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PURIFICATION AND CHARACTERIZATION OF A RECOMBINANT BETA-GLUCOSIDASE IN *ESCHERICHIA COLI*

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SUMMARY

Beta-glucosidase (BGL) is an enzyme involved in the degradation of cellulose and plays an essential part in many biological processes. Currently, most BGLs applied in the industry are derived from fungi. Exploring novel BGLs with desired properties is attractive. The recombinant BGL derived from microorganisms surrounding white-rot fungus in Cuc Phuong National Park was successfully expressed in *Escherichia coli* Rosetta 1 (denoted as the GH3S2 gene). The protein GH3S2 was purified by an affinity chromatography column using buffer PBS 50 mM (NaCl-free) pH 7, and the enzyme was collected in buffer containing imidazole 300 mM. The purity and content of the purified protein was determined. The purity of the enzyme obtained after purification reached over 95%. The result of the GH3S2 protein content in the purified sample was 1.54 mg/ml. Thus, amount of the purified GH3S2 obtained from one liter of bacterial culture was 41.80 mg. The final GH3S2 was purified approximately 7.05–fold with a purification yield of 40.06%. The purified enzyme was used to study the properties. This enzyme optimally was activated at 37^{o}C and pH 6.0. At this condition, the enzyme specific activity was 2.23 U/mg in the *p*NPG substrate, and K_m and V_{max} were, respectively, 4.55 mM and 4.91 µmol/min. Its activity increased to 200% and 119% in the presence of Ca^{2+} and Mg^{2+} and decreased to 33% and 14% when Ni^{2+} and Cu^{2+} were added. The enzyme activity was maintained at 70% when the glucose concentration was at 6 mM and then gradually decreased.

Keywords: Affinity chromatography, enzyme characteristics, *Eschericia coli* Rosetta 1, protein purification, recombinant beta-glucosidase.

INTRODUCTION

Cellulose is the most abundant polysaccharide in nature and is a major component of plant cells. This is an important source of materials for producing chemical compounds and biofuels. However, a significant amount of cellulose waste in agriculture, industry, and daily life has accumulated due to inappropriate treatment, causing environmental pollution (Ahmed *et al.*, 2017). Effective hydrolysis of cellulose requires the simultaneous interference of three main groups of enzymes, including β-1,4-endoglucanase; β-1,4-exoglucanase; and β-glucosidase. In this way, two groups of β-1,4-endoglucanase and β-1,4-exoglucanase work together to degrade natural cellulose into cellobiose, which reversely

inhibits the activity of two enzymes (Zhang *et al.*, 2017). β-Glucosidase (EC 3.2.1.21) (BGL) is widely distributed in nature and splits cellobiose disaccharides into two glucose molecules. Therefore, BGL is considered an important factor in determining the efficiency of cellulose hydrolysis. Searching for BGLs with high thermal stability is important in industrial-scale use of cellulose. Most of the BGLs in industrial use are currently derived from fungi such as *Asperginus niger* (Watanabe *et al.*, 1992), and *A. fumigatus* (Liu *et al.*, 2012). Bacterial BGLs are gaining more attention due to their fast growth rate, their ability to form a complex of multiple enzymes, and their ability to live in extreme conditions (Maki *et al.*, 2009). Studies have focused on finding BGLs originated in new sources of genes, which have the potential for industrial use, such as goat rumen, termite gut, surrounding white-rot fungus, and other objects. A metagenomic cosmid library was prepared by Feng *et al.* (2007) from DNA extracted from the contents of the rabbit cecum and screened for cellulase activities. Eleven independent clones expressing cellulase activities (four endo-beta-1,4-glucanases and seven beta-glucosidases) were isolated. Subcloning and sequencing analysis of these clones identified 11 cellulase genes. As a result, the genes encoding these enzymes had only low similarity to the known gene sequences in the GenBank database, indicating that these genes are capable of encoding new enzymes (Feng *et al.*, 2007).

In the previous study, we were successful in expressing the recombinant BGL derived from bacteria living around white-rot fungus in *Escherichilia coli* Rosetta 1. In this study, we present results of the recombinant BGL purification, enzyme activity, and stability at different temperatures and pH conditions, the influence of metal ions and chemicals on enzyme activity, and kinetic parameters of enzymes.

MATERIALS AND METHODS

Materials: strain *E. coli* Rosetta 1 harboring the recombinant vector pET22b-BGL created by the Department of Genetic Engineering of the Institute of Biotechnology was used to express the BGL gene. The sequence of the gene encoding BGL (denoted as GH3S2) was mined from metagenome DNA data of bacteria surrounding the white-rot fungus *Trametes versicolor* in Cuc Phuong National Park and used for expression in *E. coli*.

A 5 ml Histrap affinity chromatography column (Healthcare, Sweden) and a 10 kDa dialysis bag (Thermo Scientific, USA) were used for purification and salt removal of the recombinant enzyme sample. *p*NPG (Thermo Scientific, USA) was used as the substrate to determine GH3S2 activity. Other chemicals used in the study were purchased from Merck (Germany), Fermentas (Germany), and Thermo Scientific (USA).

Purification of β-glucosidase using affinity chromatography

The expression of recombinant GH3S2 in the *E. coli* Rosetta 1 cell was carried out according to the protocol described in the previous study. In brief, the recombinant strain was inoculated in Luria-Bertani liquid medium supplemented with 100 μg/ml ampicillin and incubated with shaking at 37°C overnight. After that, the overnight culture was transferred to 200 ml of modified TB media (1.2% peptone, 2.4% yeast extract, 72 mM K_2HPO_4, 17 mM KH_2PO_4, 0.24% glucose), supplemented with 100 μg/ml ampicillin to reach an initial OD_{600} of 0.1 and incubated continually. When OD_{600} reached 1, BGL expression was induced with 0.3 mM IPTG at 25°C with shaking at 200 rpm. After 4 hours, cells were recovered by centrifuging at 5000 rpm.

Cell pellets were suspended in deionized water to reach an OD_{600} of 10. The cells were disrupted by sonication for 10 minutes. Subsequently, the sample was added to PBS 300 mM without NaCl (KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM) until the final concentration of PBS was 50 mM and pH 7. Next, the supernatant was collected by centrifuging at 8000 rpm for 10 minutes. Fifteen mL of the solution were loaded onto the histrap column. Then, the column was washed with 5

column volumes (5 CV) of PBS buffer containing imidazole at 20 mM and 50 mM. The bound protein was eluted with PBS buffer 50 mM, pH 7, containing imidazole 300 mM (1 mL/faction). The purified samples were added with 10% glycerol, and then dialyzed using PBS buffer 50 mM, pH 7, and 10% glycerol overnight in a dialysis bag of 10 kD. The protein sample was evaluated for purity on a polyacrylamide gel, followed by Quantity One version 4.6 software (https://quantity-one.software.informer.com/4.6/). The protein concentration was determined by the Bradford method (Bradford, 1976).

Determination of β-glucosidase activity

BGL activity was determined by hydrolysis of *p*-nitrophenol-β-glucoside (*p*NPG), to release *p*-nitrophenol (*p*NPG) (Dashtban *et al.*, 2010). The enzyme reaction was carried out in 20 µL of enzyme diluted in PBS buffer 50 mM, pH 7, and 180 µL 5 mM *p*NPG, incubating at 37°C for 15 minutes. The reaction was stopped with 800 µL of Na_2CO_3 (0.2 M). This sample was measured with OD_{410}. The amount of *p*NP produced was calculated based on a standard curve of *p*NP concentration. One unit of BGL activity is the amount of enzyme needed to release 1 µmol *p*NP in one minute (Dashtban *et al.*, 2010).

Determination of the effect of temperature, pH, metal ions, and glucose on the enzyme activity of GH3S2

In order to determine the effect of temperature on the activity, the enzyme was incubated at temperatures of 30, 35, 37, 40, and 50°C before determining the GH3S2 activity. The effect of pH on enzyme activity was assessed with pH 5.0, pH 5.5, pH 6.0, pH 6.5, pH 7.0, and pH 8.0. The influence of metal ions such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+} on enzyme activity was determined in the reaction containing metal ions at 1 mM. The effect of glucose on GH3S2 activity was determined by adding it to the reaction and its concentration ranging from 2 to 300 mM.

Determination of enzyme stability with temperature, pH

The enzyme was incubated at 37, 40, 45, and 50°C for 1, 2, 3, 4, 6, and 12 hours to determine the temperature stability. Enzyme was prepared in PBS buffer 50 mM with different pHs, including pH 5.0, pH 6.0, pH 7.0, and pH 8.0 during 1, 2, 3, 4, 6, and 12 hours. Then, it was used for the determination of the activity according to the method described above.

Determination of kinetic parameters of the enzyme

The enzymatic activity of recombinant GH3S2 was determined at various concentrations of *p*NPG substrate ranging from 1–10 mM. The mixture contained of 1 µg enzyme in 20 µL PBS buffer 50 mM, pH 6.0 and 180 µL *p*NPG with concentrations ranging from 1–10 mM was incubated at 37 °C for 15 minutes. Then, 800 µL of 0.2 M Na_2CO_3 was added to stop the reaction. The sample was measured at OD_{410}. In order to determine K_m, V_{max}, the standard curve showing the dependence of the reaction rate on the substrate concentration, was built up with the method of Linewever–Burk (Price, 1985), where V is µmol of *p*NP released in 1 min, 1/[S] was determined as 1/[*p*NPG] with *p*NPG concentration determined as mM.

RESULTS AND DISCUSSION

The recombinant GH3S2 enzyme with a molecular weight of about 91 kDa was successfully expressed in the *E. coli* Rosetta 1 cell under optimal conditions. In this study, we purified the recombinant GH3S2, assessed the activity and kinetic parameters as well as the factors affecting GH3S2 enzyme activity.

Purification of GH3S2

Protein purification is the process of separating the target protein from the protein mixture. Normally, this process is based on differences in protein size, physicochemical properties, or affinity interactions of proteins. According to the expression vector design, the GH3S2 gene was constructed in the pET22b(+) vector at the position. Thus, the recombinant

GH3S2 protein synthesized has a His-tag tail; therefore, the GH3S2 needed to be purified by the affinity chromatography column with the Ni^{2+} ion.

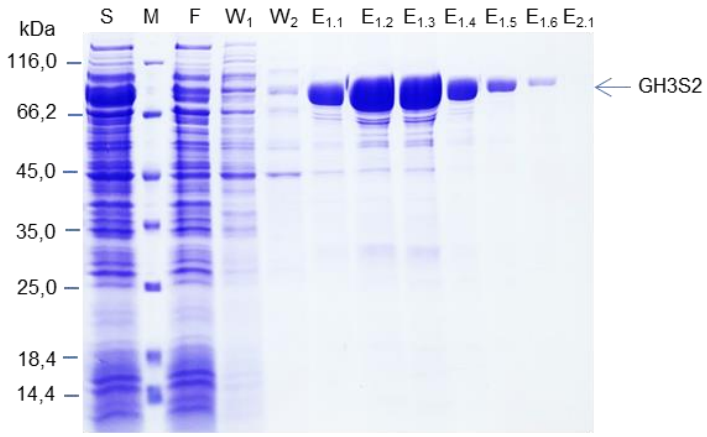

Figure 1. Assessment of GH3S2 purification using His-tag chromatography column on 12.6% polyacrylamide gel. S: Total soluble protein; M: standard protein (Fermentas, SM0431); F: Flow throught; W1, W2: Wash fractions withPBS buffer containing imidazole 20, 50 mM, respectively; E1.1 - 1.6: elution fractions with imidazole 300 mM; E2.1: retaining fraction with imidazole 500 mM.

The results shown in Fig. 1 indicated that the GH3S2 protein bound to the column very well and was eluted with imidazole at 300 mM. The GH3S2 protein was collected in 6 fractions (E1.1-E1.6) and was mainly concentrated in fractions 1, 2, 3, 4, especially 2 and 3. Protein from concentrated fractions 2, 3, and 4 were gathered to determine content and purity. The purity of the purified GH3S2 sample based on Quantity One software was above 95%. In the study by Zhao *et al.* (2004), the purity of bglA from *Bacillus polymyxa* was 92.7%. Thus, the enzyme GH3S2 was successfully purified for use in determining characterization. The result of the GH3S2 protein content in the purified sample was 1.54 mg/ml. Thus, the amount of purified GH3S2 obtained from one liter ofbacterial culture was 41.80 mg. Meanwhile, the amount of the total protein from the crude extract *E. coli* cells was 735.68 mg/L. The final GH3S2 was purified about 7.05–fold with a purification yield of 40.06% (Table 1).

Table 1. The difference in GH3S2 amount and activity between crude extract and purified protein.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	735.68	114.77	0.156	1	100
Purified GH3S2	41.80	45.98	1.10	7.05	40.06

Effect of temperature on GH3S2 activity and thermal stability of GH3S2

Microorganisms living in the soil of tropical forests such as the Cuc Phuong national forest are usually warm-loving, so their BGL enzyme can also work well at temperatures from 30 to about 45°C. Therefore, in order to evaluate the effect of temperature on the activity of GH3S2, temperatures of 30, 35, 37, 40, and 50°C were tested. The results showed that GH3S2 activity gradually increased from 30°C to 37°C, then gradually decreased at 40°C and declined sharply at 50°C. If the temperature is optimal, the

enzyme activity is 100% Then at the temperatures of 30, 35, 40, and 50ºC, the enzyme activity is 82.19, 93.66, 80.97 and 37.26%, respectively. Thus, at 37ºC, the enzyme GH3S2 showed the highest activity. This temperature is within the optimal temperature range of most cellulases of bacteria (from 35 to 50ºC). According to the research of Gomes-Pepe *et al.* (2016), 37ºC is also the optimal temperature for the BGL enzyme from the soil bacteria's metagenome. This optimal temperature is similar to that of BGL in the cattle rumen (Li *et al.*, 2014) and that of *Proteus mirabilis* VIT117 cultured on shrimp shells (Mahapatra *et al.*, 2016). However, higher optimal temperatures of BGL derived from the digestive juices of larval larvae and *A. fumigatus* Z5 have also been reported, which were respectively 55ºC (Yapi *et al.*, 2009) and 60ºC (Liu *et al.*, 2012).

In order to examine the thermal stability, GH3S2 was treated at 37, 40, 45, and 50ºC for 1, 2, 3, 4, 6, and 12 hours before incubation with the substrate. The results showed that the enzyme activity of GH3S2 was almost stable at 37ºC, and still reached 90.78% after 12 hours of treatment. At 40ºC, the enzyme maintained about 70% of its activity in 3 hours of treatment. From the 4th hour onward, the enzyme activity decreased rapidly. At temperatures of 45 and 50°C, enzyme activity was nearly halved in the first hour and then gradually decreased. This is compatible with the study of Zhang *et al.* (2017), that found BGL from *Bursaphelenchus xylophylus* was stable at temperatures below 40°C only.

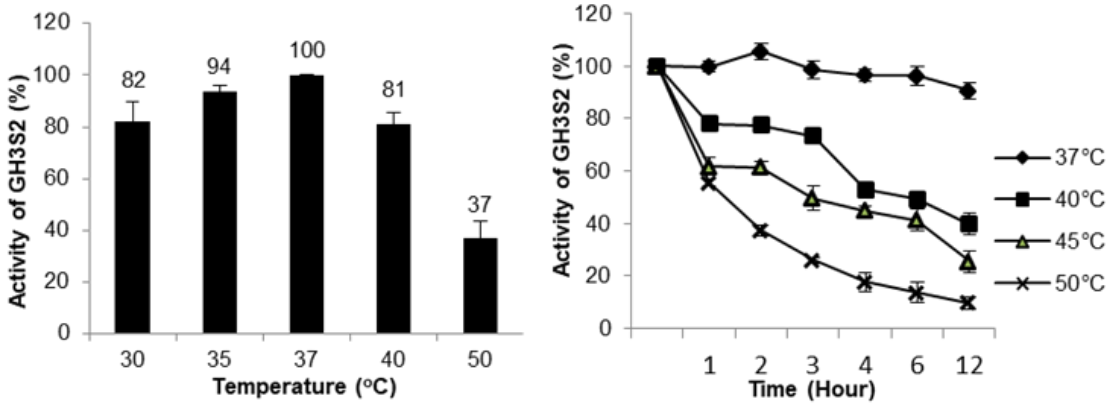

Figure 2. (A). Effect of temperature on GH3S2 activity; (B). Effect of temperature on stability of GH3S2 over time.

Effect of pH on GH3S2 activity and pH stability of GH3S2

pH is an important factor affecting BGL activity. In this study, we tested a pH range of 6 different values, from pH 5.0 to 8.0, to choose the optimal pH. The results showed that at pH 6.0, the enzyme had the highest activity. At alkaline pH (8.0) or acidic pH (5.0), the enzyme activity decreased rapidly, accounting for about 20% under the optimal condition. These results are consistent with previous publications. For example, recombinant BGL in *Bacillus licheniformis* had low activity in an acidic environment while its activity decreased dramatically at pH 8.0 (Chen *et al.*, 2017); BGL of *Caulobacter crescentus* (Justo *et al.*, 2015); BGL isolated from soil bacteria (Kim *et al.*, 2007; (Kaur *et al.*, 2007); (Li *et al.*, 2013). Meanwhile, fungi-derived BGLs worked well at pH 5.0, such as *Aspergillus niger* (Wei *et al.*, 2007) and *Sporiobolus pararoseus* (Baffi *et al.*, 2011). When testing the stability of the enzyme with different pH values, the enzyme activity was maintained at about 70% at pH 6.0 after 6 hours and also achieved at pH 7.0 after 4 hours,

then it decreased. The higher the pH, the lower the enzyme activity achieved. The previous experiment by Yin *et al.* (2019) also showed that the enzyme activity of BGL originated in subtropical soil microorganisms was maintained 70% at pH 6.0–7.5.

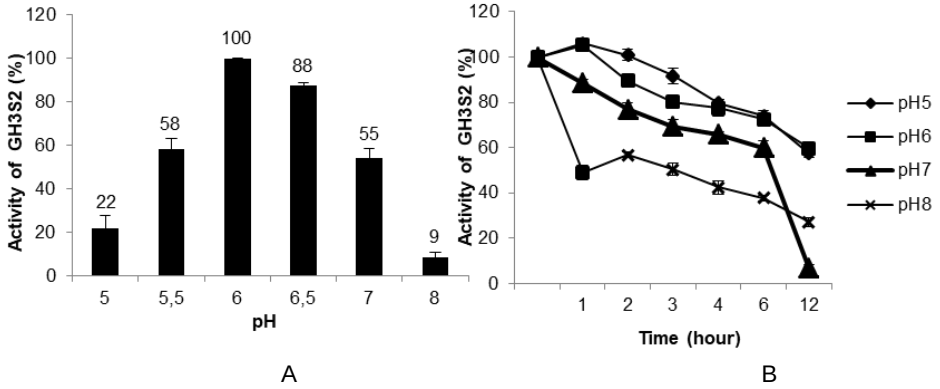

Figure 3. (A). The effect of pH on GH3S2 activity; (B). Effect of pH on the stability of GH3S2 over time

Effect of some metal ions on GH3S2 activity

Metal ions affect the enzymatic activity of BGL in the way of increasing or decreasing the activity that depends on the type of ion. In order to evaluate the influence of metal ions on the GH3S2 activity, we used eight metal ions: K^+, Na^+, Ca^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, with a final concentration of 1 mM, and the reaction determining the enzyme activity was carried out under optimal conditions at 37°C, pH 6.0, for 15 minutes. The results showed that Ca^{2+}, Mg^{2+}, and Mn^{2+} ions helped increase the enzyme activity, in which the Ca^{2+} ion increased the enzyme activity of GH3S2 robustly. If the activity of the sample without adding metal ions is considered as 100%, then when adding Ca^{2+}, Mg^{2+}, and Mn^{2+}, the enzyme activity is 208, 119, and 108%, respectively. In contrast, Fe^{2+}, Ni^{2+}, and Cu^{2+} ions reduced the enzyme activity to 37, 33, and 14%, respectively. GH3S2 activity (Fig. 4A) was not significantly affected by K+ and Na+ ions. These results could be due to the catalytic region of GH3S2 containing a divalent metal ion binding site. Increasing the BGL activity in the presence of Ca^{2+} has also been mentioned in previous studies, such as the activity of BGL derived from subtropical soil microorganisms was increased by 131% (Yin *et al.*, 2019), that of *Streptomyces griseus* was increased by 118% (Uhoraningoga *et al.*, 2019).

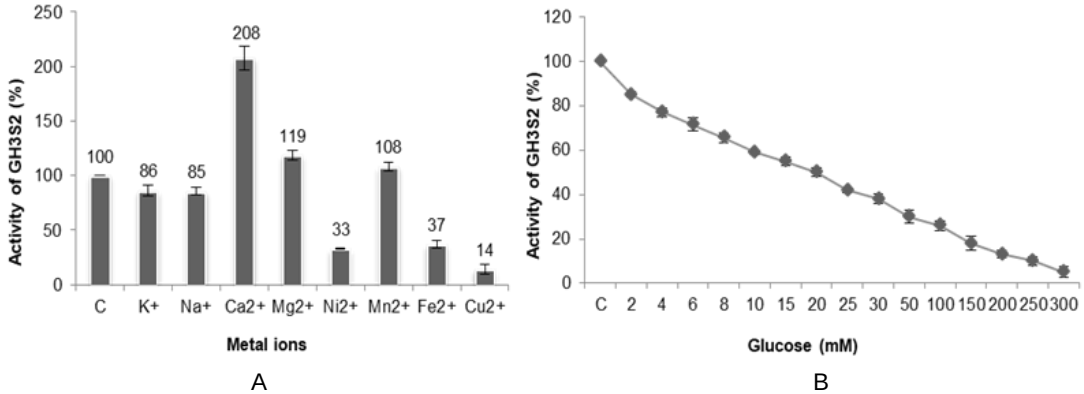

Figure 4. Effect of substances on the enzyme activity of GH3S2. (A) metal ions. (B). glucose. C: control (GH3S2 activity in the absence of substances)

Effect of glucose on GH3S2 activity

BGL is an enzyme that is sensitive to the presence of glucose. Increasing the concentration of glucose makes the enzyme activity decrease (Singhania *et al.*, 2013). BGL, which can tolerate glucose, plays an essential role in the cellulose degradation in the manufacturing industries of paper, wine, and beer. The influence of glucose on the GH3S2 activity was performed at the different glucose concentrations from 2–300 mM. The results showed that the enzyme activity achieved 70% as glucose concentration was 6 mM and was supposedly slightly affected, and then decreased to only 6% when the glucose concentration increased to 300 mM (Fig. 4B). This study showed that glucose inhibited GH3S2 during the process of degrading the *p*NPG substrate. In the previous study by Chen *et al*. (2017), it was indicated that when the glucose concentration was about 34 mM, the BGL enzyme from *B. licheniformis* was completely inactivated.

Kinetic characteristics of GH3S2

The kinetic constants of GH3S2 (K_m, V_{max}) were determined by a range of *p*NPG substrate concentrations from 1–10 mM, with an enzyme amount of 1 µg per reaction. The results showed that the dependence of the reaction rate on *p*NPG concentration follows the equation y = 0.9275x + 0.2037 with confidence R^2 = 0.9953. The K_m and V_{max} values of GH3S2 were calculated using the equation $1/v = K_m.1/V_{max}.1/[S] + 1/V_{max}$, as 4.55 mM and 4.91 µmol/min, respectively. Under these conditions, the enzyme GH3S2 had a specific activity of 2.23 U/mg with the *p*NPG substrate. Thus, the affinity for the substrate of GH3S2 was not high compared with some BGLs from other subjects, such as BGLs from soil bacteria with K_m and V_{max} of 0.16 mM and 19.10 µmol/min (Kim *et al.*, 2007); BGLs from the soil *Exiguobacterium* sp. GXG2 had K_m and V_{max} of 1.1 mM and 12.14 U/mg (Yin *et al.*, 2019). BGL of soil microorganisms around the eucalyptus tree stump had K_m and V_{max} of 0.49 mM and 10.81 U/mg (Gomes-Pepe *et al.*, 2016) and BGLX-V-Ara in *Caulobacter crescentus* represented multifunctional activities and mainly exhibited β-glucosidase with K_m and V_{max} of 0.24 mM and 0.04 U/mg (Justo *et al.*, 2015).

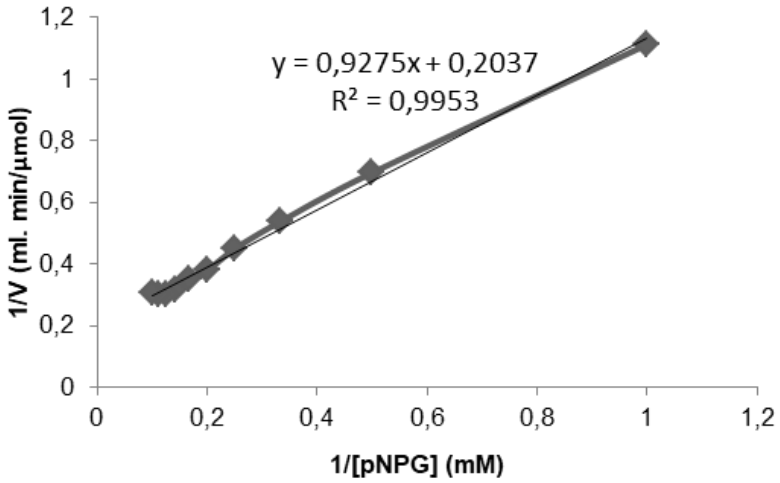

Figure 5. Reaction rate dependence of GH3S2 on pNPG substrate concentration according to Linewever–Burk

CONCLUSION

We purified the GH3S2 enzyme, which originated in the microbiota surrounding white-rot fungi, and expressed it in *E. coli* by affinity chromatography. The enzyme recovered had a purity of more than 95% and a content of 1.54 mg/ml. The enzyme worked best at 37°C in PBS

buffer 50 mM (NaCl-free) pH 6.0. Ca^{2+} and Cu^{2+} ions were two ions that changed the enzyme activity robustly, while glucose reduced the enzyme activity of GH3S2. The K_m and V_{max} of the enzyme were 4.55 mM and 4.91 μmol/min, respectively. The specific activity of the GH3S2 enzyme was 2.23 U/mg.

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REFERENCES

Ahmed A, Nasim F ul-H, Batool K, Bibi A (2017) Microbial β-Glucosidase: Sources, Production and Applications. *J Appl Environ Microbiol* 5(1): 31–46.

Baffi MA, Tobal T, Henrique J, Lago G, Leite RSR (2011) A novel β-glucosidase from *Sporidiobolus pararoseus*: characterization and application in winemaking. *J Food Sci* 76(7).

Chen Z, Meng T, Li Z, Liu P, Wang Y (2017) Characterization of a beta-glucosidase from *Bacillus licheniformis* and its effect on bioflocculant degradation. *AMB Express* 7(1).

Dashtban M, Maki M, Leung KT, Mao C, Qin W (2010) Cellulase activities in biomass conversion: Measurement methods and comparison. *Crit Rev Biotechnol* 30(4): 302–309.

Feng Y, Duan CJ, Pang H, Mo XC, Wu CF (2007) Cloning and identification of novel cellulase genes from uncultured microorganisms in rabbit cecum and characterization of the expressed cellulases. *Appl Microbiol Biotechnol* 75(2): 319–328.

Gomes-Pepe ES, Sierra EGM, Pereira MR, Castellane TCL, De Lemos EGM (2016) Bg10: A novel metagenomics alcohol-tolerant and glucose-stimulated gh1 β-glucosidase suitable for lactose-free milk preparation. *PLoS One* 11(12).

Kaur J, Chadha BS, Kumar BA, Kaur GS, Saini HS (2007) Purification and characterization of ß-glucosidase from *Melanocarpus* sp. MTCC 3922. *Electron J Biotechnol* 10(2): 260–270.

Li H, Xu X, Chen H, Zhang Y, Xu J (2013) Molecular analyses of the functional microbial community in composting by PCR-DGGE targeting the genes of the β-glucosidase. *Bioresour Technol* 134: 51–58.

Li Y, Liu N, Yang H, Zhao F, Yu Y (2014) Cloning and characterization of a new β-Glucosidase from a metagenomic library of Rumen of cattle feeding with *Miscanthus sinensis*. *BMC Biotechnol* 14(1): 1–9.

Liu D, Zhang R, Yang X, Zhang Z, Song S (2012) Characterization of a thermostable β-glucosidase from *Aspergillus fumigatus* Z5, and its functional expression in P*ichia pastoris* X33. *Microb Cell Fact* 11.

Mahapatra S, Vickram AS, Sridharan TB, Parameswari R, Pathy MR (2016) Screening, production, optimization and characterization of β-glucosidase using microbes from shellfish waste. *Biotech* 6(2).

Maki M, Leung KT, Qin W (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int J Biol Sci* 5(5): 500–516.

Justo PI, Corrêa JM, Maller A, Kadowaki MK, da C-S JL(2015) Analysis of the xynB5 gene encoding a multifunctional GH3-BglX β-glucosidase-β-xylosidase-α-arabinosidase member in *Caulobacter crescentus*. *Antonie Van Leeuwenhoek* 108(4): 993–1007

Price NC (1985) The determination of Km values from lineweaver-burk plots. *Biochem Educ* 13(2): 81–81.

Singhania RR, Patel AK, Sukumaran RK, Larroche C, Pandey A (2013) Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresour Technol* 127: 500–507.

Kim SJ, Lee CM, Kim MY , Yeo YS, Yoon SH (2007) Screening and characterization of an enzyme with beta-glucosidase activity from environmental DNA. *J Microbiol Biotechnol* 17(6): 905–12.

Watanabe T, Sato T, Yoshioka S, Koshijima T, Kuwahara M (1992) Purification and properties of *Aspergillus niger* beta-glucosidase. *Eur J Biochem* 209(2): 651–659.

Uhoraningoga A, Kinsella GK, Frias JM, Ryan BJ,

Henehan GT (2019) The Statistical Optimisation of Recombinant β-glucosidase Production through a Two-Stage, Multi-Model, Design of Experiments Approach. *Bioeng* 6(3): 61.

Wei S, Semel Y, Bravdo BA, Czosnek H, Shoseyov O (2007) Expression and subcellular compartmentation of *Aspergillus niger* β-glucosidase in transgenic tobacco result in an increased insecticidal activity on whiteflies (Bemisia tabaci). *Plant Sci* 172(6): 1175–81.

Feng Y, Duan CJ, Pang H, Mo XC, Wu CF (2007) Cloning and identification of novel cellulase genes from uncultured microorganisms in rabbit cecum and characterization of the expressed cellulases. *Appl Microbiol Biotechnol* 75(2): 319–328.

Zhao Y, Liu WF, Mao AJ, Jiang N, Dong ZY (2004) Expression, purification and enzymatic characterization of Bacillus polymyxa beta-glucosidase gene (bglA) in *Escherichia coli*. *Sheng Wu Gong Cheng Xue Bao* 20(5): 741–44.

Yapi DYA, Gnakri D, Niamke SL, Kouame LP (2009) Purification and biochemical characterization of a specific β"- glucosidase from the digestive fluid of larvae of the palm weevil, *Rhynchophorus palmarum*. *J Insect Sci* 9.

Yin B, Gu H, Mo X, Xu Y, Yan B (2019) Identification and molecular characterization of a psychrophilic GH1 β-glucosidase from the subtropical soil microorganism *Exiguobacterium* sp. GXG2. *AMB Express* 9(1).

Zhang L, Fu Q, Li W, Wang B, Yin X (2017) Identification and characterization of a novel β-glucosidase via metagenomic analysis of *Bursaphelenchus xylophilus* and its microbial flora. *Sci Rep* 7(1).