INDUCTION OF HIGHLY ACTIVE β-GLUCOSIDASE FROM BIOMASS MATERIALS AND ITS APPLICATION ON LIGNOCELLULOSE HYDROLYSIS AND FERMENTATION

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Received August 13, 2015

To obtain highly active β-glucosidase, low value agricultural residues were used to induce enzyme production. The results showed that rice bran was the best substrate to induce β-glucosidase, likely due to its high magnesium, potassium, and calcium content. The optimum temperature and pH of β-glucosidase were 65 °C and 4.8, respectively. This enzyme was thermo-tolerant and fairly stable, with a long half-life of 15 days at 50 °C. In addition, the enzyme activity was best stimulated in a concentration of 10% (v/v) ethanol. The effects of β-glucosidase on fed-batch hydrolysis of cellulose and high-temperature simultaneous saccharification and fermentation (SSF) were examined. With a cellulase loading of 10 FPU/g of substrate and a β-glucosidase loading of 15 IU/g of substrate, the final substrate loading of 35% dry mass, sugar concentration could attain 225.84 g/L after 120 h of hydrolysis. When the fed-batch SSF process was performed with *Kluyveromyces marxianus* NCYC 587 operated at 42 °C, after 72 h of fermentation, the maximum ethanol concentration was approximately 49.07 g/L, indicating that β-glucosidase was suitable for lignocellulose conversion into ethanol.

Keywords: β-glucosidase induction, biomass materials, enzyme biocatalysis, bioconversion, ethanol, fermentation

INTRODUCTION

The current global concerns regarding the growing energy demands, the depletion of fossil fuel reserves, and climate change have spurred a resurgence of interest in renewable carbohydrate energy sources.[1] Cellulosic ethanol has the potential to substantially reduce the consumption of fossil fuels and can be produced through a facile, three-step sugar-enzyme platform consisting of pretreatment, saccharification, and fermentation. Nevertheless, the high conversion costs of the required enzymes hinder its industrial application. The highest costs are incurred during saccharification, where enzymes are required to hydrolyze the polymeric carbohydrates present in the pretreated biomass solids.[2] To achieve a cost-effective production of bioethanol from lignocellulose, the availability of highly active cellulase (β-glucosidase) is one of the most important steps.[3]

The hydrolysis of cellulose to glucose is simultaneously performed by endo-β-1,4-glucanase (EC 3.2.1.4, EG), exo-β-1,4-glucanase (EC 3.2.1.91, CBH), and β-glucosidases (EC 3.2.1.21, BGL).[4] β-glucosidase is a key enzyme in the sugar-enzyme platform for bioethanol production from lignocellulose. Further, it is one of the major components of cellulase, which can hydrolyze cellobiose and cello-oligosaccharides to glucose, and can reduce the inhibition of cellobiose to

endo-β-1,4-glucanase and exo-β-1,4-glucanase, which is the rate-limiting step during lignocellulosic hydrolysis. However, the levels of β-glucosidase present in commercial cellulase and those obtained from culture filtrates are too low for practical applications.[5] Further, β-glucosidase is inhibited by processing conditions, such as low pH and high glucose and ethanol concentrations. Therefore, the conversion of cellobiose by β-glucosidase is the key factor to reduce the inhibition and enhance the efficiency of enzymes for cellulosic ethanol production. Thus, thermostable β-glucosidase production is one of the most popular research topics in bioethanol manufacturing. The application of their counterparts assures higher reaction velocity, longer half-life of enzyme activity, and decreased viscosity of the substrate solution. Moreover, the thermo-tolerance of β-glucosidase at high temperatures makes it possible to control the reaction without cooling,[6] which is particularly important in industrial applications, such as biorefinery, where the heat treatment of substrates is desirable.[7]

Various microorganisms capable of producing cellulose-degrading enzymes have been assessed and characterized.[8,9] Filamentous fungi are known to be good producers of cellulase (including β-glucosidase) and numerous fungal enzymes have been isolated and analyzed, the most studied of which are the glycosyl hydrolases of *Trichoderma reesei*,[10] *Trichoderma koningii*,[11] *Penicillium citrinum*,[3] and *Aspergillus niger*.[12] The induction of cellulase has been investigated using various substrate inducers and cellulose-rich culture media.[13] Nevertheless, fewer studies have discussed the effects of different natural lignocellulosic waste materials on the induction of β-glucosidase, and the direct involvement of mineral elements has not yet been demonstrated.

Herein, in order to assess the synthetic mechanism of β-glucosidase, the induction effect from *Hypocrea* sp. W_{63} was investigated. Further characterizations of β-glucosidase, including optimal temperature, thermo-stability, pH, and sugar and ethanol performance, were also investigated. It was found that the thermo-stability and ethanol stimulation of this enzyme played an important role in the fed-batch hydrolysis process. The objective of this work is to assess the synergetic effect of cellulase and β-glucosidase adding on fed-batch SSF.

EXPERIMENTAL

Materials

Chemicals: 4-nitrophenyl-β-D-glucopyranoside (pNPG) and 4-methylumbelliferyl-β-D-glucopyranoside (MUG) were purchased from Sigma-Aldrich. All other reagents were obtained from commercial sources and were of analytical grade. Cellulase used in the fed-batch hydrolysis and SSF process was produced from *Penicillium* sp. (200 FPU/mL) obtained from Imperial Jade Bio-technology Co. Ltd. (Yinchuan, China).

Biomass materials: sweet sorghum bagasse was provided by Beijing Tai Tian Di Energy Technology Development Co. Ltd. Sugarcane bagasse was provided by Guangxi FengHao Group Co. Ltd. (Chongzuo, China). Wheat bran and rice bran were stored at our laboratory. All the biomass materials were pre-milled and screened, and the fraction between 20 and 80 meshes was used for further experiments. The biomass components were determined according to the standardized methods of the National Renewable Energy Laboratory (NREL, Golden, CO, USA).[14]

Strains, media and growth conditions

A fungal strain identified as *Hypocrea* sp. W_{63} stored in our laboratory was originally isolated from humus soil from the nature reserves of the Wuyi Mountain (Fujian, China). This strain is known to produce high levels of β-glucosidase. The strain was prepared on a potato dextrose agar (PDA) slant cultivated at 30 °C for 4 days, and then activated in 150-mL Erlenmeyer flasks with 50 mL of liquid PDA medium. For the induction process, the inductive medium contained a carbon substrate of biomass materials, basal salt elements, and 1% (v/v) of trace element solution. Each medium contained 5% (w/v) mesh powder of different biomass materials, such as sweet sorghum bagasse, wheat bran, rice bran, rice bran (alkali pretreated), and sugarcane bagasse. Nutrients were included as basal salt elements at the following concentrations: KH_2PO_4 2 g/L, $(NH_4)_2SO_4$ 2.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.3 g/L, $CaCl_2$ 0.3 g/L, and 1% (v/v) of trace elements solution ($CuSO_4$ 1 g/L, $MnSO_4$ 1 g/L, $FeSO_4 \cdot 7H_2O$ 1 g/L, $CoCl_2$ 1 g/L). The fermentation was carried out in 500-mL Erlenmeyer flasks made up to 200 mL, incubated at 30 °C and 150 rpm for 5 days. The supernatants and crude enzymes

were then retrieved by centrifugation at 12,000×g at 4 °C for 10 min and were used for enzyme assay and protein analysis.

The thermo-tolerant yeast *K. marxianus* NCYC587 was bought from the National Collection of Yeast Cultures (UK). Yeast was grown in a static culture on liquid yeast peptone dextrose (YPD) medium containing 20 g/L of peptone, 10 g/L of yeast extract, and 20 g/L of glucose. The medium was aseptically transferred into a 250-mL Erlenmeyer culture flask containing 100 mL of liquid YPD medium. The medium had a neutral pH and was sterilized for 20 min at 115 °C. Cultivation was performed at 42 °C for 12 h under shaking at 150 rpm. Following yeast activation, the strain sludge was aseptically centrifuged and used for SSF process studies.

Molecular mass determination

SDS-PAGE was performed using 12% separating gel and 5% stacking gel to determine the induction effects of different biomass materials and the molecular mass of the enzyme with the help of standard mixtures of marker proteins. The gel was stained with Coomassie brilliant blue R-250 for 2.5 h and destained in a solution of 10% (v/v) acetic acid and 5% (v/v) absolute ethanol.[11]

Zymogram analysis

The β-glucosidase content of *Hypocrea* sp. W_{63} was determined by zymogram analysis. For this purpose, ultra-condensates (Amicon Ultra 4 mL 3 kD, Millipore) of β-glucosidase excreted from inductive medium was loaded on a native gradient acrylamide gel containing 12% separating gel and 5% stacking gel without SDS and β-mercaptoethanol. When the run finished, the gel was incubated in sodium acetate buffer (pH 5.0, 0.02 mol/L) at 60 °C for 30 min with MUG as substrate. The gel was scanned under 365 nm.[11]

Enzyme assay

The activity of β-glucosidase was assayed using pNPG and a microplate titer method, as previously described.[15] A reaction mixture (200 µL) containing 40 µL of dilution enzyme and 40 µL of pNPG (0.005 mol/L) as substrate was dissolved in sodium acetate buffer (pH 5.0, 0.02 mol/L) and incubated at 50 °C for 10 min. The reaction was terminated by the addition of 120 µL of cold Na_2CO_3 buffer (1 mol/L). The developed yellow color solutions were read at 400 nm using a microplate reader and a spectrophotometer (EON, BioTek). One unit of β-glucosidase activity was defined as the amount of enzyme required to release 1 µmol of pNPG per min in the reaction mixture. Each value was stated as an average of three parallel replicates.

Characteristics of β-glucosidase

The enzyme retrieved from *Hypocrea* sp. W_{63} was activated in 150-mL Erlenmeyer flasks with 50 mL liquid PDA medium. The activated enzyme was then transferred to an inductive medium using 5% (w/v) rice bran as substrate in a 500-mL Erlenmeyer flask containing 200 mL inductive medium and the contents were incubated at 30 °C and 150 rpm for 5 days. The supernatants and crude enzymes were retrieved by centrifugation at 12,000×*g* at 4 °C for 10 min and used for further characterization.

Optimum temperature and long-term stability

The optimal temperature was tested with standard assay conditions in the temperature range from 45 to 80 °C. The long-term stability of β-glucosidase was determined following 15 days of incubation at 40-80 °C. The residual activity was determined by enzyme assays, as described above.

Optimum initial pH of β-glucosidase

The optimum pH was assessed within a pH range from 4.0 to 5.4 achieved by the addition of 0.2 mol/L $NaHPO_4$ and 0.1 mol/L citric acid buffers. The effects of different pH values and the residual activity were analyzed following incubation at 50 °C for 10 min.

Effects of various sugars and ethanol on the activity of β-glucosidase

The effect of sugars on enzyme activity was determined using standard enzyme assay conditions in the presence of 0.01 and 0.1 mol/L of glucose, xylose, fructose, galactose, lactose, arabinose, mannose, maltose, sucrose and cellobiose. The effects of ethanol concentration (5-45% v/v) on pNPG hydrolyzing activity were also assessed using a standard assay, as described before.

Application of β-glucosidase

Alkali pretreatment

Sugarcane bagasse was mixed with 0.5 mol/L NaOH and water at a solid to liquid ratio of 1:20. The slurry was incubated at 80 °C and stirred for 2 h. After pretreatment, the residue was washed with tap water until a neutral pH was obtained, and was then dried in a forced-air oven at 60 °C until a constant weight was reached.[16]

Fed-batch hydrolysis in shake flasks

Experiments were performed in five 250-mL Erlenmeyer flasks, each containing 100 mL of buffer solution (pH 5.0, 0.2 mol/L $NaHPO_4$, and 0.1 mol/L citric acid). The dry mass (DM) loading achieved following batch enzymatic hydrolysis was 15% (w/v) of sugarcane bagasse, and was set as the concentration for the initial fed-batch process, with a cellulase loading of 10 FPU/g of substrate and β-glucosidase loading of 15 IU/g of substrate. Firstly, 5% (w/v) of fresh substrate was fed to four of the Erlenmeyer flasks after 6 h till liquefaction to achieve a final DM loading of 20%. To further increase the concentration of sugars, more fresh DM was fed after 12, 24, and 36 h to three of the Erlenmeyer flasks to reach a final DM loading of 25%, 30%, and 35% (w/v), respectively. The samples were collected at 12, 24, 48, 72, 96 and 120 h and measured by high performance liquid chromatography (Waters 2695). All experiments were carried out in duplicate.

High-temperature fed-batch SSF process

The fed-batch SSF process was performed in 250-mL Erlenmeyer flasks, each containing 50 mL of nutrient medium and different amounts of sugarcane bagasse. A nutrient medium containing $(NH_4)_2HPO_4$ 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.025 g/L, yeast extract 1.0 g/L, and buffer solution (pH 5.0, 0.2 mol/L $NaHPO_4$ and 0.1 mol/L citric acid) was also applied. All the reaction mixtures were sterilized for 20 min at 121 °C. Cellulase was loaded as10 FPU/g of substrate and pre-hydrolysis was performed for 24 h at 50 °C on a rotatory shaker at 150 rpm.[17] Thermo-tolerant yeast (0.2 g/L dry weight), pre-activated in YPD medium for 12 h, and 15 IU/g of β-glucosidase substrate were added after pre-hydrolysis. A control was set without β-glucosidase addition. Fermentations were performed at 42 °C for 72 h. The time of yeast addition was referred to as time 0 h. Samples were collected after 12, 24, 48, and 72 h of incubation and the glucose and ethanol concentrations were analyzed.

Analytical methods

The products were centrifuged at 12,000 rpm for 5 min. The sugar concentrations were measured by high performance liquid chromatography with a refractive index detector RI 2414 using a Shodex sugar SH 1011 column (Agilent) at 50 °C with 0.005 mol/L H_2SO_4 as the mobile phase at a flow rate of 0.5 mL/min.[16] The ethanol content was analyzed by gas chromatography using an HP 6820 (Agilent) with a flame ionization detector (GC-FID) and a capillary column (30.0 m × 0.25 mm × 0.25 μm). The conditions were set at an injector and detector temperature of 250 °C, argon as the carrier gas at a flow rate of 30 mL/min, and a split ratio of 1:50.

The carbon, nitrogen, and sulfur contents of the biomass materials were measured with an elemental analyzer (Vario EL cube), while other mineral contents were analyzed by inductively coupled plasma mass spectrometry (ICP, OPTIMA 8000).[18] These contents were the mass ratio of elements to constant dry materials.

RESULTS AND DISCUSSION

Different biomass materials induction on β-glucosidase

In order to reduce the cost of cellulase production, agro-industrial residues or cheap materials were chosen as induction media in this study. The use of abundantly available lignocellulosic crop residues as carbon sources of microbes producing β-glucosidase is a viable approach. The β-glucosidase activity produced from sweet sorghum bagasse, wheat straw, and sugarcane bagasse can be seen in Figure 1, yet rice bran had the strongest induction effect on β-glucosidase. The β-glucosidase specific activity of sweet sorghum bagasse, wheat bran, rice bran, rice bran (pretreated) and sugarcane bagasse was of 0.598, 1.601, 3.61, 1.14 and 1.759 U/mg-protein, respectively. The composition of different biomass materials is shown in Table 1. The alkali pretreated rice bran had the highest glucan content, but showed only a minor induction effect on β-glucosidase, indicating that the glucan content (cellulose) is not an important factor affecting β-glucosidase production. These observations may be due to a large number of inhibitors being produced during pretreatment or by the lack of essential elements during the pretreatment process. Meanwhile, the low ash content and high glucan content of rice bran (alkali pretreated) did not affect the β-glucosidase induction process.

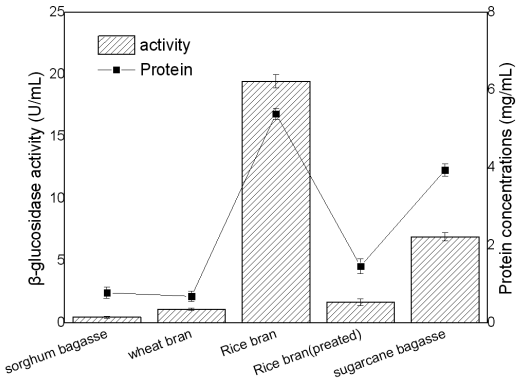


Figure 1: β-Glucosidase induction effects from different biomass materials

Table 1
Biomass material composition

Materials	Glucan, %	Xylan, %	Lignin, %	Ash, %
Sweet sorghum bagasse	38.17	21.21	25.62	15
Wheat bran	35.60	24.67	18.66	21.07
Sugarcane bagasse	41.20	20.46	20.04	18.3
Rice bran (alkali pretreated)	64.23	7.47	26.33	1.97
Rice bran	38.4	24.1	26.1	11.4

Herein, during shake flask fermentation, a maximum β-glucosidase activity of 19.46 U/mL (3.89 IU/g substrate) was achieved. Similarly, β-glucosidase activity of 2.5 IU/g was obtained with *Trichoderma atroviride* using a pretreated willow medium,[19] and when a high β-glucosidase-producing strain, *Penicillium pinophilum* KMJ601, was cultivated in media containing rice straw, a maximum β-glucosidase specific activity of 3.2 U/mL was reported.[20] Thus, rice bran appears to be a rich biomass source of nutrients, containing cellulose, proteins, and minerals, and is able to promote growth and enzyme production, following hydrolysis into glucose. Additionally, the high productivity of β-glucosidase in rice bran could also be due to the double role of biomass waste material as an induction source and as a cellulose structure support matrix for fungal adherence.

Considering that fungi are known to express functionally diverse cellulases/hemicellulases in the presence of different carbon sources,[21] the induction process is likely very complex. The biosynthesis of enzymes is known to be regulated by catabolism repression and specific induction, with regulatory genes being termed as wide domain and pathway-specific control genes, respectively. This multiplicity may occur as a result of genetic redundancy, differential mRNA processing, or post-translational modification such as glycosylation, autoaggregation and/or proteolytic digestion.[22] In the MUG-zymogram analysis (Fig. 2A), the extracellular protein band was visible in the native gel. Further, SDS-PAGE results (Fig. 2B) showed that rice bran was able to induce large amounts of protein as well as β-glucosidase, in agreement with the data described above (Fig. 1).

Previous studies have compared the diverse β-glucosidase expressions in response to different carbon sources.[23] However, a comparison of the nutrient and mineral compositions in the various biomass materials and their effect on β-glucosidase activity is yet to be performed. Solid biomass is a cheap yet rich in cellulose source material, which also contains various trace elements and vitamins. Herein, the nutrient elements of five biomass materials were analyzed

(Table 2). The nitrogen, carbon, hydrogen, and sulfur contents were similar in the various biomass waste materials analyzed. Nevertheless, the magnesium, potassium, and calcium contents in rice bran were 0.0046%, 0.01081%, and 0.00775%, respectively, and much higher than those in the other biomass materials. Magnesium, potassium and calcium were assumed to be the key minerals inducing β-glucosidase.

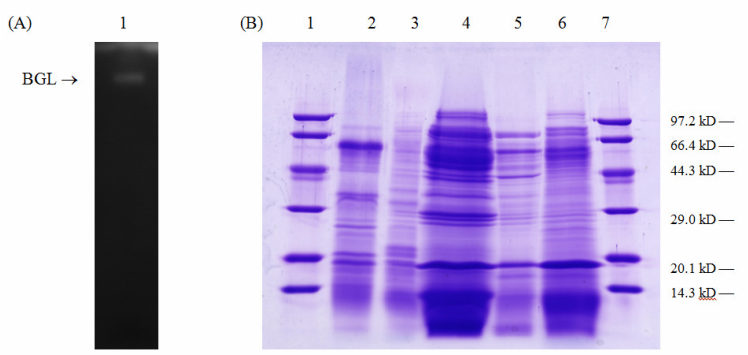


Figure 2: (A) 1: Native PAGE of extra-cellular β-glucosidase of *Hypocrea* sp. W_{63}; (B) SDS-PAGE electrophoresis of β-glucosidase induced by different biomass materials, 1: Potein marker; 2: Sweet sorghum bagasse; 3: Wheat bran; 4: Rice bran; 5: Rice bran (alkali pretreated); 6: Sugarcane bagasse; 7: Protein marker

Table 2
Nutrient and mineral element contents in the various biomass materials

Nutrients	Sweet sorghum bagasse	Wheat bran	Sugarcane bagasse	Rice bran (alkali pretreated)	Rice bran
N%	0.1228	0.0717	0.2976	0.2674	0.364
C%	10.54	10.77	13.32	10.88	11.24
H%	1.542	1.582	1.781	1.433	1.682
S%	0.136	0.0164	0.033	0.0416	0.081
Mg%	0.00139	0.000299	0.0022	0.00264	0.0046
Al%	0.000174	0.000273	0.000108	0.000396	0.00037
K%	0.006296	0.003339	0.008526	0.002618	0.01081
P%	0.00019	0.000408	0.001159	0.000819	0.000765
Ca%	0.005433	0.000794	0.00586	0.00633	0.00775
Fe%	0.000353	0.000097	0.002701	0.000629	0.000389
Cu%	0.00000567	0.000002	0.000222	0.00000533	0.00000367

In a previous study,[24] calcium was shown to provide several benefits, including protein conformation stabilization, higher affinity for the substrate, and a higher thermostability in other enzymes. Thus, the increased induction herein may be attributed to stabilization of the enzyme structure by magnesium, potassium, and calcium, since the motifs of protein and mineral elements involved in β-glucosidase regulation in upstream regions of *Hypocrea* sp. W_{63} may be affected by the mineral content.

Characteristics of β-glucosidase

Effects of temperature and initial pH on enzyme assay

The temperature adaption results showed that β-glucosidase produced by the isolated *Hypocrea* sp. W_{63} exhibited a broad temperature optimization range from 30 to 90 °C (Fig. 3A), with the maximal enzyme activity being reached at 65 °C. The optimum temperature for this enzyme is higher than that for most β-glucosidases produced by fungi, which is

usually below 60 °C.[7] β-Glucosidase was stable at 50 °C, with a long half-life of 15 days at 50 °C and retention of more than 90% of the initial activity over 4 days (data not shown). This stability may be attributed to the crucial role played by the invariant of the carbohydrate portion of the protein.[25] In comparison, β-glucosidase from *Paecilomyces thermophila* J18 was found to retain more than 95% of its initial activity after 8 h at 50 °C and β-glucosidase from *Melanocarpus* sp. MTCC 3922 retained approximately 70% after 6 h incubation at 50 °C.[26-28] Thus, the present observations of such long half-life of β-glucosidase activity make it highly suitable for industrial applications.

An initial pH value of 4.8 was shown to achieve optimal β-glucosidase activity (Fig. 3B), in agreement with the optimum pH value found in most microorganisms and plants.[26,29] Since β-glucosidase from *Hypocrea* sp. W_{63} was highly stable at 50 °C and pH 4.8, further analyses of the enzyme were performed under these conditions.

Effect of various sugars and ethanol concentration on β-glucosidase activity

The effects of various carbohydrates on pNPG activity of the crude enzyme are summarized in Table 3. Monosaccharides with different α or β links, such as xylose, fructose, galactose, and arabinose, had no impact on β-glucosidase activity or caused a little increase. Similar results were observed with disaccharides such as mannose and sucrose. However, glucose, cellobiose, and maltose inhibited the enzyme activity. When β-glucosidase was studied in a sugar concentration of 0.1 mol/L, only 19.74%, 45.95% and 58.72% of enzymes were left, respectively.

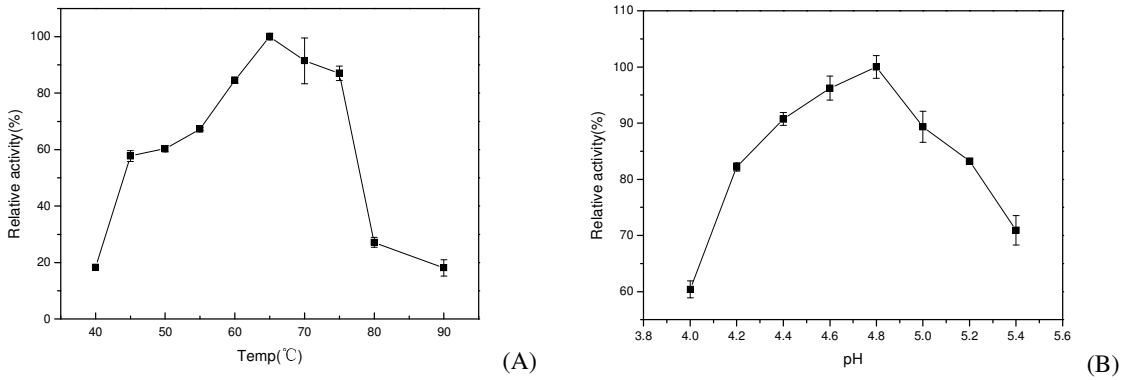


Figure 3: Effects of (A) temperature and (B) pH on the activity of the crude β-glucosidase

Table 3
Effect of carbohydrates on the activity of β-glucosidase

Carbohydrate	Related activity, 0.01 mol/L	Related activity, 0.1 mol/L
Glucose%	65.27±3.36	19.74±0.16
Xylose%	104.59±1.64	113.19±1.28
Fructose%	103.11±1.64	102.7±1.23
Lactose%	111.55±0.25	128.42±4.01
Arabinose%	110.81±3.85	113.43±1.31
Mannose%	107.04±3.68	95.17±3.93
Maltose%	93.69±2.13	58.72±2.62
Sucrose%	105.73±1.47	96.23±2.05
Cellobiose%	88.04±3.03	45.95±2.54

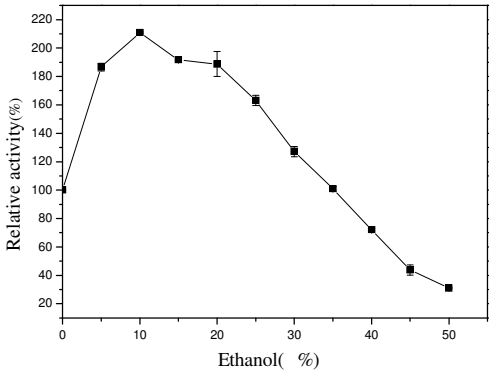


Figure 4: Effects of ethanol concentration on β-glucosidase activity

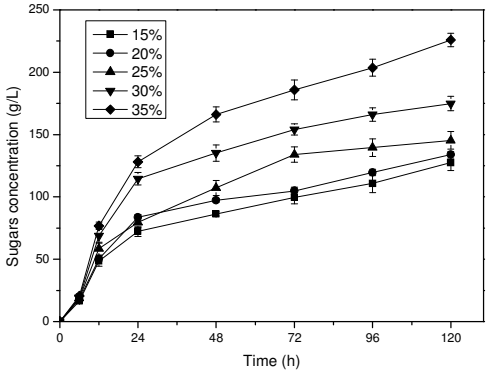


Figure 5: Concentration of sugars with different substrate loadings

β-Glucosidase catalysis is a reversible reaction, and glucose and cellobiose both acted as inhibitors. Surprisingly, lactose had a slight increasing effect on enzyme activity, likely due to disaccharides acting as glucose-acceptors in the transglucosylation reaction.[28] From a biotechnological perspective, the high catalytic efficiency associated with good thermostability and tolerance to elevated concentrations of monosaccharides and disaccharides make the β-glucosidase induced herein highly attractive for industrial applications.[26]

β-Glucosidase is likely to be affected by protein denaturation.[22] However, the presence of ethanol had a positive influence on the hydrolytic activity of β-glucosidase from *Hypocrea* sp. W_{63} (Fig. 4). It was previously ascertained that the specific activity of β-glucosidase was stimulated by the addition of ethanol up to 30% (v/v) to the crude extract. However, the most simulating effect of enzyme activity was observed at a 10% (v/v) concentration, with a two-fold relative enzyme activity improvement (Fig. 4). The relative activity can be improved up to 196.44%. Stimulating effects of organic solvents have also been described for β-glucosidase from *Rhizomucor miehei*,[30] *Aspergillus oryzae*,[31] *Melanocarpus sp.*,[26] *Thermoascus aurantiacus*,[15] *Fusarium oxysporum* and *Pichia anomala*.[24]

It was proposed that the stimulation of enzyme activity by 10% (v/v) concentrations of ethanol occurs due to the more pronounced nucleophilicity of ethanol with respect to water, resulting in a higher rate of reactions involving nucleophilic substitutions.[32,33] For lignocellulose-based ethanol production, the minimum economically viable industrial-scale distillation is above 4% (v/v) in the fermentation broth,[34] with many researchers preferring ethanol concentration of more than 8% (v/v).[35] However, too high an ethanol concentration or too long a reaction time could hamper the performance of yeast and enzymes.[36] Herein, β-glucosidase proved to be more tolerant to glucose in reaction mixtures containing 10% (v/v) of ethanol. Thus, it can be concluded that the β-glucosidase produced from *Hypocrea* sp. W_{63} has great potential applications in bioethanol production, where the ethanol concentration threshold is compatible with a good level of enzyme activity.

Application of β-glucosidase

Fed-batch hydrolysis effects of sugarcane bagasse

The total sugars release from sugarcane bagasse, such as arabinose, cellobiose, glucose and xylose, is shown in Figure 5. During the first 6 h, the initial DM loading was 15% (w/v). When all of the enzymes were added after the first 24 h for pre-hydrolysis, there was greater sugar release at higher enzyme loadings, while after 36 h, all the hydrolysis systems from 15-35% (w/v) were added up to the final substrate loading and liquefied. The total sugars consist of arabinose, cellobiose, glucose and xylose. In the final stage of 120 h, the sugar concentration of 15%-35%

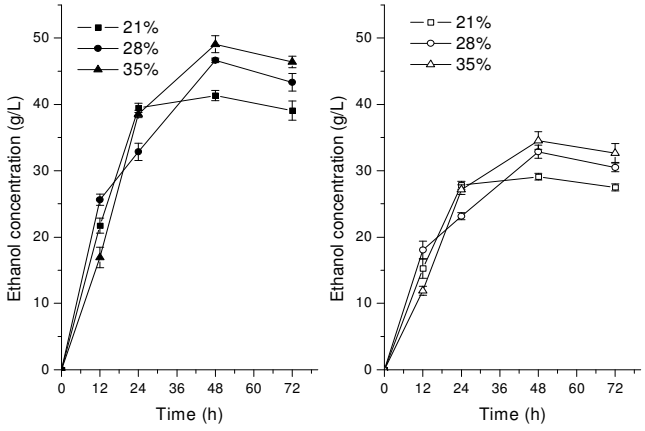
(w/v) was 127.48 g/L, 133.78 g/L, 145.30 g/L, 174.79 g/L and 225.84 g/L, respectively. Finally, at 120 h, the highest sugar concentration was achieved (225.84 g/L, consisting of 3.35 g/L arabinose, 32.78 g/L cellobiose, 132.53 g/L glucose, and 57.18 g/L xylose). With the synergistic effect of β-glucosidase in this fed-batch process, the conversion rate of 35% DM was up to 55.17%.

High-temperature fed-batch SSF

The feeding points used to obtain higher ethanol concentrations are shown in Table 4. The highest concentration of DM 35% (w/v) reached the maximum ethanol concentration (49.07 g/L; Fig. 6A) in 48 h, but 24.58 g/L of glucose was detected as residual sugar at the end of the process. In other *K. marxianus* systems used in the high-temperature SSF process,[37-39] the maximum ethanol concentrations ranged from 16.8 to 36.2 g/L. This was the first time that such high ethanol production was acquired in high-temperature fed-batch SSF at such high DM loading. Also, at a DM loading of 21% (w/v) and 28% (w/v), ethanol concentrations of 41.34 and 46.66 g/L were obtained, respectively. On the contrary, in the blank control (Fig. 6B), ethanol production was only 29.11, 32.86 and 34.55 g/L at a DM loading of 21%, 28%, and 35%, respectively. Thus, without β-glucosidase, a much lower ethanol production was observed.

Table 4
Prehydrolysis conditions by fed-batch mode

Dry mass	Feeding amount (w/v)			
	0 h	6 h	12 h	24 h
21%	14	7		
28%	14	7	7	
35%	14	7	7	7



(A) (B)

Figure 6: Effects of β-glucosidase addition on high-temperature fed-batch SSF; (A) with 10 FPU/g substrate cellulase and 15 IU/g substrate β-glucosidase addition; (B) Control; with 10 FPU/g substrate cellulase, without β-glucosidase addition

Different inhibitor concentrations in the broth led to constant glucose concentration in the final fermentation process, indicating that there was yeast cessation in the final fermentation at such high DM. This may be due to inhibitory effects and an increase in end-product inhibition as the sugar consumption rate was lower, and it could also be a reflection of less efficient mass

transport.[40] Furthermore, the increased concentration of inhibitors, such as acetic acid, furfural, and ethanol, would hamper the performance of the yeast and enzymes.[36] Herein, the addition of β-glucosidase effectively transformed cellobiose and cello-oligosaccharides into fermentable sugars. The inhibitors such as ethanol production resulted in a better configuration in most of the SSF processes assessed. β-Glucosidase functioned in the high-temperature fed-batch SSF process, enhancing bioethanol performance, indicating that the presence of a suitable β-glucosidase, such as *Hypocrea* sp. W_{63}, is necessary in such processes.

CONCLUSION

In order to reduce the cost of cellulase production, agro-industrial residues or cheap materials were chosen as induction media. Rice bran was found to be the best inducing material for β-glucosidase production. The analysis of mineral elements revealed that the magnesium, potassium, and calcium content was higher in rice bran, and these were assumed to be the key minerals inducing β-glucosidase. β-glucosidase from *Hypocrea* sp. W_{63} was highly stable at 50 °C and pH 4.8. Furthermore, a two-fold enzyme activity improvement was achieved with 10% (v/v) of ethanol. The synergistic effect of β-glucosidase on the fed-batch SSF process showed that the highest sugar concentration (225.84 g/L) was achieved at a DM loading of 35% (w/v). High-temperature fed-batch SSF was operated with *K. marxianus* NCYC 587 with the aim of obtaining higher ethanol concentrations. Ultimately, the maximum ethanol production achieved 49.07 g/L at a high DM loading of 35% (w/v) in 48 h. Thus, these desirable properties make β-glucosidase a promising material for application in bioethanol production.

ACKNOWLEDGEMENTS: This research was financially supported by the Program of National Natural Science Foundation of China (grant numbers 21506215, 51606203), Bureau of International Cooperation, Chinese Academy of Sciences (grant number 182344KYSB20160056), National High Technology Research and Development Program of China (863 Program) (grant number 2015AA020202) and Science & Technology Project of Guangdong Province (grant number 2017A010105018).

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