on the β-glucose. Consequently, the method described by Genta and his collaborators [20] and adapted to β-glucosidase assessment is based on the comparison of the amount of glucose liberated by the β-glucosidase, determined by GOD (Glucose Oxidase kit) either directly or after a heat treatment. In addition, the amount of pNP is also measured spectrophotometrically, in order to compare it to the GODs determined amount of glucose, without heating. We found that these latter amounts are similar whereas, after boiling, the amount of β-glucose is roughly nil. Indeed, the comparison of these amounts proves that pNP concentration (40 μg/mL) is equal to the non boiled glucose sample, β form (38 μg/mL). The amount of boiled glucose, α form, was 0.08 μg/mL. Consequently, bglG could be considered as a retaining-enzyme. According to these findings and the 3D structure of all known retaining β-glucosidases, the distance between the two bglG catalytic acids should be around 5.5 Å.

4. Conclusions

In summary, we have produced, purified and characterized an atypical β-glucosidase (bglG) from a local filamentous fungus (*S. microspora*). Regarding its several unusual properties, bglG should be studied further on both basic and applied levels. The highlighting of Fe^{2+} in the bglG structure is a very important clue to explain the bglG inhibition/activation and others structural properties. Not only Fe^{2+} but also other compounds presented enhancing capacity of bglG activity such as xylose, DTNB and mercaptoethanol. If the latter two compounds should act on the status of cystein residues, xylose effect is more difficult to explain. We advanced the hypothesis of trans-glucosilation via xylose as acceptor, which somehow pumps the liberated glucose. This would sequestrate such strong inhibitor and indirectly enhances the bglG enzyme activity.

Another peculiarity of our enzyme consisted on its ability act on sucrose, a property rarely reported for β-glucosidases except the almond one [10]. Such capacity would be understood once the bglG gene isolated and/or the 3D structure determined. Meanwhile, this capacity could be of great benefits in some applications such the bioconversion of molasses into fermentable sugars, which can be valuable sources for bio-ethanol production. In this field, we showed recently that the addition of bglG to crude cellulolytic juice enhances its hydrolytic of cellulosic substrates, resulting in the enhancement of glucose yield and hence the bio-ethanol yield after fermentation.

Acknowledgments

This work was supported by grants from the Ministry of Higher Education, Scientific Research and Technology, Tunisia. The authors would deeply thank Prof. Hafedh Belghith and Dr. Khmais Benhaj for helpful discussions, advice and English improvement, Kamel Walha and Hajer hssairi for their expert help in chromatographic analysis.

References

[1] M. Gruno, P. Valjamae, G. Pettersson, G. Johansson, Biotechnology and Bioengineering 86 (2004) 503–511.
[2] B. Henrissat, Protein Sequences & Data Analysis 4 (1991) 61–62.
[3] H.W. Xu, G.F. Dai, G.Z. Liu, J.F. Wang, H.M. Liu, Bioorganic & Medicinal Chemistry 15 (2007) 4247–4255.
[4] A. Esen, Beta glucosidases: Biochemistry and Molecular Biology 533 (1993) 1–13.
[5] R. Yu, L. Wang, X. Duan, P.F.B. Gao, Biotechnology Letters 29 (2007) 1037–1043.
[6] G.D. Haki, S.K. Rakshit, Bioresource Technology 89 (2003) 17–34.
[7] T.K. Ghose, V.S. Bisaria, Biotechnology and Bioengineering 21 (1979) 131–146.
[8] W. Saibi, B. Amouri, A. Gargouri, Applied Microbiology and Biotechnology 77 (2007) 293–300.

[9] J.J. Yoon, K.Y. Kim, C.J. Cha, Journal of Microbiology (Seoul Korea) 46 (2008) 51–55.
[10] A. Papalazaridou, L. Charitidou, A. Sivropoulou, Journal of Endotoxin Research 9 (2003) 215–224.
[11] H. Fang, C. Zhao, X.Y. Song, Bioresource Technology 101 (2010) 4111–4119.
[12] M. Lever, G. Ho, R. Cord-Ruwisch, Bioresource Technology 101 (2010) 7083–7087.
[13] R.K. Sukumaran, R.R. Singhania, G.M. Mathew, A. Pandey, Renewable Energy 34 (2009) 421–424.
[14] J. Zhou, Y.H. Wang, J. Chu, Y.P. Zhuang, S.L. Zhang, P.F.B. Yin, Bioresource Technology 99 (2008) 6826–6833.
[15] S. Takashima, A. Nakamura, M. Hidaka, H. Masaki, T. Uozumi, Journal of Biochemistry 125 (1999) 728–736.
[16] Q. Yan, X.W. Zhou, W. Zhou, X.W. Li, M.Q. Feng, P.F.B. Zhou, Journal of Microbiology and Biotechnology 18 (2008) 1081–1089.
[17] P. Turner, A. Pramhed, E. Kanders, M. Hedstrom, E.N. Karlsson, D.T. Logan, Acta Crystallographica 63 (2007) 802–806.
[18] T. Akiyama, H. Kaku, N. Shibuya, Phytochemistry 48 (1998) 49–54.
[19] Y. Han, H.F.B. Chen, Bioresource Technology 99 (2008) 6081–6087.
[20] F.A. Genta, A.F. Dumont, S.R. Marana, W.R. Terra, C. Ferreira, Biochimica et Biophysica Acta 1774 (2007) 1079–1091.
[21] P. Turner, D. Svensson, P. Adlercreutz, E.N.F.B. Karlsson, Journal of Biotechnology 130 (2007) 67–74.
[22] B. Amouri, A. Gargouri, Biochemical Engineering Journal 32 (2006) 191–197.
[23] W. Saibi, S. Abdeljalil, A. Gargouri, World Journal of Microbiology and Biotechnology (2010) 1–10.
[24] F. Brini, W. Saibi, I. Amara, A. Gargouri, K. Masmoudi, M. Hanin, Bioscience, Biotechnology, and Biochemistry (2010).
[25] M. Mandels, E.T. Reese, Journal of Bacteriology 73 (1957) 269–278.
[26] M. Mandels, J. Weber, Advances Chemistry Ser 95 (1969) 391.
[27] M.M. Bradford, Analytical Biochemistry 72 (1976) 248–254.
[28] C. Lee, A. Levin, D. Branton, Analytical Biochemistry 166 (1987) 308–312.
[29] D. Wang, J.K. Dzandu, M. Hussain, R.M. Johnson, Analytical Biochemistry 180 (1989) 311–313.
[30] J.K. Dzandu, J.F. Johnson, G.E. Wise, Analytical Biochemistry 174 (1988) 157–167.
[31] J. Lewis, Biochemical Education 25 (1997) 55–56.
[32] M. Messer, M. Griffiths, P.D. Rismiller, D.C. Shaw, Comparative Biochemistry and Physiology 118 (1997) 403–410.
[33] U.K. Laemmli, Nature 227 (1970) 680–685.
[34] R. Spiro, V. Ginsburg, Academic Press 28 (1972) 3–43.
[35] M.S.M. Ahmadi Moghadam, M.M. Esfahani Iranian, Journal of Environmental Science and Health 2 (2005) 1–5.
[36] H. Lineweaver, D. Burk, Journal of the American Chemical Society 56 (1934) 658–666.
[37] J. Lin, L.M. Ndlovu, S. Singh, B. Pillay, Biotechnology and Applied Biochemistry 30 (Pt 1) (1999) 73–79.
[38] M.A. Abdel-Naby, M.Y. Osman, A.F. Abdel-Fattah, Applied Biochemistry and Biotechnology 76 (1999) 33–44.
[39] M.L. Patchett, R.M. Daniel, H.W. Morgan, The Biochemical Journal 243 (1987) 779–787.
[40] S.J. Lin, E. Yoshimura, H. Sakai, T. Wakagi, H. Matsuzawa, Biochimica et Biophysica Acta – Protein Structure and Molecular Enzymology 1433 (1999) 132–138.
[41] M. Rhimi, M. Juy, N. Aghajari, R. Haser, S. Bejar, Journal of Bacteriology 189 (2007) 3556–3563.
[42] M.A. Borgi, K. Srih-Belguith, M. Ben Ali, M. Mezghani, S. Tranier, R. Haser, S. Bejar, Biochimie 86 (2004) 561–568.
[43] F.R. Harmon, N.H. Goss, H.G. Wood, Biochemistry 21 (1982) 2847–2852.
[44] Y. Hu, H. Luan, H. Liu, G. Ge, K. Zhou, Y. Liu, L. Yang, Bioscience, Biotechnology, and Biochemistry 73 (2009) 671–676.
[45] R.M. Wright, M.D. Yablonsky, Z.P. Shalita, A.K. Goyal, D.E. Eveleigh, Applied and Environmental Microbiology 58 (1992) 3455–3465.
[46] J.A. Perez-Pons, X. Rebordosa, E. Querol, Biochimica et Biophysica Acta 1251 (1995) 145–153.
[47] G. Ellman, Archives of Biochemistry and Biophysics 82 (1959) 70–77.
[48] R. Sengar, H. Srivastava, Biologia Plantarum 37 (1995) 147–151.
[49] S.P.a.B.L.H.J. Fernando, Archives of Biochemistry and Biophysics 129 (1969) 370–376.
[50] M. Hrmova, G.B. Fincher, Carbohydrate Research 342 (2007) 1613–1623.