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PURIFICATION AND CHARACTERIZATION OF β-GLUCOSIDASE PRODUCED BY *PAECILOMYCES SP*

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ABSTRACT

β-glucosidase (EC 3.2.1.21) produced by *Paecilomyces* sp. in a medium containing 5% ($^{w}/_{v}$) corn cob has been purified 11.7 folds through a three step procedure involving acetone precipitation, ion exchange chromatography on DEAE-Sephadex and gel filtration on Sephadex-G-200. The relative molecular weight was estimated to be 55,000 Daltons. The enzyme had a narrow acidic pH stability range and was maximally active at pH 5.0. It was optimally active at 60°C, had a half-life of 81min at 70°C and retained 100% of its original activity at 50°C for 2 h. It was completely inhibited by Zn^{2+}, EDTA, glycine and mercuric chloride but was stimulated by Ba^{2+}, Ca^{2+} and Co^{2+}. The Michaelis constant (*km*) of the enzyme for salicin was 25 mg/ml.

KEY WORDS: β-glucosidase, corn cob, gel filtration, half life, ion exchange chromatography, *Paecilomyces* sp.

INTRODUCTION

β -glucosidase (EC 3.2.1.21) is one of the three enzymes of the cellulase complex, which act synergistically to effect cellulose hydrolysis (Lynd *et al.*, 2002). Since cellulose is the world's most abundant naturally occurring renewable resource, its hydrolysis would help alleviate the shortage of food and feeds, solve waste disposal problems and diminish enormous dependence on fossil fuels by providing a convenient and renewable source of energy (Kumakura, 1997). Much attention has been focused on β -glucosidase because of its central role in the hydrolysis. It acts on cellobiose, which at high levels inhibits other enzymes of the cellulase complex. Despite its importance, extracellular levels of the enzymes in microbial culture filtrates, especially of *Trichoderma reesei*, an acknowledged cellulase producer are too low to be of practical use (Lynd *et al.*, 2002). Furthermore during a fermentation reaction, large quantities of the enzyme would be lost through inactivation at unfavorable conditions. Part of the solution to these problems include the isolation of organisms that produce increased extracellular levels of the enzyme and supplementation with exogenously added purified and characterized β-glucosidase in immobilized form.

Numerous studies have been carried out on the enhancement of β-glucosidase production and the characterization of the enzyme from different microorganisms (Nogawa *et al.* 2001; Pardo *et al* 2000; Lin *et al.*1999). This paper describes the purification and characterization of β-glucosidase from *Paecilomyces* sp., a soil fungus. The characterisation of the endo-B-glucanase enzyme produced by the same organism has been previously reported (Okolo *et al.*, 1998).

MATERIALS AND METHODS

Microorganism: *Paecilomyces* sp. was isolated from a Nigerian soil and identified by the mycology laboratory of the University of North Carolina, USA. Cultures were maintained on potato dextrose agar (PDA) slants and stored at 4°C.

Enzyme production system: The fermentation medium was modified from that developed by Mandels and Weber (1969) and consisted of the following (g/l): $(NH_4)_2SO_4$, 1.0; Corn cob, 50.0; KH_2PO_4, 2.0; CaCl, 0.3; $MnSO_4.7H_2O$, 0.3; and trace metal salts (mg/l); $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.56; $ZnSO_4.7H_2O$, 1.4; CoCl, 2.0; pH 6.0. Triton–X–100 was added to give a final concentration of 0.2% ($^{v}/_{v}$). Four agar plugs (1.6cm diameter) of a 96h PDA culture of *Paecilomyces* sp. grown at 35°C were inoculated into 250 ml Erlenmeyer flasks each containing 50 ml medium. The flasks were incubated in an orbital shaker (150 rpm, 28 ± 2°C) for 6 days. Thereafter, the medium was centrifuged (3000g, 15min, 4°C) and the clear supernatant used as the enzyme source.

Assay procedures: β-glucosidase activities were measured in a 2 ml reaction mixture containing 1 ml each of culture supernatant and 1.0 % ($^{w}/_{v}$) salicin (Dickson & Co.) in 0.2M sodium acetate buffer, pH 5.0. Reducing sugar released was determined after 30min incubation at 50°C by the 3, 5-dinitrosalicylic acid (DNS) method (Bernfeld, 1955). One unit of β-glucosidase activity is defined as the amount of enzyme required to release one micromole of reducing sugar expressed as glucose per minute under the assay conditions. Protein was estimated according to the method of Lowry *et al.*, (1951) using bovine serum albumin as standard.

Enzyme purification: Two volumes of acetone (-10°C) were added drop-wise with stirring to the culture supernatant (200ml) maintained at 4°C in a cooled incubator. The precipitate recovered after 12h by centrifugation (3000g, 15min, 4°C) was resuspended in 30ml of 0.2M sodium acetate buffer, pH 5.0 (hereafter referred to as the buffer). β-glucosidase activity and protein were assayed. The solution was applied on a pre-equilibrated DEAE - Sephadex A-50 column (2 x 24.5cm). Proteins were eluted with the buffer and linear gradients of NaCl (0-0.5M) at a flow rate of 40 ml/h. Thirty-five fractions of 10ml portions were collected. Fractions 3-7 with high β-glucosidase activity were pooled, concentrated by acetone precipitation and resuspended in 12ml of the buffer. β-glucosidase activity and protein were assayed. The enzyme solution was loaded onto a Sephadex G-200 column (1 x 35.5cm) pre-equilibrated with the buffer. Thirty fractions of 10ml portions were collected. Fractions 3-8 which showed high activities of β-glucosidase were pooled and concentrated by acetone precipitation as described above to 10ml volume in the buffer.

Estimation of molecular weight: The method of Andrews (1964) was used on a Sephadex G-200 column (1 x 35.5cm) equilibrated with the buffer. The standard proteins used with their molecular weights (Da) were lysozyme (14,000); trypsin (23,000); egg albumen (42,000) and bovine serum albumen (68,000).

pH activity and stability profiles: The pH activity profile was determined in a reaction mixture consisting of 0.05 ml each of the enzyme solution and 1% ($^{w}/_{v}$) salicin prepared in buffers of

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Table 1: Purification summary of β-glucosidase from *Paecilomyces* sp.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification
Culture supernatant	1976	817.3	2.4	100	1
Acetone precipitate	738.6	68.8	10.7	37.4	4.5
DEAE-Sephadex	105.8	7.1	14.9	5.4	6.2
Sephadex G-200	36.4	1.3	28	1.8	11.7

Table 2: Effects of metal ions and enzyme modulators on β-glucosidase activity.

Metal ions			Enzyme modulators		
Metal ion (10^{-3}M)	Specific activity (U/mg protein)	Relative activity (%)	Enzyme modulator (mM)	Specific activity (U/mg protein)	Relative activity (%)
Control	28.15	100	Control	14.62	100
Ba^{2+}	38.38	140	EDTA[a] (50)	0.00	0
Ca^{2+}	35.08	124	Glycerol (50)	13069	94
Co^{2+}	42.31	186	Glycine (50)	0.00	0
Cu^{2+}	6.77	24	$HgCl_2$ (0.1)	0.00	0
K^{+}	23.54	84	Sodium azide (5)	14.15	97
Mg^{2+}	28.08	99			
Zn^{2+}	0.00	0			

[a]*EDTA: Ethylene diamine-tetra acetic acid.*

different pH values. The reaction was terminated by the addition of 3,5-DNS and enzymes activities determined as described. For the pH stability profile, equal volumes of the enzyme and the respective buffers were incubated for 2h at 50°C. The reaction was terminated by the addition of 3,5-DNS and enzyme activities determined as described above.

Temperature activity and stability profiles: Temperature activity profile was determined by incubating 0.05 ml each of the enzyme solution and 1% ($^w/_v$) salicin in 0.2M sodium acetate buffer, pH 5.0 for 30min at various temperatures (30-100°C). The reactions were terminated by the addition of 3,5-DNS and enzyme activities determined as described previously. Thermal stabilities at 50°C, 60°C and 70°C were studied by incubating the enzyme in thin walled test tubes at the various temperatures and withdrawing 0.05 ml of enzyme at each temperature at 30min interval for 3h. Withdrawn samples were cooled to room temperature (28 ± 2°C) and residual enzyme activities determined by the addition of 0.05 ml of 1%($^w/_v$) salicin in the buffer and incubating at 50°C for 30min. Reaction were stopped by the addition of 3,5-DNS and enzyme activity determined.

Effects of metal ions: Various metal salts (Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, K^{+}, Mg^{2+} and Zn^{2+}) at a concentration of 1mM were examined for their effects on enzyme activity. The reaction mixture consisted of 0.05ml each of enzyme solution, metal solution and 1% ($^w/_v$) salicin in the buffer, incubated at 50°C for 30min. Reactions were stopped by addition of 3,5-DNS and enzyme activities determined as described above. Results were compared with a control treated in the same manner as the test but which contained the buffer in place of the metal solution.

Effects of enzyme modulators: The effects of some enzyme

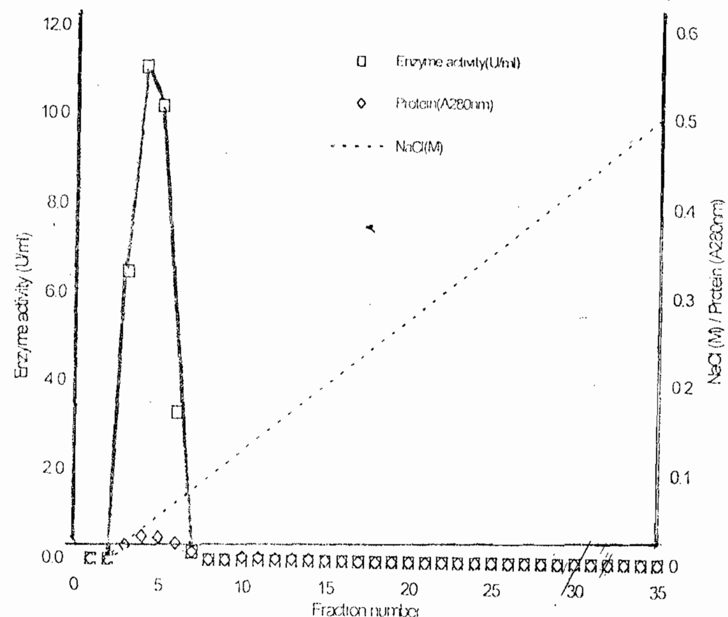

Fig. 1: Elution profile of β-glucosidase on DEAE-Sephadex column

modulators on β-glucosidase activity were examined at various concentrations (Table 2). Reaction mixtures consisted of 0.05ml each of enzyme solution and a solution of each modulator made in the buffer kept at room temperature (28 ± 2°C) for 30min. Thereafter 0.05ml of 1% ($^w/_v$) salicin was added and the mixture incubated at 50°C for 30min. Reactions

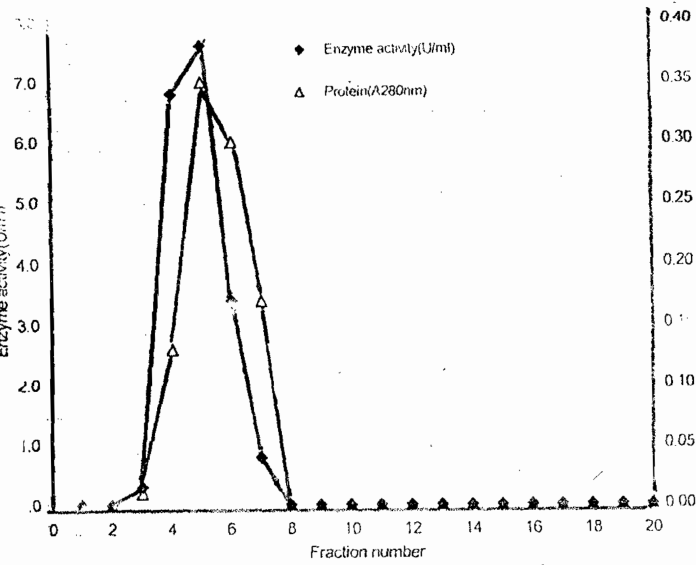

Fig.2: Elution profile of β-glucosidase on Sephadex G-200

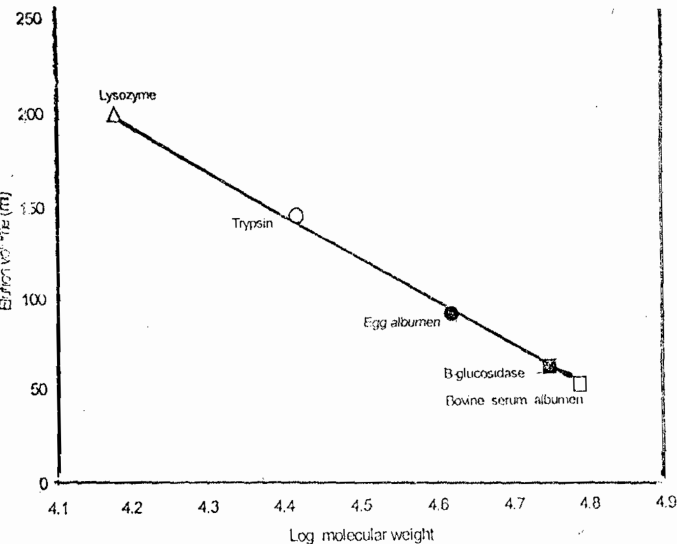

Fig. 3: Estimation of molecular weight of *Paecilomyces* sp β-glucosidase by gel filteration on Sephadex G-200

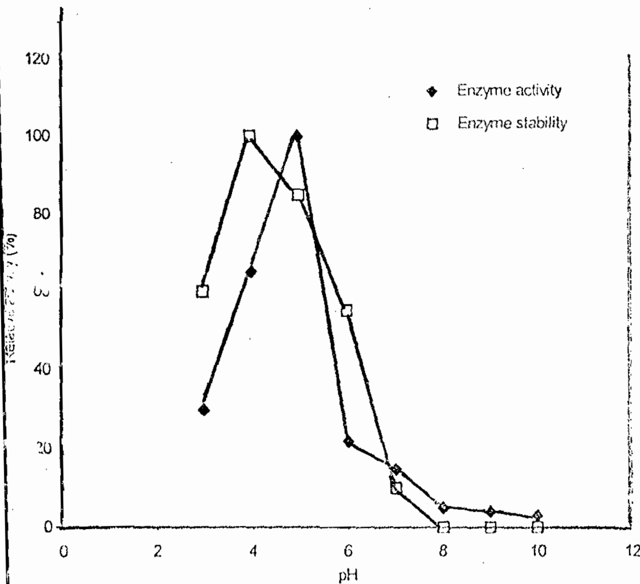

Fig.4: pH activity and stability profiles of β-glucosidase

were stopped by the addition of 3,5-DNS and enzyme activities determined in the same manner as the test but which contained the buffer in place of the modulator.

Determination of the Michaelis constant (*km*): This was determined by the linear transformation of the Michaelis Menten equation (Line-weaver and Burk, 1934) using salicin at concentrations of 0.2 – 1.4% ($^{w}/_{v}$) in 0.2M sodium acetate buffer, pH 5.0.

RESULTS AND DISCUSSION

A quantitative evaluation of the results obtained from the consecutive purification steps is shown in Table 1. The specific activity of the purified enzyme was estimated to be 28 U.mg/Protein, which is about 11.7 folds higher than that of the crude culture supernatant. The column profile for the β-glucosidase from DEAE-Sephadex is shown in Fig. 1. Two unequal protein peaks were detected, but only the major one contained β-glucosidase activity. The minor protein peak might represent another component of the cellulase enzyme complex since more than one component has often been reported from the culture supernatant of many cellulolytic organisms (Nogawa *et al.*, 2001; Dong *et al*, 1997). This is because cellulase enzyme systems act synergistically to effect complete cellulose hydrolysis (Lynd *et al.*, 2002). A further purification of the β-glucosidase active fractions on Sephadex-G-20 showed only one peak with β-glucosidase activity (Fig.2). This indicates that the β-glucosidase produced by *Paecilomyces* sp. has only one component. This is contrary to the multiplicity of the same enzyme reported for many organisms. Iwashita *et al.* (1998) reported three β-glucosidases from *Aspergillus kawachii* while Lin *et al.* (1999) reported two β-glucosidases from *Thermomyces lanuginosus* – SSBP.

The apparent molecular weight of the enzyme was estimated to be 55,000 Da (Fig. 3). This is the same as that of β -glucosidase 1 from *Robilarda* sp (Uziie and Sasaki, 1987), but higher than 38,000 Da reported for the two β-glucosidases from *Trichoderma koningi* (Wood, 1988).

The enzyme exhibited narrow pH activity and stability profiles. It was optimally active at pH 5.0 and maximally stable at pH 4.0 (Fig. 4). It retained over 55% of its maximum activity in the acid pH ranges (pH 3-6) for 2h, but lost all its activities in the alkaline pH ranges (pH 8-10) after 2h. Optimal activity at the acidic pH range has also been reported for many fungal β-glucosidases (Tong *et al.*, 1980, Lin *et al.*, 1999)

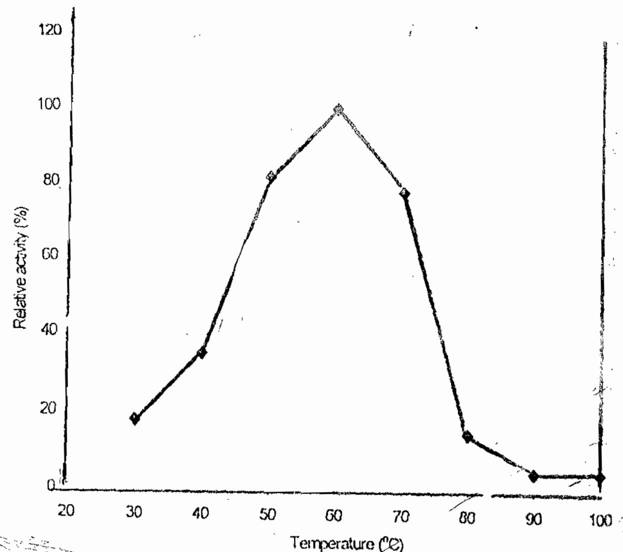

Fig. 5: Effects of temperature on the activity of β-glucosidase

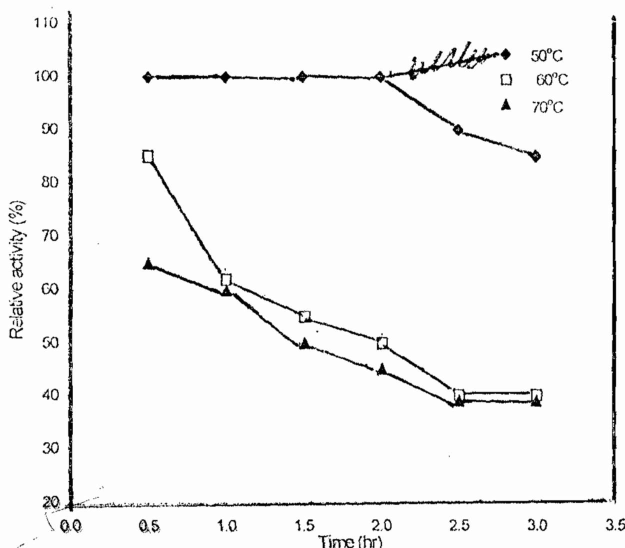

Fig. 6: Temperature stability profiles of β-glucosidase

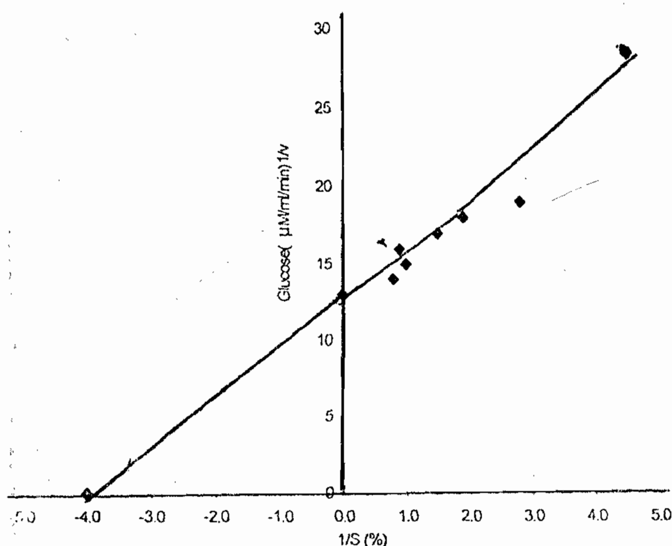

Fig.7: Lineweaver-Burk plot for the determination of the *Km* of β-glucosidase

The enzyme was optimally active at a temperature of 60°C and a significant ($P<0.05$) reduction in activity was observed at 100°C (Fig.5). It however retained 100% of its original activity at 50°C for 2h and had half-lives of 93min and 81min at 60°C and 70°C respectively (Fig. 6). Although *Paecilomyces* sp. is a mesophilic fungus, the temperature stability and activity ranges of its β-glucosidase enzyme were higher than those of many mesophilic organisms and comparable to those of many thermophilic cellulolytic organisms. Lin *et al.* (1999) reported optimal activity at 65°C for the β-glucosidase of *T. lanuginosus -SSBP*. At 60°C, the β-glucosidase of *Paecilomyces* sp. was more thermostable than that of *Thermo-monospora* sp. with a half-life of 60min (Hagerdale *et al.*, 1978) but less thermostable than that of *Aspergillus ustus* with a half-life of 2.7 day (Macris and Galiotou-Panayotou, 1986). This fairly high thermal stability of the β-glucosidase of *Paecilomyces* sp. is a desirable attribute needed for the retention of specific activity during the lengthy incubation period required for the solubilization of recalcitrant cellulose materials. It also encourages high temperature fermentations, which limits microbial contamination.

The Michaelis constant (*km*) of the enzyme with salicin was 2.5 mg/ml (Fig.7). This value was low compared with 0.11mg/ml reported for the β-glucosidase of *Robillarda* sp. (Uziie and Sasaki, 1987).

The activities of β-glucosidase was completely inhibited by Zn^{2+}, EDTA, glycine and $HgCl_2$ but was stimulated by Ba^{2+}, Ca^{2+} and Co^{2+} (Table 2). With respect to Zn^{2+} and EDTA inhibition, and the non-inhibitory effects of Mg^{2+}, the β-glucosidase of *Paecilomyces* sp resembles that of the yeast *Dekkera intermedia*, however both enzymes differ since Ba^{2+} and Ca^{2+} which stimulated the *Paecilomyces* sp. enzyme inhibited that of the yeast (Blondin *et al.*, 1983). The inactivation of the enzyme by mercuric chloride suggests that the enzyme requires reduced thiol groups for activity and complete inhibition by EDTA indicates that the enzyme is a metallo-protein or that they require metal ions for activation and/or stability (Au and Chan, 1987).

This study has shown that *Paecilomyces* sp., a mesophilic fungus produced β-glucosidase, which has only one component with acidic pH activity and stability optima and thermal stabilities comparable to those of many thermophilic fungi. The enzyme therefore lends itself suitable for acidic fermentation systems at fairly high temperatures.

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