Pertanika J. Sci. & Technol. **17 (1)**: 137 – 147 (2009)
ISSN: 0128-7680
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Physicochemical Characterisation and Substrate Specificity of Purified β-1,6-glucanase from *Trichoderma longibrachiatum*

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ABSTRACT

The β-1,6-glucanases are ubiquitous enzymes which appear to be implicated in the morphogenesis and have the ability to become virulence factor in plant-fungal symbiotic interaction. To our knowledge, no report on ß-1,6-glucanases purification from *Trichoderma longibrachiatum* has been made, although it has been proven to have a significant effect as a biocontrol agent for several diseases. Therefore, the aim of this study was to purify β-1,6-glucanase from *T. longibrachiatum* T28, with an assessment on the physicochemical properties and substrate specificity. β-1,3-glucanase enzyme, from the culture filtrate of *T. longibrachiatum* T28, was successively purified through precipitation with 80% acetone, followed by anion-exchange chromatography on Neobar AQ and chromatofocusing on a Mono P HR 5/20 column. (One β-1,6-glucanase) band at 42kDa in size was purified, as shown by the SDS-PAGE. The physicochemical evaluation showed an optimum pH of 5 and optimum temperature of 50°C for enzyme activity with an ability to maintain 100% enzyme stability. Enzyme activity was slightly reduced by 10-20% in the presence of 20 mM of Zn^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+} and Fe^{2+}. The highest β-1,6-glucanase hydrolysis activity was obtained on pustulan due to the similarity of β-glucosidic bonds followed by laminarin, glucan and cellulose. Therefore, it can be concluded that the characterization of ß-1,6-glucanase secreted by *T. longibrachiatum* in term of molecular weight, responsed to selected physicochemical factors and the substrate specificity are approximately identical to other *Trichoderma sp.*

Keywords: β-1,6-glucanase, characterisation, metal ion, pH, purification, substrate specificity, *Trichoderma longibrachiatum*

INTRODUCTION

Filamentous fungi of the genus *Trichoderma* have long been recognized as the agents for the biocontrol of plant diseases (Ridout *et al.*, 1988) which can directly impact mycelia or the survival propagules of other fungi through the production of toxic secondary metabolites (Benitez *et al.,* 2004), formation of specialized structures (Sarrocco *et al.,* 2006) and secretion of cell wall degrading enzymes (Noronha and Ulhoa, 1996). The most common cell wall degrading enzymes are chitinase, ß-1,3-glucanase, ß-1,6-glucanase and proteinases (Haran *et al.*, 1995; Haran *et al.*, 1996; Lorito *et al.*, 1996). The secretion of these enzymes was proposed to be regulated by catabolite repression (Lorito *et al.,* 1996) and the presence of fungal cell walls (Djonovic *et al.,* 2006). Chitin and ß-1,3-glucans were considered as the major components in the cell wall, while β-1,6-glucan represented relatively minor components of

Received: 11 January 2007
Accepted: 8 April 2008
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the fungal cell walls (Lora *et al.*, 1995) and plants were found to lack β-1,6-glucan altogether (Inbar and Chet, 1994). Due to this factor, chitinase and ß-1,3-glucanase have been proposed as the key enzymes in mycoparasitism against phytopathogenic fungi (Elad *et al.,* 1982), whereas less attention has been given to the function of ß-1,6-glucanase.

The existence of ß-1,6-glucanase has been known for almost five decades (Delgado-Jarana *et al.*, 2000). Endo-ß-1,6-glucanase was shown to act cooperatively with chitinase to hydrolyse fungal cell walls of *B. cinerea, Gibberella fujikuroi, Phytophthora syringe* and *Saccharomyces cerevisiae* (de la Cruz *et al.*, 1993). The β-1,6-glucanases are ubiquitous enzymes which appear to be implicated in the morphogenesis such as the synthesis of β-1,6-glucan of cell walls in *S. cerevisiae* (Cid *et al.*, 1995) and *S. commune* (Khun *et al.,* 1990), mobilisation of an extracellular storage glucan (Stasinopoulos and Seviour, 1989), and digestive metabolism in invertebrates (Papavizas, 1985). In plant-fungal symbiotic interaction, ß-1,6-glucanase was identified as being secreted by *Trichoderma* sp. into the apoplast of the host grass; *Poa ampla*, upon attack by its endophytic fungus, *Neotyphodium* sp. (Moy *et al.,* 2002). Recently, ß-1,6-glucanase has also been shown to be a virulence factor in the interaction between the mycopathogen *Verticillium fungicola* and its host, *Agaricus bisporus* (Amey *et al.,* 2003).

Even though ß-1,6-glucanases have been purified from several filamentous fungi, including *Penicillium brefeldianum* (Schep *et al.,* 1984), *Acermonium* sp. (Martin *et al.*, 2006), *Acremonium persicinum* (Pitson et al., 1996) *Saccharomycopsis fibuligera* (Mulenga and Berry, 1994) and *T. harzianum* (de la Cruz *et al.,* 1995), their physiological function has not been conclusively studied. The current knowledge on ß-1,6-glucanases is still limited to the studies on biochemical and lytic properties of purified enzymes (Montero *et al.*, 2005).

To our knowledge, no report on ß-1,6-glucanases purification from *T. longibrachiatum* has been made, although it has been proven to have a significant effect as a biocontrol agent for groundnut rot (Sreenivasaprasad and Manibhusanrao, 1990; 1993) and coffee pulp (Onsando and Waudo, 1992). Therefore, the aim of this study was to purify β-1,6-glucanase from *T. longibrachiatum* T28. In the current study, the physicochemical properties and the substrate specificity of the purified β-1,6-glucanase were also assessed.

MATERIALS AND METHODS

Strains and Growth Conditions

The *T. longibrachiatum* T28 was obtained from Plant Systematic and Microbe Laboratory, Biology Department, Universiti Putra Malaysia and maintained on the Malt Yeast Glucose Agar (MYG) slant.

The Preparation of Crude Culture

The preparation of the test strain culture was carried out using the sub-cultured *T. longibrachiatum* T28 from the slant agar to the MYG plate culture and incubated at 32°C. After 4 days, $1x10^7$ spores.ml^{-1} of *T. longibrachiatum* T28 were prepared and added into Trichoderma Complete Medium (pH 5.5), which was supplemented with 0.5% (w/v) glucose. The seed cultures were then shaken (180rpm) at 32°C for 24 hours, before the seed cultures were filtered and washed thoroughly with sterile distilled water. After that, the seed cultures were transferred into the Trichoderma Minimal Medium (pH 5.5; 1.0% w/v *Pleurotus sajor-caju* mycelium) and shaken at 180rpm at 32°C for another 72 hours. The culture filtrate was collected, dialysed against distilled water, for at least 24 hours at 4°C before it was ready to be used for enzyme purification. The β-1,6-glucanase activity and protein concentration were determined as below.

Enzyme Assays

β-1,6-glucanase activity was determined using the method described by Somogyi (1952) in measuring the amount of reducing sugars released from pustulan. One enzymatic unit (U) of β-1,6-glucanase activity is defined as the amount of enzyme which releases 1μmol of glucose in 1 hour at 45°C.

The determination of protein was performed using Bio-Rad protein assay kit, based on the method by Bradford (1976). The mixture of distilled water and Bio-Rad reagent was used as control, and the standard curve for the protein was plotted using bovine serum albumin in the range of 0-20μg.

β-glucanase Purification

Unless indicated, all the steps in the purification process were carried out according to the method used by Muskhazli *et al.* (2005) and performed at 4°C. The purification process was performed in three steps; these comprised of acetone precipitation, followed by anion-exchange step using Neobar AQ column and ended with chromatofocusing step performed on a Mono P HR 5/20 column. In each step, all the active fractions were pooled and dialysed against distilled water for 24h at 4°C before they were assayed for β-1,6-glucanase activity and protein content. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was also carried out on the collected fraction, according to Laemmli (1970), i.e. using 10% acrylamide gels and stained with Coomasie R-250 brilliant blue (Sigma). Low molecular range standard proteins were used to determine the molecular mass.

Physicochemical Parameters and Substrate Specificity

The optimal temperature for β-1,6-glucanase activity, was determined by measuring the reducing sugars released, after 30 minutes of incubation at the temperatures between 25°C-75°C, i.e. at an increment of 5°C. Meanwhile, the temperature stability for the β-1,6-glucanase activity was examined by maintaining the purified β-1,6-glucanase for 1 hour at the temperatures between 25°C-75°C, at the increment of 5°C, before the β-1,3-glucanase activity was determined.

The effect of pH on the enzyme activity was determined by varying the pH of the reaction mixture between pH 4-8, at the increment of 1 pH unit. The pH of the mixture was adjusted to the intended pH, with 50mM sodium citrate buffer. The stability of the pH was determined by incubating the purified β-1,6-glucanase enzyme, at pH 4-8 for 1 hour at 37°C, before the pH was changed to pH 5, prior to the activity determination of β-1,6-glucanase.

In addition, the effects of several metal ions on the activity of β-1,6-glucanase were also investigated. The metal ions used for this study were Zn^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+} and Cu^{2+}. Twenty millimolar (20mM) of metal ion solution, in sodium acetate buffer pH 5.5, was prepared and added into the purified β-1,6-glucanase enzyme before the activity was determined.

The β-1,6-glucanase activity, on several substrates, was also determined. These included pustulan (1.0% w/v), laminarin (1.0% w/v), cellulose (1.0% w/v) and glucose (1.0% w/v). Each substrate was prepared in 0.05M sodium acetate buffer pH 5.5.

RESULTS AND DISCUSSION

β-glucanase purification

Acetone precipitation, at 80% saturation, was found to be capable of recovering 58.70% of the total β-1,6-glucanase activity from the culture filtrates. The precipitated protein was

dialysed, dissolved in distilled water, and labelled as crude enzyme. The crude enzyme, from *T. longibrachiatum* T28, showed β-1,6-glucanase activities at 20.61U. The elution pattern for the anion exchange chromatography of the crude enzyme fraction is shown in *Fig. 1a*. Based on the data presented in the figure, fractions 4-10 is shown to correspond in protein and β-1,6-glucanase activity. This pooled fraction was later designated as T28 (G1), and it has β-1,6-glucanase activity of 8.85U, i.e. equivalent to 25.21% of the total activity (Table 1). The analysis of the pooled fractions, using the SDS-PAGE, showed that 5 bands virtually appeared for T28 (G1), with the molecular weight aproximately at 31kDa, 45kDa, 66.2kDa, 96kDa and 97.4kDa *(Fig. 1c)*. Meanwhile, the elution profiles for T28 (G1), during chromatofocusing, are depicted in *Fig. 1b*, and from this, only one major peak for β-1,6-glucanase activity was obtained from fractions 49-51, which comprised 8.43% of the total enzyme activity (2.96U). The analysis of the pool, corresponding to these fractions using the SDS-PAGE, revealed only one major band with a calculated molecular mass of about 45kDa (*Fig. 1c*). The existence of the purified β-1,6-glucanase, in the range of 41kDa to 53kDa, has been reported previously. The molecular weight sizes of ß-1,6-glucanase, from different species, have been reported to be in the range of 41kDa to 53kDa. These include ß-1,6-glucanase from *T. viride* with a molecular weight of 45kDa (Nobe *et al.*, 2003), *T. harzianum* at 46kDa (Moy *et al.*, 2002) and 47kDa (Montero *et al.*, 2005). Several other isoforms or sub-units of β-1,6-glucanase from *T. harzianum* with different catalytic activities, molecular weights and substrate specificities have also been previously reported; these were with different molecular weights of 43kDa and 51kDa (de La Cruz, 1995).

TABLE 1
Summary of the purification steps and enzyme activity for each step involved in a typical purification of β-1,6-glucanase

Steps	Fractions	Enzyme activity (U)	Yield (%)
Culture filtrate		35.11	100
Acetone precipitation		20.61	58.70
Anion exchange	4-10	8.85	25.21
Chromatofocusing	49-51	2.96	8.43

However, the difference in the molecular weight of the purified β-1,6-glucanase is not new because the molecular mass of β-glucanase has appeared to vary between species and also within species (Pitson *et al.*, 1993). Nevertheless, it is not known whether the existence of the isoform or different molecular weights of this enzyme was the product of or as a result of the same or separated β-1,6-glucanase genes. According to Mrsa *et al.* (1993), one of the reasons for the differences is attributed to the anomalous migration of protein in the gels rather than to the post-translation processing of the polypeptide chain, and sometimes, the type of growth substrate used can also influence the number of bands on the SDS-PAGE (Vazquez-Garciduenas *et al.,* 1998) or the activation of a specific gene (Montero *et al.*, 2005). Furthermore, according to Matsuzawa *et al.* (1996), species, the type of reactions (exo- or endo-) and the method of purification may also impose effect on characterization, even for the purified β-glucanase of the same type.

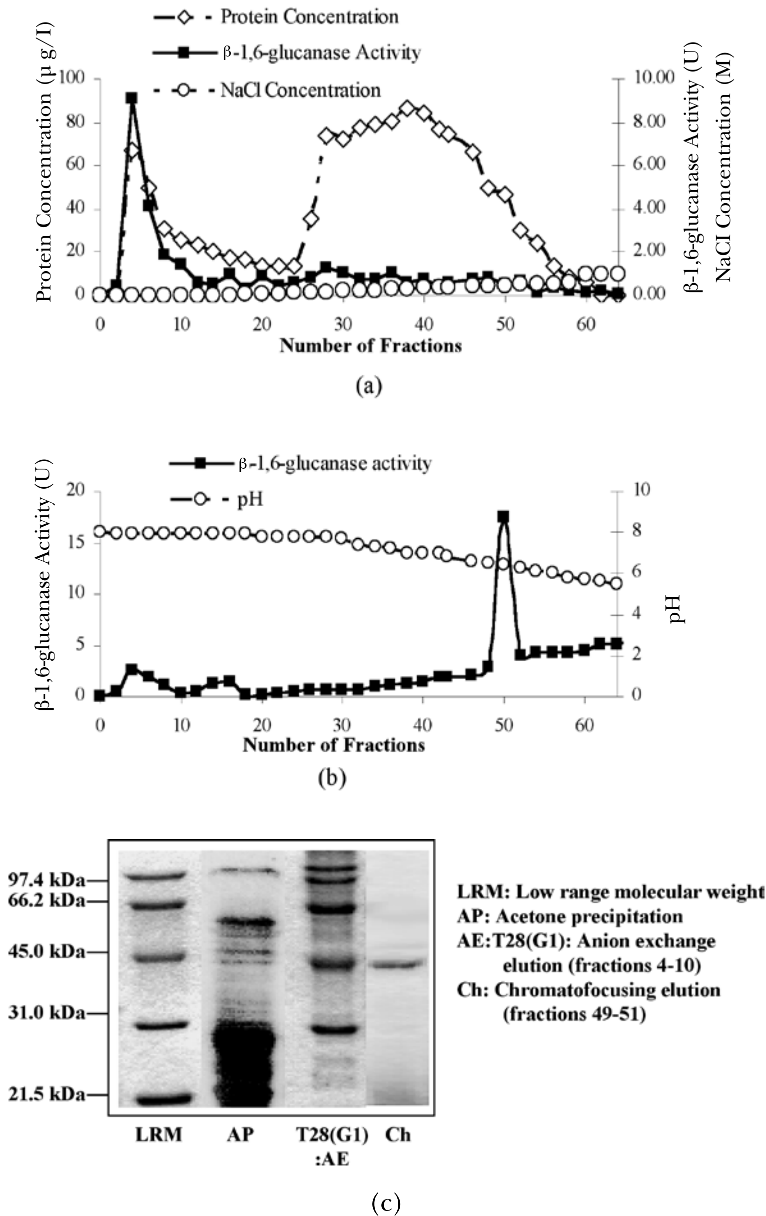

Fig. 1: The purification of T. longibrachiatum *T28* β*-1,6-glucanases (a) Elution profile of the crude culture after anion exchange (Neobar AQ exchanger column), eluted with a 0 to 0.5M NaCl gradient with fractions 4-10 as T28 (G1); (b) Elution profile of T28 (G1) after chromatofocusing on mono P HR 5/20 with a 8.5 to 5.5 pH gradient; (c) SDS-PAGE (10%) of protein from pooled peaks and stained with Coomassie blue. LRM=Low range standard molecular weight; AP=Acetone precipitation; AE:T28(G1)=Anion exchange elution fractions 4-10; Ch= Chromatofocusing elution fractions 49-51*

Physicochemical Parameters and Substrate Specificity

In this study, the optimum temperature for the β-1,6-glucanase activity was found to be 50°C. The enzyme showed a rapid reduction in its stability, i.e. from 100% to only 40%, when the temperature was increased from 50°C to 55°C. Enzyme was found to be inactive and unstable at the temperature above 70°C (*Fig. 2a*), and was completely inactivated at 75°C; this was probably due to the thermal denaturation of the enzyme. Meanwhile, the optimal activity, for the short-term incubation, was often seen at the temperatures ranging from 30°C to 50°C; nevertheless, many fungal β-glucanases appeared to be stable at the temperatures up to 50°C to 60°C (Pitson *et al.*, 1993). β-1,6-glucanase for *T. longibrachiatum* T28 was shown to have the same optimal temperature and stabilisation temperature to the purified β-1,6-glucanase from *T. harzianum* (de la Cruz *et al.*, 1995; Hiura *et al.*, 1987) and *T. viride* (Nobe *et al.*, 2003). One simple conclusion which could be made from this was that even though *T. longibrachiatum* was categorised as warm climate fungi (Danielson and Davey, 1973), the enzyme itself could not function in extreme temperatures. However, Bodenmann (1985) explained that the effect of temperature could be stabilised by the substrate in the culture through the 'padding effect' which resisted the heat.

The optimal activity of the fungal β-glucanase usually appears in acidic conditions, which is often between pH 4.0 to 6.0, and according to Pitson *et al.* (1993), most of the fungal β-glucanases have a broad optimum pH. As shown in *Fig. 2b*, there was a clear influence of pH on the enzyme activity, with β-1,6-glucanase enzyme shown to be the most active at pH

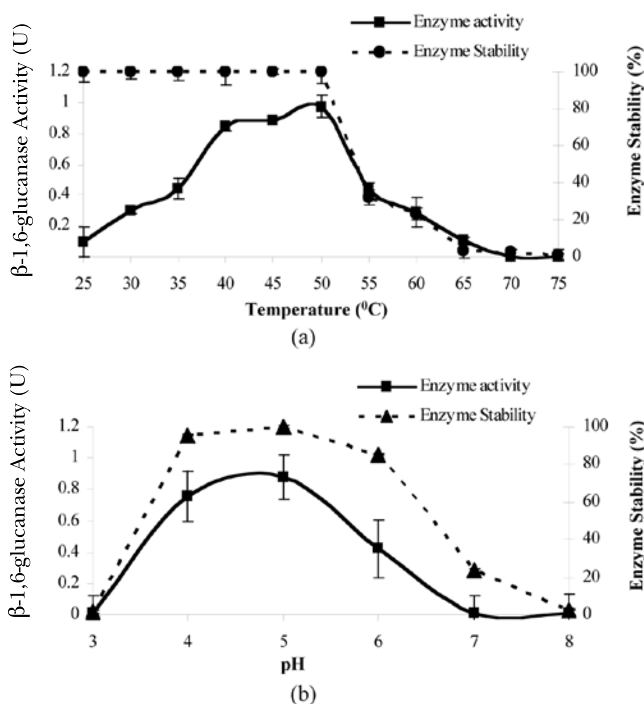

Fig. 2: The effect of physicochemical parameters, (a) temperature and (b) pH, on the activity and stability of the purified β*-1,6-glucanase in* T. longibrachiatum *T28. Each value of the* β*-1,6-glucanase stability is represented as a percentage compared to control, which is taken as 100%*

5.0. Meanwhile, this enzyme was able to sustain the activity above 80% over a pH range of approximately 4-6. However, in some cases, enzyme stability can be sustained up to pH 9 in the absence of a substrate (Tangarone *et al.*, 1989). An acidic or alkaline concentration may have an impact on the nutrient transport mechanism, it may also cause enzyme structure or disintegrate while in extreme condition (Kubicek-Pranz, 1998).

Metal ion is necessary for the enzyme activity as an additional factor which is involved in the catalytic process, as oxidizing or reducing agent, and to maintain the enzyme configuration (Deane *et al.,* 1998). The effects of several known metal ion inhibitors, on the activity of the purified enzymes, are shown in Table 2. In overall, the β-1,6-glucanase activity was inhibited by these metal ions but only in the range of 10-20%. In particular, Cu^{2+} ion in the assay mixture gave the highest inhibition, at 34.5% for β-1,6-glucanase. Watanabe *et al.* (1988) explained that the inhibition effect in the context of enzyme-substrate complex. In certain conditions, the position of the metal ion in enzyme-substrate complex prevented the substrate to form a firm bound to the recognition site in β-1,6-glucanase enzyme for the substrate hydrolysis, and thus brought to a halt or reduction in the enzyme activity. The inhibition effect of the metal ions on the enzyme activity, which is similar to one in the finding of the current study, has also been reported previously. In specific, the enzyme activity was slightly inhibited (20-35%) when 1mM of Co^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+} where added into the culture (Hiura *et al.,* 1987), while a significant inhibition on the enzyme activity was established with the presence of 1mM $HgCl_2$, $MnCl_2$, $KMnO_4$ (Tangarone *et al.,* 1989); 1% (w/v) SDS and 1% (w/v) β-mercaptoethanol (Thrane *et al.,* 1997).

Mycoparasitism in *Trichoderma* species was regulated by catabolite repression and carbon source starvation (Lorito *et al.,* 1996), which means that the β-1,6-glucanase activity was controlled by the carbon source as shown in Table 2. The purified β-1,6-glucanase has the ability to hydrolyse pustulan because most of the linkages are β-1,6-linked glucan. It also was found to be able to split the linkages for the non-specific substrates such as laminarin (β-1,3-glucan bonds) and cellulose (β-1,4-glucan bonds), but in relatively low capacity of 41.6% and 17.5% respectively, as compared to pustulan. A similar result, on the ability of β-1,6-glucanase from other *Trichoderma* species to hydrolyse the non β-1,6-linked substrate, has also been reported previously (de la Cruz *et al.*, 1995; Moy *et al.*, 2002; Nobe *et al.*, 2003). The ability to hydrolyse the non β-1,6-linked substrate may be due to the enzyme amino acid sequence (de la Cruz *et al.*, 1993). However, not all purified β-glucanase has this ability; for this, Vazquez-Garciduenas *et al.* (1998) found that the purified β-1,3-glucanase could only hydrolyse laminarin. Nonetheless, the existence of glucose in media caused almost 98% reductions in the enzyme activity due to the catabolite repression which led to less β-1,6-glucanase being secreted, while the accumulation of other carbon sources caused their starvation and this further led to a high enzyme activity (Montero *et al.,* 2005). Although purified enzyme has been indicated to have the ability to hydrolyse all the substrates (as shown in the results of this study), the type of linkages in the substrate or substrate specificity still possesses some influences on the enzyme activity. The highest activity was obtained when the enzyme hydrolyse substrate contained the same β-glucosidic bonds specific for the enzymes. The substrate specificity of each enzyme has a significant effect on the mycoparasitism performance for *Trichoderma* species (de la Cruz *et al.,* 1993) and as demonstrated by Djonovic *et al.* (2006), a high β-1,6-glucanse activity was found to be lower in *Rhizoctonia solani* as compared to *Pytium ultimum*, due to its cell wall, which is mainly fabricated with chitin and β-1,3-linked glucan (Bartnicki-Garcia, 1968). On the other hand, *P. ultimum* attained a high content of β-1,6-linked glucan in its cell wall, giving a significant effect on the β-1,6-glucanse activity.

TABLE 2
The effect of metal ions (20mM) and 1% (w/v) of different substrates on β-1,6-glucanase activity in *T. longibrachiatum* T28

		Enzyme activity (U) ± s.d*	Relative activity (%)**
Metal ions (20mM)	None	0.97 ± 0.300	100
	Zn^{2+}	0.87 ± 0.180	89.6
	Ca^{2+}	0.87 ± 0.764	90.2
	Co^{2+}	0.73 ± 0.461	75.5
	Mg^{2+}	0.69 ± 0.277	71.2
	Cu^{2+}	0.63 ± 0.731	65.5
	Mn^{2+}	0.79 ± 0.688	81.9
	Fe^{2+}	0.78 ± 0.423	80.6
Substrate (1% w/v)	Pustulan	0.97 ± 0.300	100
	Laminarin	0.04 ± 0.539	41.6
	Cellulose	0.17 ± 0.216	22.5
	Glucose	0.0001 ± 0.132	0.12

*The results are the mean values of triplicate tests ± standard deviation.

**Relative activity (%) is expressed as a percentage compared to control, which is taken as 100%.

CONCLUSIONS

In conclusion, all the results obtained in this experiment showed that ß-1,6-glucanase secreted by *T. longibrachiatum,* possessed a molecular weight size and it also responded to selected physicochemical factors and substrate, which are almost similar to other *Trichoderma sp.*

ACKNOWLEDGEMENTS

The authors gratefully acknowledged the Ministry of Science, Technology and Innovation Malaysia for the research grant, which was given under the Fundamental Research Program (05-11-02-0060F) and the staff of PS&M Laboratory, Biology Department, Universiti Putra Malaysia.

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