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Purification and characterization of a highly glucose tolerant β-glucosidase isozyme from *Paecilomyces variotii* MG3.

Purificación y caracterización de una isoenzima β-glucosidasa altamente tolerante a la glucosa de Paecilomyces variotii MG3

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ABSTRACT

Isolation and purification of a highly glucose tolerant β -glucosidase by a novel isolate *Paecilomyces variotii* MG3 was achieved through a three steps purification strategy. The purification steps used were ammonium sulphate fractionation, column chromatography using Sephacryl S 300 HR and anion exchange chromatography using DEAE-cellulose. The fungus produced two isozymes of β –glucosidase. Vmax values of isozyme I and II were 0.2497U/ml and 0.3519U/ml respectively. The Km value of isozyme I was 0.08839mM and that of isozyme II was 0.2710mM. Isozyme I exhibited a Ki of 901.6mM and was found to be insensitive to glucose concentration as high as 2M. The isozymes were tolerant to ethyl alcohol concentration up to 30% and displayed synergism with commercially available cellulase in cellulose degradation.

Keywords:- β-glucosidase; *Paecilomyces variotii*; glucose tolerant isozyme; ethyl alcohol tolerant

RESUMEN

El aislamiento y la purificación de una β-glucosidasa altamente tolerante a la glucosa mediante un nuevo aislamiento de *Paecilomyces variotii* MG3 se logró a través de una estrategia de purificación de tres pasos. Los pasos de purificación utilizados fueron fraccionamiento con sulfato amónico, cromatografía en columna con Sephacryl S 300 HR y cromatografía de intercambio aniónico con DEAE-celulosa. El hongo produjo dos isoenzimas de β-glucosidasa. Los valores de Vmax de las isoenzimas I y II fueron 0,2497U/ml y 0,3519U/ml respectivamente. El valor Km de la isozima I fue de 0,08839 mM y el de la isozima II fue de 0,2710 mM. La isozima I mostró una Ki de 901,6 mM y se encontró que era insensible a una concentración de glucosa de hasta 2 M. Las isoenzimas fueron tolerantes a una concentración de alcohol etílico de hasta el 30 % y mostraron sinergismo con la celulasa disponible comercialmente en la degradación de la celulosa.

Palabras clave:- β-glucosidasa; *Paecilomyces variotii;* isoenzima tolerante a la glucosa; tolerante al alcohol etílico

INTRODUCTION

Hydrolysis of cellulose to glucose is the most important process in bio-ethanol production and enzymatic hydrolysis is the most preferred method of cellulose degradation as it requires less energy and mild environmental conditions (Hong *et al.,* 2009; Rosales-Calderon *et al.,* 2014, Ahamed *et al.,* 2017). Cellulose degradation requires synergetic action of three major groups of enzymes. 1,4-β-D glucan-4-glucanohydrolases or endoglucanases act on the amorphous regions of cellulose and generates oligosaccharides of various lengths and consequently new chain ends. 1,4-β-D glucan glucanohydrolases or exoglucanases act on the reducing or non-reducing ends of cellulose chains and liberate either glucose or cellobiose as major products. β-glucoside glucohydrolases or β-glucosidase cleaves the cellobiose into glucose (Jørgensen *et al.,* 2007; Annette *et al.,* 2013). β-glucosidase is generally responsible for the regulation of the total cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose (Woodward and Wiseman, 1982

β-glucosidases occur universally in plants, animals, fungi and bacteria (Esen, 1993) and have been purified from various sources. But most of the reported β-glucosidases are inhibited by glucose. This limits their application in commercial scale cellulose degradation ventures (Gunata and Vallier 1999; Riou *et al.,* 1998). Therefore the search for β-glucosidases insensitive to glucose inhibition has increased recently and enzymes with this characteristic would improve the process of saccharification of lignocellulosic materials (Bhatia *et al.,* 2002). In this paper we report the presence of a highly glucose tolerant β-glucosidase isozyme from *Paecilomyces variotii* MG3.

Other applications attributed to β-glucosidases include (1) synthesis of glucosides and fucosides with various applications in pharmaceutical, cosmetic and detergent industries (Ducret *et al.,* 2006; Turner *et al.,* 2007; Zhang *et al.,* 2007), (2) hydrolytic removal of aglycone moiety from flavanoid and isoflavanoid glycosides (Briante *et al.,* 2000; Ribeiro *et al.,* 2007), (3) flavor improvement of fruit juices and wine through the release of aroma rich compounds from natural non-odorous and nonvolatile glycoside precursors (Gueguen *et al.,* 1997), and (4) biosynthesis of oligosaccharides (Bruins *et al.,* 2003).

MATERIAL AND METHODS

Microorganisms: In the present investigation, *P. variotii* MG3, a fungal isolate from Vaghmon grassland soil, Kerala, India, was used. The organism was maintained on potato-dextrose-agar (PDA) slants at 4^0C and sub cultured every 4 weeks (Job *et al.,* 2010).

Inoculum preparation: The spores were scraped down from a four day-old PDA slant of *P.variotii* with 5ml physiological saline containing 0.1% (w/v) Tween 80 using a sterile inoculation loop under aseptic conditions (Lee and Chen, 1997). 1ml of the spore suspension prepared in this manner contained approximately 3 X 10^6 spores.

Production of β-glucosidase: The enzyme was produced through solid state fermentation (ssf) using wheat bran as the solid matrix. The medium used for the production of β-glucosidase, inoculum concentration and incubation period were optimized by applying a two step statistical approach; Plackett–Burman design followed by Box–Behnken design (Job *et al.,* 2010).The optimized production medium contained (g/L) $(NH_4)_2SO_4$ 1.4; KH_2PO_4 2; CaCl 0.3; $MgSO_4.7H_2O$ 0.3; peptone 2; cellobiose 2.5 and (mg/L) $FeSO_4.7H_2O$ 5; $MnSO_4.7H_2O$ 1.6; $ZnSO_4.7H_2O$ 1.4 and $CoCl_2$ 2. pH of the medium was adjusted to 7 with 0.1N NaOH. An inoculum concentration of 1.2 X 10^6 spores/ml and an incubation period of 96 h were used for the production of the enzyme. 10 g of wheat bran taken in 250ml Erlenmeyer flasks were moistened with the production medium to attain a final moisture content of 80% (w/v) after inoculation. The contents were mixed thoroughly and autoclaved at 121^0C for 20 min.

β-glucosidase assay: β-glucosidase was assayed by using a reaction mixture containing 900µl 1mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) in 100mM citrate-phosphate buffer (pH 6) and 100µl appropriately diluted enzyme (Martino *et al.,* 1994). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µM of *p*-nitrophenol /min into the reaction mixture under the above assay conditions.

The amount of glucose released from cellobiose as a result of the activity of β-glucosidase was assayed by using the glucose oxidase–peroxidase-*o*-dianisidine (GOPOD) system (Bergmeyer *et al.,* 1974). Protein content was determined according to the method of Lowry *et al.,* (1951) as milligram protein per milliliter of sample (mg/ml).

Extraction and purification of the enzyme: After 96 h of incubation, 25ml, 50mM citrate-phosphate buffer (pH 6) was added to the culture, mixed thoroughly and the slurry was squeezed through two layers of cheesecloth. The filtrate was centrifuged at 10000 rpm for 20 min at 4^oC. The supernatant obtained was used as the crude enzyme extract.

β-glucosidase was purified from the crude enzyme extract prepared through solid state fermentation using ammonium sulphate fractionation, column chromatography using Sephacryl S 300 HR (prequilibrated with 0.1M citrate phosphate buffer, pH 6) and anion exchange chromatography using DEAE-cellulose (prequilibrated with 0.1M citrate phosphate buffer, pH 5, eluted with a linear gradient of 0 to 0.6M NaCl in 0.1M citrate-phosphate buffer, pH 5). For concentrating the active fractions at various steps of the purification protocol Amicon Ultra–4 50K centrifugal filter device (Millipore) was used.

Polyacrylamide Gel Electrophoresis (PAGE): Electrophoresis under native conditions (non-denaturing PAGE) was performed on polyacrylamide gels having a resolving gel composition of 10% and stacking gel composition of 4% according to Pan *et al.,* (1989).

Electrophoresis under denaturing conditions (SDS-PAGE) was performed according to Laemmli (1970) at 10% polyacrylamide gel concentration. Protein band pattern was visualized by staining with Coomassie Brilliant Blue.

Activity staining: Activity staining of β-glucosidases in the slab gel was done with 1mM 4-methylumbelliferyl-β-D-glucopyranoside (MUG) (Hu *et al.,* 2007). After the completion of electrophoresis on a 10% non-denaturing PAGE, the slab gel was washed three times with 0.1M citrate-phosphate buffer (pH 6). It was then incubated at room temperature (28 ± 2^0C) for 10 min in a solution containing 1mM MUG in 0.1M citrate-phosphate buffer (pH 6), and the β- glucosidase bands were visualized under UV.

Determination of Km ,Vmax and Ki: The Michaelis-Menten constant (*Km*) and the maximal reaction velocity (*Vmax*) were determined for both the isoenzymes of β-glucosidases by using the reaction system with different concentrations of *p*NPG ranging from 0.05 to 0.75mM. The reaction mixtures with enzyme were incubated for 20 min at 50^0C. The reaction was stopped after 20 min and the amount of *p*-nitrophenol released was measured. The data obtained were utilized for the determination of *Km* and *Vmax.*

The inhibitory effect of glucose on the enzyme activity was measured by adding various concentrations of glucose (0.5, 1, 1.5, 2M) into the reaction mixture with 1mM pNPG as substrate and performing the enzyme assay as detailed above.

The inhibitory effect of glucose on the enzyme activity was also monitored using electrophoresis under native conditions. Electrophoresis was performed on polyacrylamide gels having a resolving gel composition of 10%. Activity staining of β-glucosidase in the slab gel was done with 1mM MUG. After the completion of electrophoresis the slab gel was washed three times with 0.1M citrate-phosphate buffer (pH6). It was then incubated at room temperature (28 ± 2^0C) for 10 min in a solution containing different concentrations of glucose and 1mM MUG in 0.1M citrate-phosphate buffer (pH6) and the β-glucosidase bands were visualized under UV.

The inhibition constant (*Ki*) due to the effect of glucose on the enzyme activity was determined by measuring the enzyme activity in presence of various concentrations of glucose (200-1000mM) in reaction mixtures with different concentrations of *p*NPG (0.2-0.45 mM) as substrate. The data obtained were utilized to determine the *Ki* .

Km, *Vmax* and *Ki* were determined using nonlinear regression and curve fitting functions of the software GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Determination of the effect of alcohol on enzyme activity: The effect of ethyl alcohol on the enzyme activity was measured by adding various concentrations of ethyl alcohol (3-30%) into the reaction mixture with

1mM *p*NPG as substrate and was expressed as residual activity. Reaction mixture without ethyl alcohol was used as a control.

Testing of synergism with cellulase in cellulose hydrolysis: The effect of supplementation of purified β-glucosidase on microcrystalline cellulose (Sigma; 2% w/v) hydrolysis at Ph 5 and 50^0C by cellulase (SRL, India) was examined by comparing the amount of glucose released in the absence and presence of the β-glucosidase enzyme. A reaction mixture containing 10ml, 2% (w/v) microcrystalline cellulose suspension in 0.1M citrate-phosphate (pH 5) buffer and 1ml cellulase enzyme (5U/ml) was mixed with 1 ml (~ 3U) β-glucosidase enzyme (isozyme I). The same mixture with 1 ml buffer instead of β-glucosidase was used for comparison. The reaction mixtures were incubated at 50^oC for 24 h with occasional shaking. After the incubation samples were taken and the amount of glucose released was determined using the GOPOD method.

RESULTS

Paecilomyces variotii MG3 isolated from the soil of Vagmon grasslands, Kerala was used in the present study. The strain was reported earlier as an efficient producer of the enzyme under ssf conditions (*Job et al.,* 2010)

Purification of β-glucosidases

Ammonium sulphate fractionation resulted in the formation of two peaks one at 40% and the other one from 60 to 80% ammonium sulphate concentrations (hereafter called AS40 and AS 60-80 respectively). The concentrated protein solutions were purified further using Sephacryl S 300HR columns. Pooled fractions obtained from column chromatography of AS 40 and AS 60-80 when subjected to native PAGE and activity staining using MUG showed single bands. This indicated the presence of one isozyme each in each pooled fraction (fig. 1). Hence for further discussions the pooled fractions obtained from AS 40 and AS 60-80 were named as isozyme I and II respectively.

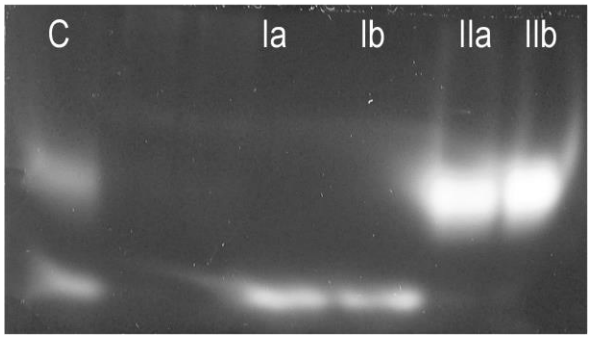

Fig. 1. Native gel after activity staining using 1mM MUG (C-crude extract, I – Isozyme I, II – Isozyme II, a – after column chromatography, b – after anion exchange chromatography)

Isozyme I and II were further purified using DEAE-cellulose. The β-glucosidase from AS 40 was eluted at a salt concentration of 0.5M, while that from AS 60-80 was eluted at 0.3M NaCl. The purified fractions were then subjected to SDS-PAGE analysis (fig 2). Both the fractions indicated the presence of single band when stained with Coomassie brilliant blue. Summary of the purification strategy used and the fold purification attained were given in table 1.

The molecular weight of the native β-glucosidases estimated by SDS-PAGE using molecular weight markers revealed that the molecular weight of Isozyme I and II were approximately 40kDa and 90 kDa respectively (fig 2). SDS-PAGE analysis also showed that both the isozymes were monomers.

Table 1. Purification profile of β-glucosidase

Fraction	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude Extract	2435	15000	6.2	1	100
$(NH4)_2SO_4$					
AS 40	472	1690	3.6	0.6	11.27
AS 60-80	713	8860	12.4	2	59.1
Sephacryl-S 300HR					
Isozyme I	165	1375	8.33	1.3	9.2
Isozyme II	352	7950	22.6	3.7	53
DEAE-Cellulose					
Isozyme I	115	1285	11.2	1.8	8.6
Isozyme II	249	7255	29	4.7	48.4

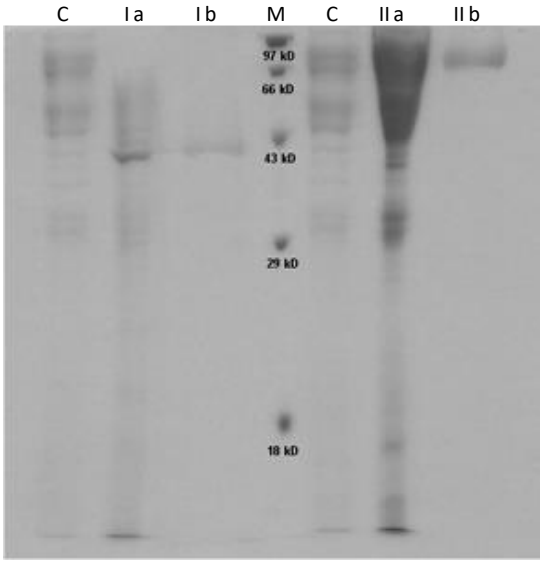

Fig. 2. SDS-gel after Coomassie brilliant blue staining. (I- isozyme I, II- isozyme II, C- crude extract, a- after ammonium sulphate fractionation, b- after gel filtration and anion exchange chromatography, M- molecular weight markers)

The Michaelis-Menten constant (*Km*) and the maximal reaction velocity (*Vmax*) were determined for both the isozymes of β-glucosidases by using the software, GraphPad Prism version 5.03. Nonlinear regression curves were fitted to the data obtained with Isozyme I and II and varying substrate concentrations. *Vmax* and *Km* values of both the isozymes were obtained from the best fit values of the models (tables 2 and 3). As per the model the *Vmax* values of isozyme I and II were 0.2497U/ml and 0.3519 U/ml respectively. The *Km* value of isozyme I was 0.08839mM and that of isozyme II was 0.2710 mM.

Table 2. Best-fit values of the model's parameters. (Nonlinear regression model is fitted to the data obtained by using isozyme I with increasing concentrations of the substrate)

Analysis type - Michaelis-Menten	
Best-fit values	
Vmax	0.2497
Km	0.08839
Std. Error	
Vmax	0.008289
Km	0.01045
95%Confidence Intervals	
Vmax	0.2306 to 0.2688
Km	0.06428 to 0.1125
Goodness of Fit	
Degrees of Freedom	8
R square	0.9912

Table 3. Best-fit values of the model's parameters. (Nonlinear regression model is fitted to the data obtained by using isozyme II with increasing concentrations of the substrate)

Analysis Type - Michaelis-Menten	
Best-fit values	
Vmax	0.3519
Km	0.271
Std. Error	
Vmax	0.005867
Km	0.01164
95%Confidence Intervals	
Vmax	0.3393 to 0.3645
Km	0.2461 to 0.2960
Goodness of Fit	
Degrees of Freedom	14
R square	0.9979

Effect of different concentrations of glucose on the activity of β-glucosidase and determination of: the effect of increasing concentrations of glucose on the enzyme activity was measured by adding various concentrations of glucose (0.5, 1, 1.5, 2M) into the reaction mixture with 1mM pNPG as substrate. The relative β-glucosidase activity was determined with respect to the control reaction mixture without glucose. As evident from table 4, 0.5 to 2M glucose showed no inhibitory effect on isozymes I. But isozyme II was completely inhibited by glucose concentrations 1M and above. This was further confirmed by activity staining using 1mM MUG (fig. 3).

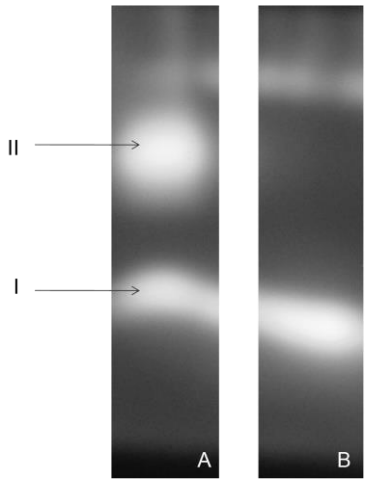

Fig.3. Native gel after activity staining using 1mM MUG (A-gel incubated in reaction mixture without glucose, B-gel incubated in reaction mixture with 2M glucose, I – Isozyme I, II – Isozyme II)

Nonlinear regression curves were fitted to the data obtained with Isozyme I and II and varying concentrations of glucose. Inhibition constant (*Ki*) of both the isozymes were obtained from the best fit values of the models. As per the model the *Ki* values of isozyme I and II were 901.6 mM and 22.65 mM respectively.

Effect of varying concentrations of ethyl alcohol on the activity of β-glucosidase: As evident from the present study β-glucosidase produced by *P. variotii* was insensitive to ethyl alcohol at all the concentration tried (table 5) and lower concentrations of alcohol (3 and 6%) even slightly stimulated the enzyme activity. The activity of both the isoenzymes decreased progressively beyond 30% concentration of ethyl alcohol.

Synergism with cellulase: The ability of β-glucosidase and cellulase to act in combination to degrade microcrystalline cellulose was analyzed. A mixture of isozyme I and cellulase showed a high degree of cooperation in solubilising microcrystalline cellulose (table 6). The mixture of enzymes could almost double the amount of glucose released.

Table 4. The effect of glucose on the activity of isozymes I and II

	Residual Activity (%)	
Alcohol (%)	Isozyme I	Isozyme II
3	106.0 ± 2.5	104.0 ± 1.9
6	105.4 ± 2.2	103.1 ± 2.1
9	100.0 ± 3.1	100.0 ± 2.9
12	100.0 ± 2.9	100.0 ± 3.4
15	100.0 ± 1.8	99.3 ± 3.6
18	99.8 ± 2.7	98.9 ± 2.0
21	99.7 ± 3.4	98.2 ± 3.9
24	99.5 ± 2.9	97.8 ± 3.1
27	99.5 ± 3.5	97.8 ± 0.1
30	99.5 ± 2.9	97.8 ± 3.5

Table 5. Effect of varying concentrations of ethyl alcohol on the activity of β-glucosidase

	Relative activity (%)	
Glucose(M)	Isozyme I	Isozyme II
0.5	100.00	85.2 ± 0 .9
1.0	99.92 ± 0.07	0
1.5	99.90 ± 0.10	0
2.0	99.93 ± 0.06	0

DISCUSSION

Paecilomyces spp. were reported from a wide range of habitats, substrates and materials. It is commonly found in the air and soil in the tropics and has been utilized in the industrial process known as 'Pekilo' to produce microbial protein (Cabib et al., 1983). Besides a few reports regarding the production of tannase (Battestin and Macedo, 2007) and xylanase (Kumar et al., 2000; Li et al., 2006; Yang et al., 2006) from the genus Paecilomyces, these fungi have received only a little attention in enzyme production despite their abundance. In the present study, during the secondary screening using minimal medium itself P.variotii strain MG3 produced approximately double the amount of β-glucosidase produced by the Aspergillus sp. and Penicillium sp. isolated.

Through a three step purification strategy we could isolate two isozymes, with molecular weight 40 and 90 kDa, from the crude extract. Both the isozymes were monomeric. Most fungal β-glucosidases reported are either

monomers or dimers of a reduced molecular weight between 43 and 131 kDa. β-glucosidases with high molecular weight reported in the literature are those of Botrytis cinerea, Monilia sp., Aureobasidium pullulans and Trichoderma longibrachiatum which have values of 350, 480, 340 and 350 kDa, respectively (Mamma et al., 2004). The isozymes purified in the present study belong to the larger category of β-glucosidases with low molecular weight.

β-glucosidase form different source exhibits a wide range of variation in the case of Km. In the present study isozyme I has a low Km compared to that of isozyme II. Low Km value is a reflection of a higher specificity towards a substrate. The remarkable resistance exhibited by the isozyme I towards glucose inhibition may be due to the high specificity it owes towards its substrate.

Competitive inhibition by glucose is a common characteristic of fungal β-glucosidases although there are exceptions like β-glucosidases produced by several Aspergillus species (Mamma et al., 2004). Most of the microbial β-glucosidases exhibit glucose inhibition constant (Ki) values ranging from 0.5 to no more than 100mM (Zanoelo et al., 2004). This limits their application in commercial scale cellulose degradation venture. Therefore the search for β-glucosidases insensitive to glucose has increased recently. The β-glucosidases isozyme I from P. variotii possessed an unusually high Ki of 901.6mM. The high glucose tolerance of the β-glucosidase produced by this fungal strain projects it as a potent candidate for various biotechnological applications especially in the processes of saccharification of lignocellulosic materials.

The effect of ethyl alcohol on the activity of the isozymes was also studied and it was found that both the isozymes were highly ethanol tolerant. Among all processes for bioethanol production from lignocellulosic biomass, simultaneous saccharification and fermentation appears as a promising alternative. In this process, the enzymatic hydrolysis of cellulose and the fermentation of monomeric sugars are performed in one single step. Since the sugar produced through the saccharification of lignocellulosic, material is meant for a simultaneous conversion to ethyl alcohol, use of an alcohol insensitive β-glucoisidase will be of added advantage. The ethyl alcohol tolerance of the P.variotii β-glucoisidases make them suitable candidates for simultaneous saccharification and fermentation.

It was also found that isozyme I when mixed with commercial cellulase preparation could increase the yield of glucose from cellulose. Synergism between cellulase components during the hydrolysis of cellulose was first demonstrated by Giligan and Reese (1954). Subsequently several researchers showed the cooperative action between endo-, exo-giucanases during the solubilization of crystalline cellulose and the release of various higher cello-oligosaccharides and cellobiose (Mandels and Reese, 1964; Selby and Maitland, 1967; Wood and MeCrae, 1972). The β-glucosidase completes the hydrolysis by cleaving the cello-oligosaccharides and cellobiose to glucose (Coughlan, 1985). β-glucosidase is generally responsible for the regulation of the entire cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose, since both endoglucanse and exoglucanase activities are often inhibited by cellobiose (Woodward and Wiseman, 1982). β-glucosidase not only produce glucose from cellobiose but also reduces cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently(Rani et al.,2014). The results of the present study on the synergetic action of cellulases and β-glucosidase are consistent

with the above mentioned facts. Our result showed that the P.variotii β-glucosidase can be supplemented to commercially available cellulase preparations to increase the rate of cellulose digestion.

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