Original Article

ASSESSMENTS ON THE CATALYTIC AND KINETIC PROPERTIES OF BETA-GLUCOSIDASE ISOLATED FROM A HIGHLY EFFICIENT ANTAGONISTIC FUNGUS *Trichoderma harzianum*

AVALIAÇÕES DAS PROPRIEDADES CATALÍTICAS E CINÉTICAS DA BETA-GLUCOSIDASE ISOLADAS DE UM FUNGO ANTAGONÍSTA ALTAMENTE EFICIENTE Trichoderma harzianum

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ABSTRACT: Due to the toxicity and inefficiency of chemical fungicides to control infestation of *Macrophomina phaseolina* (Tassi) Goid which causes charcoal rot in plants, a biotechnological approach using β-glucosidase (EC.3.2.1) as the alternative bioactive ingredient in fungicide is hereby, proposed. The extracellular enzyme was isolated from a highly efficient fungal antagonist, *Trichoderma harzianum* T12. The highly similar molecular masses obtained using SDS-PAGE (96 kDa) and MALDI-TOF mass spectrometry (98.3 kDa) affirmed that the β-glucosidase was purified to homogeneity. Consequently, optimum catalytic parameters that rendered the highest enzyme activity were found to be: 45˚C, pH 7, inoculum size of 10 % (w/v), supplementation with metal ions Zn^{2+} and Mn^{2+} ions, and Tween 80. Addition of wheat bran and $(NH_4)_2SO_4$ as carbon and nitrogen sources also improved enzyme activity. BLASTn showed the sequence of β-glucosidase T12 was highly identical to other β-glucosidases *viz. T. harzianum* strain IOC-3844 (99%), *T. gamsii* and *T. virens* bgl1 (86 %) as well as *T. reesei* strain SJVTR and *T. viride* strain AS 3.3711 (84 %). Kinetic assessment showed that β-glucosidase T12 catalyzes hydrolytic activity is characterized by a K_m of 0.79 mM and V_{max} of 8.45 mM min^{-1} mg^{-1} protein, with a corresponding k_{cat} of 10.69 s^{-1}.

KEYWORDS: β-glucosidase. *Trichoderma harzianum.* Kinetic assessment. M*acrophomina phaseolina.*

INTRODUCTION

Charcoal rot is a disease prevalent in commercial crops caused by a pathogenic root fungus *Macrophomina phaseolina* (Tassi) Goid which led to substantial losses in crop yields such as corn (*Zea mays* L.), sorghum (*Sorghum bicolor*), sesame (*Sesamum indicum* L.) and soybean (*Glycine max* L.) (RAYATPANAH et al., 2012; KHALILI et al., 2016) worldwide. Although, various pest management strategies *viz.* cultural, regulatory, physical, chemical fungicides and biological have been suggested, they are mainly as precautionary measures (JANA et al., 2003; GANESHAMOORTHI et al., 2010). Utilization of chemical fungicides against *M. phaseolina* is also inefficient due to the soil-borne nature of the fungus (ANIS et al., 2010). Aside from being expensive and detrimental towards the environment and human health, continuous use of chemical fungicides can interfere with the delicate balance of beneficial microbes within the soil (ABOSHOSHA et al., 2007). One of the major concerns is that it can perpetually increase incidences of fungicide resistant phytopathogenic microorganisms (ANIS et al., 2010; KHALILI et al., 2016).

In view of the shortcomings in current pest management strategies, the study hereby suggests a greener approach to better control charcoal rot caused by *M. phaseolina*. The alternative technique suggested here focuses on the use of an enzyme, β-glucosidase (EC.3.2.1) naturally secreted by the antagonistic fungal isolate, *T. harzianum*, as a bioactive ingredient in a fungicide. β-glucosidase belongs to a group of enzymes called hydrolases which specifically hydrolyse O- and S-glycosyl compounds (EXPASY., 2017). It is known that among the well-described antagonistic mechanisms for the fungal isolate, *T. harzianum* (nutrient competition, antibiotic production and mycoparasitism) (KUBICEK et al., 2001; VITERBO et al., 2002; GAJERA et al., 2010; KHALILI et al., 2016), its mycoparasitic behaviour is particularly interesting. *T. harzianum* has evolved the capability to secrete a type of β-glucosidase that digests the ordered layers of amorphic β-1, 3-glucan (GAJERA et al., 2010; KHALILI et al., 2017) in *M. phaseolina*. The hydrolytic activity of this β-glucosidase targets the sugar-based polymer that

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forms the filling material in the chitin-based cell wall of the pathogenic fungus (GAJERA et al., 2010; KHALILI et al., 2017). Driven by this knowledge, we believe that the enzyme may prove useful as an alternative bioactive component in a fungicide to inhibit proliferation of *M. phaseolina*.

Like any other formulations used in detergents or in enzyme-assisted manufacturing processes (WAHAB et al., 2012a; WAHAB et al., 2014; MOHAMAD et al., 2015a; MANAN et al,. 2016), the constituents proposed in our enzyme-based formulation is considerably less toxic than that found in chemical fungicides. Moreover, manufacturing processes that use natural enzymes as catalysts are typically carried out under near ambient conditions i.e. (1 atm, 30-50^{o}C, pH 6-8) (KARBOUNE et al., 2008; MARZUKI et al., 2015a; MARZUKI et al., 2015b; MOHAMAD et al., 2015b; MARZUKI et al., 2015c; MANAN et al., 2016; KHALILI et al., 2017), similar to conditions under agricultural settings. For these reasons, an enzyme-based fungicide i.e. β-glucosidase T12 from *T. harzianum* was employed for managing *M. phaseolina* infestation. The technique proposed in this study appears feasible, prospectively greener as well as being expedient and practical since its application follows the usual agricultural practices.

To boost the catalytic activity of the extracellular β–glucosidase, a comprehensive optimization of the catalytic parameters is, therefore, necessary. This study was aimed in optimizing the relevant parameters that rendered the highest activity in the enzyme. The parameters assessed included temperature of reaction, pH of buffer, concentration of metal ions and inoculum size. Various types of carbon and nitrogen sources as well as surfactants that can further improve enzyme activity were also examined. Nucleotide sequence analysis, and kinetic assessment were carried out on this enzyme and the results are also presented in this paper.

MATERIAL AND METHODS

Collection of *T. harzianum* T12 and *M. phaseolina* fungal isolates

The colonies of *T. harzianum* (T12) fungal antagonists were isolated from agricultural soil samples collected from the Mazandaran province, Iran. The antagonistic efficacy of the T12 isolate utilized in this study was reported in our previous work (KHALILI et al., 2016). Upon investigating the virulence (pathogenicity, genetic diversity and pathogenic variability tests), *M. phaseolina* M2 isolate obtained from crown segments of infected soybean (*Glycine max* L.) root picked from Mazandaran province was found to be the most virulent.

Growth conditions of fungal cultures

Following the method described by Goujon and co-workers, the pure culture of *T. harzianum* T12 was grown. Potato dextrose agar (PDA) (Oxoid Ltd, England) slants were incubated in darkness at 30 ˚C for 5 days and subsequently stored at 4 ˚C prior to use. For the culture media, a 5-day old culture of each fungus was grown in sterile potato dextrose broth (PDB, Oxoid Ltd, England) and inoculated into an Erlenmeyer flask containing 30 mL of sterile PDB as carbon source. The mixture was incubated at 30 ˚C ± 1 ˚C for 5 days under stationary condition for developing the fungal spore suspension.

Sporulation Medium and Inoculum Development

Sporulation medium (compositions (%, w/v): Trisodium citrate, 0.5; KH_2PO_4, 0.5; NH_4NO_3, 0.2; $(NH_4)_2$ SO_4, 0.4; $MgSO_4$, 0.02; Peptone, 0.1; Yeast Extract, 0.2; Glucose, 2; Agar, 2.5) was prepared and its pH was adjusted to pH 5.5 before autoclaving for 15 min at 121 ˚C. The fungal culture was transferred to sporulation slants under sterile conditions and incubated for 48 h at 35 ˚C to allow sporulation and subsequently stored at 4 ˚C. The inoculum medium (100 mL) was prepared according to the following compositions (% w/v): Trisodium citrate, 0.5; KH_2PO_4, 0.5; NH_4NO_3, 0.2; $(NH_4)_2$ SO_4, 0.4; $MgSO_4$, 0.02; peptone, 0.1; yeast extract, 0.2; glucose, 2; in 500 mL Erlenmeyer flask containing glass beads for uniform growth. The inoculum medium was adjusted to pH 5.5 prior to autoclaving for 15 min at 121˚C. A loopful of *T. harzianum* T12 culture from the sporulation medium was transferred aseptically into the flask and incubated on an orbital shaker (120 rpm) for 24 h at 30 ˚ C (IQBAL et al., 2011).

Production and Extraction of β-glucosidase

A 10 g banana waste was mixed with 2% HCl at room temperature in an Erlenmeyer flask for 2 h, autoclaved (15 min at a pressure of 15 lb/in2 and 121˚C) and the resultant slurry was filtered through a Whatman No 1 filter paper (Whatman® Grade 1 Qualitative Filtration Paper, Sigma plot) and used as the fungal growth medium. Next, a solution consisting of nutrient salts $(NH_4)_2SO_4$ 3.0, $FeSO_4 \cdot 7H_2O$ 0.005, KH_2PO_4 1.0, $MnSO_4 \cdot H_2O$ 0.0016, $MgSO_4 \cdot 7H_2O$ 0.5, $ZnSO_4 \cdot 7H_2O$ 0.0017, $CaCl_2$ 0.1, $CoCl_2$ 0.002, NaCl 0.1 (g/L) was used to moisten the banana waste (10 g) prior to inoculation

with a freshly prepared *T. harzianum* T12 spore suspension (5 mL, 5 %, w/v) and incubated at 30˚C for 24 h (SHAHZADI et al., 2014). For the β-glucosidase extraction process, sodium citrate buffer (0.05 M, pH 4.8) at a 1:10 ratio was added into the culture medium. The flask was agitated at 120 rpm for 30 min before filtering through a Muslin cloth. The filtrate was centrifuged for 10 min (10,000 *x*g) and the supernatant was tested for β-glucosidase activity (IRSHAD et al., 2013).

Purification of β-glucosidase

Purification of β-glucosidase was carried out by ammonium sulfate precipitation executed at room temperature unless specified otherwise. The crude extract exhibiting high β-glucosidase activity was precipitated by treatment with ammonium sulfate at different saturation levels (20-80%) (w/v) while on ice with continuous stirring for 1 h.

The precipitate fractions were centrifuged (10,000 *x*g, 15 min at 4˚C), the supernatant decanted and a small volume of 0.1 M citrate buffer (pH = 7.5) was added into the precipitated enzyme protein. The precipitate was left to stand for 4-6 h at 4°C and subsequently centrifuged (10,000 *x*g, 15 min at 4˚C), the supernatant decanted and sediment was dissolved in Tris-HCl buffer (0.2 M, pH 8). The solution was transferred into a pre-soaked (0.1 M Tris-acetate buffer, pH 7.5 solution overnight) dialysis bag (12 kDa), sealed and dialyzed against distilled water with four regular changes of the water at every 6 h interval. Total protein and activity of purified β-glucosidase were determined before and after the dialysis using ammonium sulfate precipitation. The purified β-glucosidase was lyophilized and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for further purification and molecular weight determination (AHMAD et al., 2011).

Determination of β-glucosidase Activity and Protein Content

β-glucosidase activity was examined using *p*-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma Plot) as substrate in a microtiter plate. A 100 µL reaction mixture (2.5 U/mL of enzyme, 25 µL of *p*NPG (10 mM) and 50 µL of phosphate buffer (pH 7.0) was incubated at 45˚C for 30 min and the reaction was terminated by adding 100 µL of NaOH-glycine buffer (0.4 M, pH 10.8). The developed color due to liberation of *p*-nitrophenol (*p*NP) was read at 412 nm in an ELISA Reader (MULTISKAN; Labsystems) absorbance spectrometer. The concentration of liberated *p*NP was calculated by comparing the reading corrected for blanks against a *p*NP standard calibration curve. One unit (U) of β-glucosidase activity was defined as the amount of enzyme needed to liberate 1µM of pNP per min under standard assay conditions. The protein content of the enzyme extracts was determined by the Bradford method using Bovine serum albumin (BSA) as the standard and measured at 595 nm (BRADFORD, 1976).

Determination of Molecular Weight of β-glucosidase T12 and degree of purity
SDS-PAGE

SDS-PAGE (TGX Fastcast, Bio-Rad) on a 5% stacking and a 12% separating gel, respectively, was performed to determine the molecular weight of the purified β-glucosidase (LAEMMLI, 1970). A 50 µL buffer sample (0.05% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol, and 1% SDS in 0.25 M Tris–HCl buffer; pH 6.8) was added into a 100 µL of protein sample in an eppendorf tube, boiled in hot water bath for 5 min, cooled to room temperature and electrophoresed. The protein bands were visualized by staining with Coomassie Brilliant Blue G (Sigma) and destained in diluted acetic acid overnight. The molecular weight of the purified β-glucosidase was determined in comparison to marker protein (standard protein marker, 15-350 kDa; Sigma, USA).

HPLC Analysis of β-glucosidase T12

High performance liquid chromatography (HPLC) using a hypersial ODS column (4.6 mm × 100 mm) equipped with Waters 2690 Alliance Separation Model (Agilent Technologies, USA) was used to cheek the degree of purity of the enzyme. A 5 µL sample volume was injected and separated using a solvent system consisting of acetonitrile - water (70:30) maintained at a flow rate of 1.0 mL/min. A UV detector equipped with a Supelco C-18 column (250 × 4.6 mm, the particle size of 5µm, product code: 963967-902) at a flow rate of 2 mL/min was used to detect the sample (BAI et al., 2013).

(MALDI-TOF)/TOF-Mass Spectrometry (MS) of β-glucosidase T12

Molecular mass of the enzyme was determined by matrix-assisted laser desorption ionization /time of flight (MALDI-TOF) recorded using KOMPACT SEQ (Kratos Analytical Inc) MALDI mass spectrometer in positive ion mode. α-Cyano-4-hydroxy cinnamic acid and sinapinic acid dissolved in a mixture containing 60% (v/v) acetonitrile, 40% (v/v) water, and 1% (v/v) trifluoroacetic acid were used as matrices.

The purified enzyme was resolved on HPLC and the eluted sample (100 pmol) was spotted along with matrix solution on MALDI target plate and evaporated. The spectrum was recorded by averaging 3000 shots at 100% laser intensity in detection range of 20,000-100,000 Da. The final spectrum was subjected to smoothing and baseline subtraction. A linear time-of-flight detector (1.7 m) using a pulsed N_2 laser (337 nm) was employed for data collection. Ions were extracted into the linear TOF using an extraction potential of 20 kV in high-mass detection mode (TIWARI et al., 2013).

Characterization of the Catalytic Activity of β-glucosidase T12

The assessments on the catalytic activity of the purified T12 β-glucosidase was executed using a standardized protein concentration (14.65 mg/mL. Activity of each enzyme sample was assayed using *p*-nitropheny-β-D-glucopyranoside (*p*NPG) as the substrate and stirred at 200 rpm.

Effect of Temperature

Assessments were performed by incubating the enzyme-substrate mixture for 30 mins at varying temperatures (25-90 ˚C) in sodium acetate buffer (20 mM) at pH 7.0. Aliquots were withdrawn at regular intervals, cooled in an ice bath and the residual activity was calculated using *p*NPG assay. The thermal stability study was conducted by pre-incubating the enzyme solution at 25˚C to 90 ˚C at regular periods of 30–240 mins.

Effect of pH

The purified enzyme was incubated in a variety of buffers for 30 mins (100 mM sodium citrate buffer: pH 3.0- pH 6.0, 100 mM sodium phosphate buffer: pH 7.0 - 8.0, 100 mM glycine-NaOH buffer: pH 9.0 and 100 mM sodium hydroxide buffer: pH 10.0) in dissolved *p*NPG, respectively. For evaluation of pH stability, the purified enzyme was incubated at optimum temperature in buffers of various pH (pH 3-10) with incubation time ranging from 30-240 mins.

Effect of Metal Ions and Surfactants

Various divalent metal ions (Li^+, Na^+, K^+, Ca^{2+}, Mg^{2+} , Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{3+}) (10 mM) were added into the reaction mixtures prior to 30 min incubation at 25 ˚C, followed by the activity assay. For the effect of surfactants, a 50 µL of enzyme was incubated in 50 µL of surfactants (Tween 20, Tween 40, Tween 80, Tween 100 and Triton X-100) (1.0 %, w/v) for 1 h under optimum pH and temperature conditions and the residual activity was determined. The activities assayed in the absence of metal ions and surfactants were designated as the negative controls, expressed as 100 %.

Effect of Natural Carbon Sources

The effect of carbon sources evaluated included pectin, wheat bran, copra meal, wheat husk and potato peel. Individually, each carbon source 1.0 % (w/v) was added into a flask, inoculated with *T. harzianum* T12 culture and incubated for 5 days under optimum pH and temperature well as optimum concentrations of metal ions and surfactants.

Effect of Nitrogen Sources

Various nitrogen sources (1.0 %, w/v) that consisted of ammonium chloride, NH_4Cl, ammonium sulfate $(NH_4)_2SO_4$, ammonium nitrate, NH_4NO_3, sodium nitrate, $NaNO_3$ and potassium nitrate, KNO_3, were added to the mineral salt medium prior to activity assay under optimum pH and temperature conditions.

Effect of Inoculum Size

To determine the optimum inoculum level that would give the best enzyme activity, duplicate flasks that contained production media (50 mL) inoculated with varying volumes (2-10 mL) of freshly prepared co-cultures inoculums incubated for 6 days under optimum pH and temperature conditions were used.

Amplification for Obtaining Full Sequence of β-glucosidase T12

Genomic DNA Amplification

For the purpose of amplifying the full sequence of β-glucosidase gene from *T. harzianum* T12, we designed two specific primers using Primer3 (v. 0.4.0) online application. The degenerate primers were designed based on sequence similarity of existing β-glucosidase genes present in the database, and ordered from 1st BASE (Malaysia) (Table 1). Fungal chromosomal DNA was prepared as described by Sun and co-workers (SUN et al., 2001).

Table 1. The designed specific oligonucleotide primers used to amplify the gene sequence of β-glucosidase T12.

Name Primers	Oligonucleotides sequence
TFbgl2	5'-GCTCTAGAATGTTGCCCAAGGACTTTCAG-3'
TRbgl2	5'-GCTCTAGATCAAGCTCTTTGCGCTCTTCTTG-3'

Polymerase Chain Reaction (PCR) of β-glucosidase T12 and Purification of PCR Product

Amplification of the DNA fragments encoding *T. harzianum* (T12) bgl12 gene was performed using polymerase chain reaction (PCR). The PCR was carried out in a reaction mixture (100 µl) containing DNA template (10-100 ng), 10 mM deoxynucleotide triphosphates (dNTPs) (0.2 mM), 10 X PCR buffer (10.0 µl), 25 mM MgCl2 (2 mM), oligonucleotide primers: TFbgl2 (30 pmol) and TRbgl2 (30 pmol), and Taq DNA polymerase (2 U). The gene was amplified using a thermocycler (Gene Amp PCR system 2400, Perkin Elmer, Foster, CA) with a pre-denaturation temperature at 94 °C for 5 min, 30 cycles PCR of 30 s denaturation at 94 °C, 45s annealing at 50 °C and 50s extension at 72 °C. The final elongation step at 72 °C was for 10 min and preservation was at 4 °C. The amplified products were electrophoresed on 1.0 % agarose gel (w/v) at 70 mA for 30 min and then subjected to staining with ethidium bromide (1 µg/µl) for 10 min (SARAH et al., 2007).

For the purification, aliquots of the PCR product were loaded onto 1.0 % agarose gel (w/v) and electrophoresed. Agarose strips containing the desired DNA fragment were excised with a sterile scalpel, and kept in a sterilized Eppendorf tube. PCR products were purified and sequenced by First Base Laboratory Co., Ltd. Identical sequences were searched in the NCBI database using BLASTn. β-glucosidase T12 gene sequencing and analysis of the PCR products were carried out at 1st BASE Laboratories (Malaysia). Retrieved β-glucosidase T12 sequence contained the proposed complete β-glucosidase gene sequence.

Determination of Kinetic Parameters of β-glucosidase T12

The kinetic parameters (K_m and V_{max}) measurements were carried out in phosphate acetate buffer (100 mM, pH 7, 45°C) by varying the concentrations of *p*NPG (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg/mL). The values of K_m and V_{max} were calculated by Michelis-Menten plot from experimental steady-state data (Equation 1). K_m is Michaelis constant, is defined as concentration of substrate needed to reach half maximum velocity – measure of substrate affinity and the V_{max} is maximum velocity – directly proportional to enzyme concentration (BHATTI et al., 2013).

$$V1 = \frac{V_{max}[S]}{K_m[S]} \quad \text{(Equation 1)}$$

Where $V1$ = reaction rate, [S] = substrate concentration, V_{max} = maximal velocity, and K_m is the Michaelis constant. The k_{cat} value was calculated by applying the equation $k_{cat}=V_{max}/[E]$, where [E] is the enzyme concentration employed in the assay.

Statistical Analysis

Statistical analyses were conducted using the IBM SPSS version 20.0 software and the level of significance of less than 0.05 was used for assigning significant differences among groups. Since the data violated the assumption of normality following the use of Kolmogorov-Smirnov and Shapiro-Wilk tests, the non-parametric test of Kruskal-Wallis H with pairwise comparison using Mann-Whitney U test was used for investigating statistical differences among groups. Unless specified otherwise, the data meant for statistical inference are presented as median ± interquartile range.

RESULTS AND DISCUSSION

Production and Purification of β-glucosidase T12

The β-glucosidase T12 (accession number KY199423) was successfully produced from the culture supernatant of *T. harzianum* T12 (total activity = 756 U, total protein = 338 mg) and subsequently purified using ammonium sulfate fractionation. The protein in the crude enzyme was precipitated with gradual increment of ammonium sulfate that ranged from 20-80% and the results are tabulated in Table 2. The specific activity of the enzyme was found to improve from 2.24 U/mg in the crude extract to 10.52 U/mg in the dialyzed fraction that corresponded to an acceptable 4.70 purification fold. Based from the obtained results, it can be inferred that the protocol used to purify β-glucosidase T12 yielded a purified and concentrated form of the enzyme.

Table 2. Summary of the purification of β-glucosidase T12 derived from *Trichoderma harzianum*.

Fraction	Total Activity of β-glucosidase (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude extract	756.0	338.0	2.24	1.00	100.00
20% $(NH_4)_2SO_4$	630.0	225.9	3.76	1.67	83.33
40% $(NH_4)_2SO_4$	532.5	130.4	4.61	2.05	70.37
60% $(NH_4)_2SO_4$	360.8	85.6	5.87	2.62	47.61
80% $(NH_4)_2SO_4$	342.0	52.0	6.58	2.94	45.23
Dialysis	263.0	25.0	10.52	4.70	34.79

Determination of Molecular Weight of β-glucosidase T12 and degree of purity

SDS-PAGE

Results show that purified β-glucosidase appeared to be homogeneous in the F3 and F4 on SDS-PAGE (Figure 1) as estimated from the obtained 96 kDa molecular mass (represented by single bands), indicating that the 60 and 80% saturation fractions of ammonium sulfate contained pure β-glucosidase, respectively. However, fractions precipitated from F1 (20% $(NH_4)_2SO_4$) and F2 (20% $(NH_4)_2SO_4$) showed presence of additional protein bands. So only fractions F3 and F4 were combined and used in subsequent enzyme characterization studies (Table 2).

Noteworthily, the estimated molecular mass of the purified β-glucosidase T12 was found comparable with those produced by *Pseudomonas purpurogenous* KJS 506 (89.6 kDa), *T. harzianum* T3 (98 kDa) and *T. viride* (99 kDa) (JEYA et al., 2010; RAMANI et al., 2012) although its molecular mass was a slightly lower as compared to β-glucosidase from *Sechium edule* (116 kDa) (MATEOS et al., 2015). The 16S rDNA nucleotide sequence of *T. harzianum* T12 was deposited in the GenBank database with accession number KY004113.

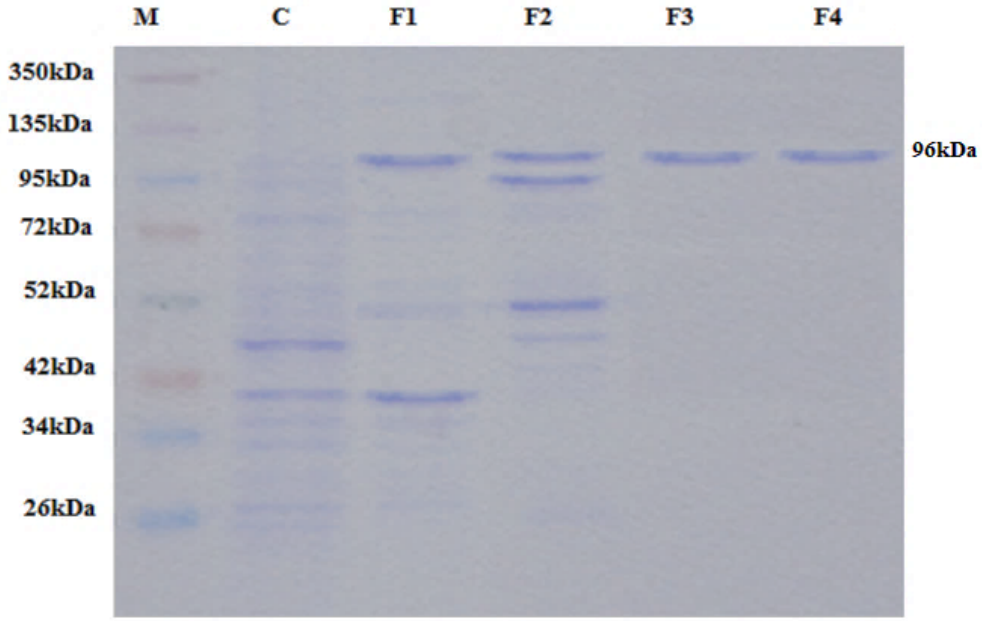

Figure 1. SDS-PAGE of the fractions obtained from the purification of crude β-glucosidase enzyme T12 using ammonium sulfate; M: 1 kB Protein ladder, C: Protein precipitations at various percentages of $(NH_4)_2SO_4$ are as follows: F1; 20%, F2:40 %, F3: 60 % (96 kDa) and F4: 80 % $(NH_4)_2SO_4$ (96 kDa).

HPLC Analysis of β-glucosidase T12 and MALDI-TOF-MS

The purity of β-glucosidase T12 was further checked using HPLC and the result (Figure 2) revealed that the enzyme showed a single peak at retention time of 1 min. The obtained molecular mass of the protein presented here agrees well with the single protein band (96 kDa) seen on SDS-PAGE, suggesting that the purification protocol used in this study yielded a pure enzyme solution.

Molecular mass of β-glucosidase T12 was also determined by MALDI-TOF. It can be seen that the purified enzyme has a major peak showing a molecular mass of 98.3 kDa (Figure 3). We realised that accomplishing good MALDI spectra for our β-glucosidase T12 protein may prove quite challenging as the process involves close attention to salt and denaturant concentrations, matrix/analyte ratios, and the T-leap, all of which were crucial in maintaining protein solubility. However, the obtained result insinuated that we were successful in obtaining a good spectra. It appears that the active form of the enzyme is monomeric as the observed molecular mass in MALDI-TOF-MS (98.3 kDa) agreed relatively well with that acquired by SDS-PAGE (96 kDa).

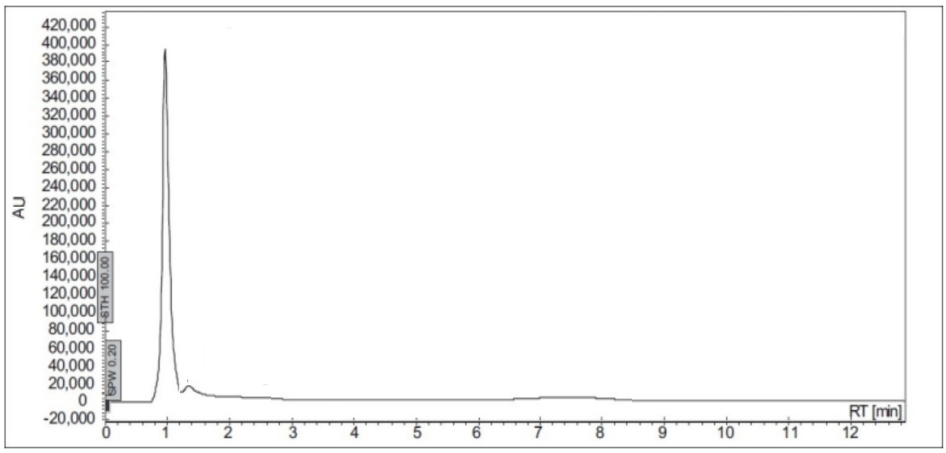

Figure 2. Purity check of β-glucosidase T12 by HPLC chromatogram

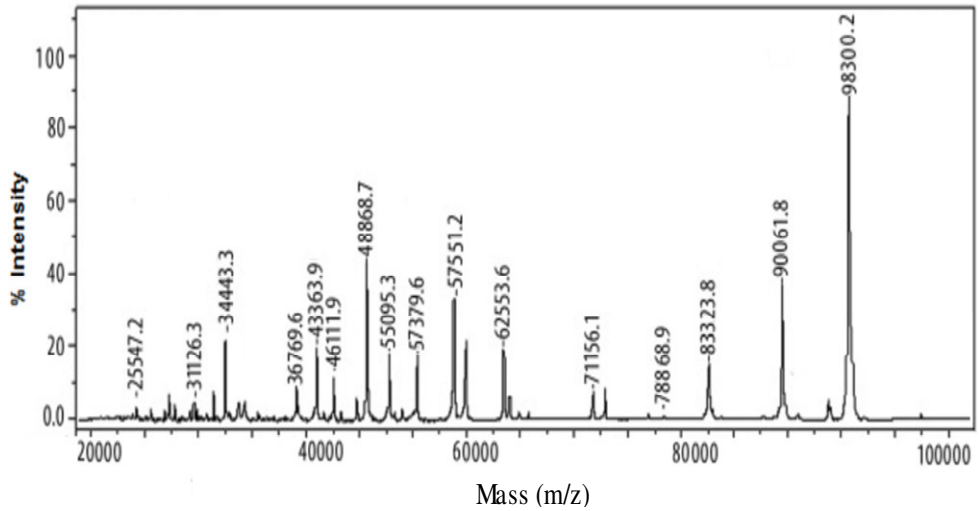

Figure 3. MALDI-TOF-MS spectrum of purified β-glucosidase T12.

Optimal Conditions for the Activity of β-glucosidase T12

Effect of temperature

It has been indicated that a wide range of applications would require an enzyme to have a relatively high thermal-stability (GOUJON, 2010). Considering that the surrounding temperature can significantly influence activity and the stability of an enzyme (MOHAMAD et al., 2015a), such factor was evaluated in this study and the significant data are tabulated in Figure 4a. It was observed that the activity profile for the enzyme was increased with increment of the temperature and peaked at 45°C (75.0 U/mL), before a decline was observed. Activity

of β-glucosidase T12 was at its lowest at 25 °C (18.5 U/mL) and 60 °C (20.0 U/mL), possibly attributable to rigidity of the enzyme structure that has yet to achieve its catalytically active form, and the greater unfolding of its protein at a high temperature, respectively (WAHAB et al., 2012a; WAHAB et al., 2014). The catalytic behaviour of β-glucosidase T12 seen here is consistent with those reported for mesophilic organisms (25-45 °C) (WAHAB et al., 2015). Similarly, the highest activity of β-glucosidase derived from *Debaryomyces vanrijiae* occurred at 40 °C. Although optimum activities of various fungal β-glucosidases have been reported to range between 40°C and 50°C, mesophilic strains producing thermally stable β-glucosidases have also been reported (GARCIA et al., 2015).

In this context, the fact that the optimum temperature within the *in vivo* setting of an enzyme would be markedly lower than that of *in vitro* (plate, greenhouse, open field), attaining the optimum activity of β-glucosidase T12 *via in vivo* process for agricultural management of charcoal rot disease is a slow process as the fungus requires a longer duration to sufficiently mature and to begin producing the enzyme, in order to exert its antagonistic activity against *M. phaseolina*. Therefore, harvesting the β-glucosidase T12 from *T. harzianum* as a component of biofungicide to control *M. phaseolina* infestation *via* an *in vitro* process (direct spraying) reported here appears feasible and deserves consideration.

Interestingly, the time course profile for the stability of β-glucosidase T12 (Figure 4b) revealed the enzyme was quite resistant to high temperature denaturation even for up to 45 ˚C (70 U/mL) and remains quite active for long durations of incubation (up to 240 mins). Pertinently, β-glucosidase T12 exhibited remarkable half-life (240 min) for most of the assessed temperature, except for 25 and 60˚C.

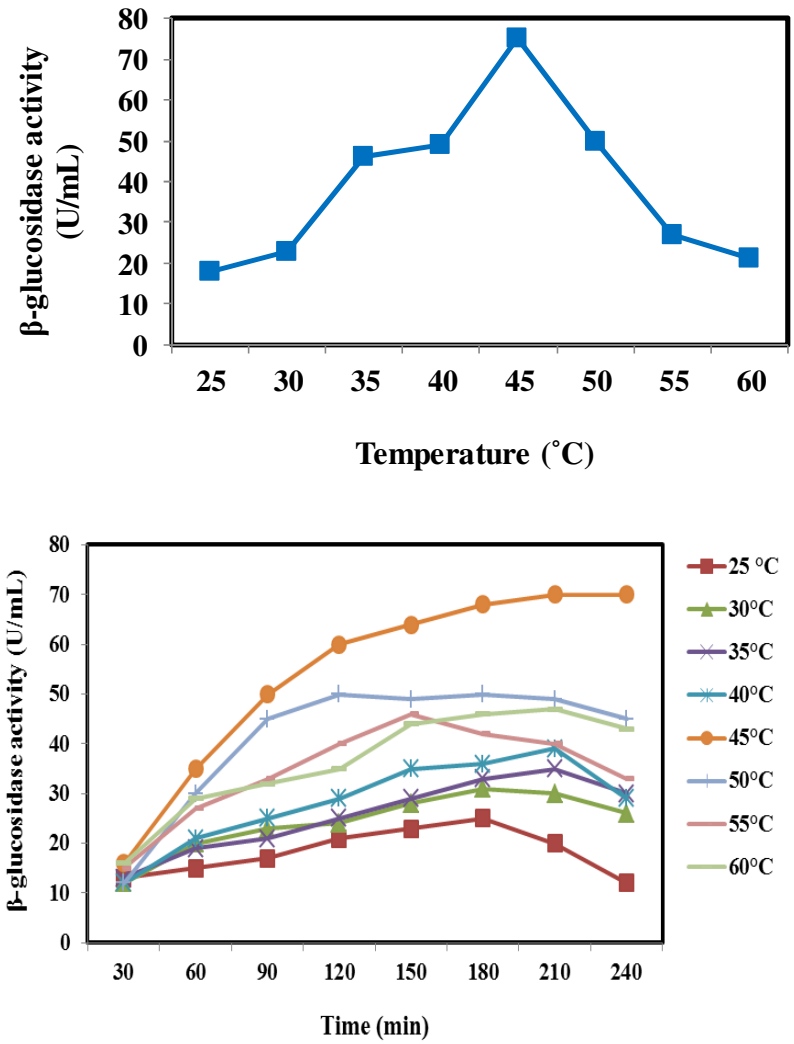

Figure 4. Effect of different temperatures on activity of purified β-glucosidase T12. (a) activity and (b) thermostability [Assessment condition: up to 240 min, pH 7.0 at 200 rpm].

The low enzyme activity at 25 ˚C may be explained by the sub-optimal of the surrounding to permit efficient catalysis, while 60˚C may be nearing the denaturation temperature of the enzyme. Likewise, the results corroborate the fact that the enzyme was relatively thermostable, possibly suited for other commercial applications requiring prolonged reaction times aside for agricultural applications. In general, researchers have indicated that the thermal stability of β-glucosidases varies between 30-50 ˚C (VITERBO et al., 2002) and last within a short span of time, in which substantial reductions in the overall enzyme activity occur beyond 40 ˚C (YUN et al., 2001). Hence, β-glucosidase T12 reported here is considerably more stable, suggesting its wide applicability for industrial and agronomical usages. Results of thermal stability and durability of β-glucosidase T12 indicate the suitability of its use for controlling infestation of *M. phaseolina*, presumably occur by disruption of their protective cell wall (VITERBO et al., 2002). The outcome seen here is not uncommon for β-glucosidases as it mirrors a rather robust regulation of the enzyme protein at the cellular level (VITERBO et al., 2002).

Effect of pH

Enzymes are poly-ionic polymers that are sensitive to changes in pH and, hence vulnerable to alterations in the distribution of charges within the active site as well as on the enzyme surface (ILLANES, 2008). Figure 5a represents the reaction profile for the effect of pH on activity of β-glucosidase T12. Enzyme activity was found to increase from pH 3.0 (13 U/mL) to pH 7.0 (77 U/mL), beyond which a large decline was observed. Researchers have indicated that the optimum pH ofor β-glucosidases tend to vary from one fungal species to another (pH 4.0-6.0) (KUBICEK et al., 2001; KAUR et al., 2007; BAI et al., 2013; GARCIA et al., 2015), it can be seen that pH 7 is optimum for β-glucosidase T12. Activity of the enzyme was at its lowest at pH 3 (U/mL) and pH 9 (16 U/mL), which may be due to the partial loss of its catalytically active structure induced by the extreme pHs. This may be explained by (a) reversible protonation of the surface functional groups of the amino acids at high pH (resulting in more positively charged enzymes), (b) deprotonation at very low pH (producing more negatively charge enzymes) and (c) irreversible denaturation of its poly-ionic three-dimensional structure. Furthermore, the surrounding environment pH has been known to directly influence the ionization of the substrate as well as its binding to the enzyme (BISWANGER, 2014).

As the catalytic behavior of enzymes is significantly dependent on the surrounding pH, specific evaluation focusing on the long-term effect of various pH on the activity of enzyme may provide empirical evidence on their tolerance. In this present study, the pH stability of the β-glucosidase T12 was examined by pre-incubating the enzyme solution at pH 3.0-9.0 for 12 h at room temperature (30 ˚C) and the results are presented in Figure 5b. Results revealed that β-glucosidase T12 remained structurally stable particularly at its optimal pH 7.0 (70 U/mL, up to 240 mins) followed by pH 8.0 (60 U/mL, up to 210 mins). β-glucosidase activities observed here are marginally higher than those reported by previous researchers (pH 2.5-6.0) (KAUR et al., 2007; JAYA et al., 2010; GARCIA et al., 2015). The remarkably long half-lives showed by β-glucosidase T12 (exceeded 4 h) in a wide range of buffers (pH 7-9), were comparatively longer than those previously reported by Souza et al. (2010) and Jabbour et al. (2012), inferring good resilience of the enzyme. Moreover, considering that soil pH is type-specific, and the fact that the β-glucosidase T12 produced here is effective within a wide range of pH, its application to control infestation of *M. phaseolina* may attract broader agricultural application.

Effect of Metal Ions

The influence of various monovalent and divalent metal ions (10 mmol/L) on the activity β-glucosidase T12 derived was monitored by incubating the enzyme solutions for 30 min at 50 °C prior to activity assay. Except for Cu^{2+} (45 %) and Ca^{2+} (66 %), other divalent metal ions *viz.* Zn^{2+}, Mn^{2+}, Mg^{2+} were found to enhance activity of β-glucosidase T12 as compared to the negative control (100 U/mL, 100 %), resulting in higher residual activity corresponding to 122, 103 and 102 %, respectively. These results suggest that supplementation with these metal ions can prospectively improve efficacy of the enzyme in the formulation to control *M. phaseolina*. While β-glucosidase T12 was inhibited by monovalent ions *viz.* Li^{+} (79 %), K^{+} (77 %), Na^{+} (66 %), inhibition of enzyme activity was more evident in reactions supplemented with trivalent Fe^{3+} ions (55 %) (Figure 6).

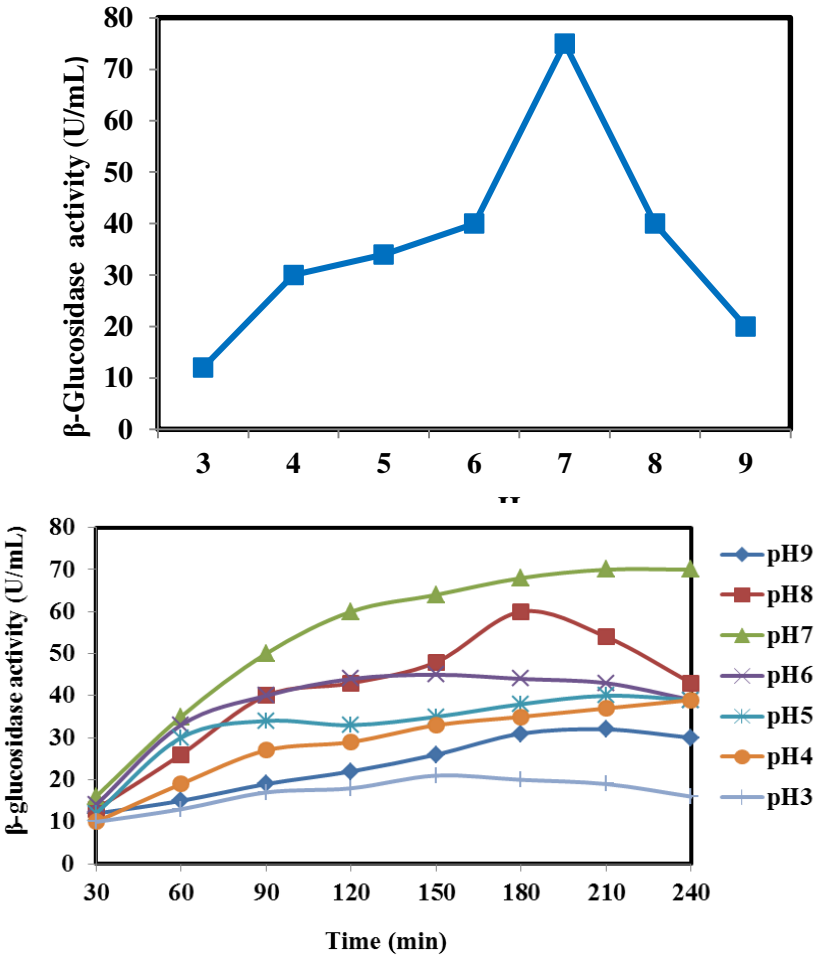

Figure 5. Effect of different pH on activity of purified β-glucosidase T12 (a) activity and **(b)** pH stability [Assessment condition: Up to 240 min at 45 °C, pH 7.0 at 200 rpm.

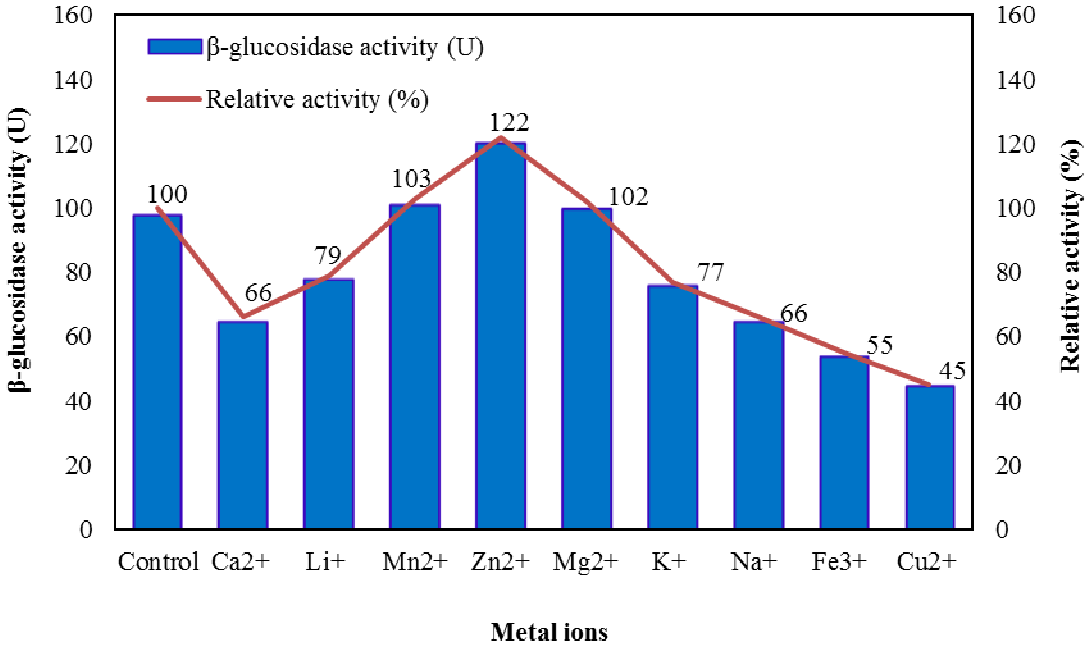

Figure 6. Effect of metal ions (10 mM) on activity of purified β-glucosidase T12. The data labels depict the relative activities of the enzyme in comparison to the negative control. [Assessment condition: 45°C, pH 7 at 200 rpm].

Overall, the study shows that β-glucosidase T12 is similarly inhibited like other fungal β-glucosidases and hydrolases assessed for divalent metal ions such as Zn^{2+} (JABBOUR et al., 2012), Fe^{2+} (SUN et al., 2009), Cu^{2+} (BHIRI et al., 2008; KARBOUNE et al., 2008) and Ca^{2+} (KARBOUNE et al., 2008). Equally, β-glucosidases are generally inhibited by monovalent ions i.e. Li^{+}, possibly due to interaction with the SH group of cysteines within the vicinity or substrate binding site of the enzyme. The phenomenon becomes enhanced when there is an insufficient difference in electronegativity to form a full ionic bond. Additionally, interaction between monovalent ions and β-glucosidases can displace the hydrogen atom in disulphide and consequently altering the folding of the protein structure. These changes can effectively inhibit activity of some enzymes (MATEOS et al., 2015).

Effect of Surfactants

Assessments on the effects of various surfactants on β-glucosidase T12 showed that significantly higher ($p \leq 0.05$) specific activities were achieved after 120 h of incubation in Tween 80 (170 U/mL, 184 %), Tween 100 (134 U/mL, 146 %), Tween 40 (120 U/mL, 130 %) and Tween 20 (101, U/mL, 110 %), respectively. Levels of enzyme activity were markedly lower in reaction mixtures supplemented with Triton X-100 (90 %) (Figure 7a). The generally positive influence of surfactants of the Tween series (Tween 20, Tween 40, Tween 80, Tween 100 and Triton X-100) on activity of β-glucosidase T12 (Figure 7a) was largely due to the non-ionic nature of these surfactants. Their rigid and bulky non-polar heads tend to be less denaturing as their molecules are unable to penetrate the three-dimensional structure of the water-soluble protein (WAHAB et al., 2012a), thereby preserving the native conformation of β-glucosidase T12 protein. Supplementation with Tween 80 showed positive improvements presumably due to the surfactant having the largest hydrophobic surface that can better bind to the surface of the proteins and induce structural changes (WAHAB et al., 2012a), hence stimulating enzyme activity. Similarly, the use of 0.01 % (v/w) Tween 80 has been reported beneficial in improving activities of several types of enzymes such as exoglucanase, endoglucanase and β-glucosidase (cellulases) by increasing availability of the nutrients to the active sites for catalysis (EL-HAWARY and MOSTAKA, 2011).

Effect of Natural Carbon and Nitrogen Source

Earlier studies have reported that different agricultural products such as wheat straw, rice bran and soybean meal have been used for producing cellulosic enzymes such as β-glucosidases (EL-HAWARY and MOSTAKA, 2011). The effects of various natural carbon sources (potato peel, pectin, wheat husk, copra meal and wheat bran) on activity of β-glucosidase T12 were investigated and the data are presented in Figure 7b. The activity of β-glucosidase T12 in the presence of wheat bran was significantly the highest ($p \leq 0.05$) when compared to the rest of the natural sources tested in this present research. Specific activity of β-glucosidase T12 was the highest in mixtures supplemented with wheat bran corresponding to 180 U mL (189 %) ($p \leq 0.05$), possibly attributable to presence of adequate amounts of macronutrients i.e. proteins, carbohydrates, fats, fiber as well as micronutrients calcium, magnesium, phosphorus, potassium and sulfur, essential for high enzyme production and cell mass (IQBAL et al., 2011). Specific activity was second highest in copra meal (161 U/mL, 168 %) while, mixtures containing potato peel gave the lowest specific activity at 58 U/mL (61 %) (Figure 7b). Supplementation with wheat husk and pectin were found to negatively affect enzyme properties and caused reduction in specific activity of β-glucosidase T12 to 92 U/mL (96 %) and 79 U/mL (83 %), respectively (Figure 7b).

Being the major component of proteins, nitrogen is required for fungal growth and consequently to produce enzymes. Specific activity of β-glucosidase T12 was maximum after 120 min of incubation in the presence of $(NH_4)_2SO_4$ (160 U/mL) ($p \leq 0.05$), while the lowest in the presence of NH_4NO_3 (79 U/mL) ($p \leq 0.05$) (Figure 7c). No significant difference in enzyme activity was observed in mixtures supplemented with KNO_3 (90 U/mL), NH_4Cl (88 U/mL) and $NaNO_3$ (80 U/mL) (Figure 7c) ($p > 0.05$). Likewise, previous researchers (GOYARI et al., 2014) have described that $(NH_4)_2SO_4$ was an appropriate nitrogen source for cultivating fungal cultures followed by NH_4NO_3, KNO_3, peptone and urea (CRUCELLO et al., 2015; GOYARI et al., 2014); hence their findings appear to be supported by the results in this study (Figure 5c). However, using the abovementioned carbon and nitrogen sources as growth media to culture *T. harzianum* can be a rather costly approach to produce β-glucosidase T12, hence the use of other natural nitrogen sources i.e. banana wastes may promise a more feasible solution. Moreover, the banana biomass can be put to good use in producing a more value-added product i.e. β-glucosidase T12.

Effect of Inoculum Size

It has been indicated that an inoculum size can affect the overall microbial growth in the culture, a determinant factor in enzyme activity. While utilization of inadequate inoculum sizes would result in a shorter fungal lag phase, utilization of inoculum sizes beyond the optimum can increase moisture factor and cause an undesirable overcrowding of fungal spores in the growth culture (IRSHAD et al., 2013). Results revealed that the maximum enzyme activity of 119 U/mL was attained in experimental flasks receiving 5 mL (10 %) of inoculum density of co-culture (*T. harzianum* T12), beyond which the activity started to reduce (Figure 7d). Beyond the optimum inoculum size, enzyme activity declined as described by a previous work, generally due to poor development of fungal machinery to produce high concentrations of the enzyme or overproduction of fungal spores (ZHANG et al., 2014).

Determination of Kinetic Parameters of β-glucosidase T12

The kinetic parameters K_m, V_{max} and k_{cat} of the T12 β-glucosidase were estimated by the Michaelis-Menten plot (double reciprocal plot) and determined as 0.79 mM, 8.45 mM min^{-1} mg^{-1} protein and a turnover number (catalytic efficiency) of 10.69 s^{-1}. The high value of coefficient of determination (R^2 = 0.9999) signified the kinetic plot was accurate and reliable. K_m value for β-glucosidase T12 was lower than a same enzyme produced by *Fusarium solani* (K_m 1 mM) (RAMANI et al., 2010), but was seen higher than β-glucosidases from other fungi such as *T. harzianum* fungus (K_m 0.057 mM), *T. viride* (K_m 0.09 mM) (RAMANI et al., 2010) and *Penicillium occitanis* (K_m 0.37 mM) (BHATTI and BATOOL, 2013). A low disassociation constant, K_m, seen here indicated a strong affinity of β-glucosidase T12 towards *p*-NPG. Most importantly, the data agreed with the perceptively rapid proliferation of the *T. harzianum* T12 in the banana waste media used initially for growing the fungal starter culture. The exceptionally high V_{max} value (8.45 mM/min/mg^{-1}) of β-glucosidase T12 as compared to β-glucosidases from *T. viride* (V_{max} 76 µmole $min^{-1}mg^{-1}$) (KUBICEK et al., 2001) and *Fusarium solani* (V_{max} 55.6 µmole min^{-1}) (RAMANI et al., 2010) also affirmed that the enzyme was reasonably efficient in hydrolysing *p*-NPG as the substrate.

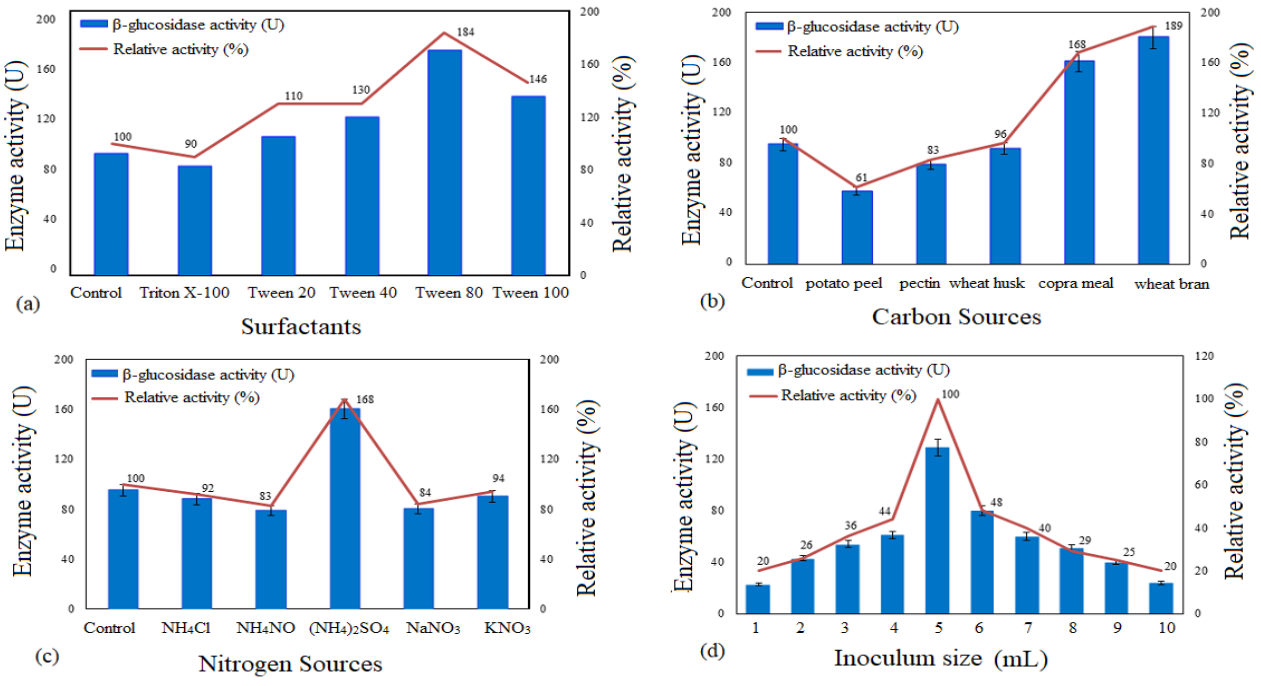

Figure 7. Effect of various parameters on activity of purified β-glucosidase T12. A) Effect of surfactant (1% w/v) depicted as relative activity. [Assessment condition: 45°C, pH 7 at 240 min]. B) Effect of natural carbon sources [Assessment condition: 45°C, pH 7 at 240 min]; C) Effect of nitrogen sources (1% w/v) [Assessment condition: 45°C, pH 7 at 240 min]. D) Effect of inoculum size [Assessment condition: 45°C, pH 7 at 240 min].

β-glucosidase T12 Sequence and Analysis

PCR product was sent to 1st BASE Laboratories (Malaysia) for sequencing and the primers were able to amplify the putative β-glucosidase T12 gene. The result showed that the complete β-glucosidase T12 sequence was found in positions 1−1367 (Figure 8).

1	atgttgccca	aggactttca	gtggggtttt	gcgacggctg	cctaccagat	tgaaggcgcc
61	atcgacaaag	acggccgagg	ccccagcatc	tgggacacct	tttgcgccat	ccccggcaag
121	attgccgatg	gaacctccgg	cgtcacggcc	tgcgactcgt	acaaccgcac	cgccgaagat
181	attgcgctac	tcaagtcgct	tggagcaaag	tcgtaccgct	tctccatctc	ctggtcccga
241	ctggatttcg	agggcggccg	cgacgaccct	gtgaatcagc	tgggaatcga	ccattatgca
301	cagtttgtcg	acgacctgct	ggaggcgggc	atcacgccct	tcatcacgct	gttccactgg
361	gatctgcccg	aggagctgca	tcagcgatat	ggtggcttgt	tgaaccgcac	cgagttcccg
421	ctggatttcg	aaaactatgc	gcgcgtcatg	ttcaaggcat	tgcccaaggt	gaggaactgg
481	atcaccttca	acgagccgct	gtgctctgcc	atccccggtt	acggctctgg	cacttttgcc
541	cctggccgcc	agagcaccac	cgagccttgg	atcgttggcc	acaaccttct	tgtcgcccac
601	ggccgtgctg	tcaaggtgta	ccgcgacgag	ttcaaggacc	tcaacgatgg	ccagatcggc
661	atcgtcctca	atggcgactt	tacctatccc	tgggactcgt	ctgatccccc	cgacagagag
721	gccgccgaga	ggcgattgga	gttcttcacg	gcgtggtatg	cggatcccat	ctacctgggc
781	gactaccctg	cctctatgcg	caagcagctg	ggcgaccgcc	tgccagagtt	tacgcccgag
841	gagaaggcct	ttgtccttgg	ctccaacgac	ttctacggca	tgaaccacta	cacgtccaac
901	aagattctgg	accgcacctc	gcctgccacc	gcggacgaca	ctgttggcaa	cgtcgatgtc
961	ttgttctaca	acaaggaggg	ccagtgcatc	ggcccagaga	cggaatcctc	gtggcttcgt
1021	ccttgtcccg	ctggcttccg	cgatttcctc	gtgtggatca	gcaagcgtta	caactacccc
1081	aagatctacg	tcaccgagaa	cggcacgagt	ctcaaggggg	agaacgacct	gcccaaggag
1141	aagattctgg	aggatgattt	ccgcgtcaac	tactacaacg	agtatatccg	cgccatgttc
1201	accgccgcga	cgctagacgg	agtgaacgtc	aagggatact	ttgcctggtc	gctgatggac
1261	aactttgagt	gggctgatgg	ctacgtgaca	agatttggtg	tgacttatgt	ggattacgag
1321	aatggacagc	agcgattccc	gaagaagagc	gcgaagagct	tgaagcctt	ttgatt 1376

Figure 8. Nucleotide alignment for the full sequence of DNA of the β-glucosidase T12 fragment amplified using designed primers TFbgl2 and TRbgl2, start and end codons are shown in the box.

The invariant positions in the nucleotide sequence were removed and the nucleotide sequence of the β-glucosidase T12 gene has been deposited in the NCBI GenBank database under accession number KY199423. A noteworthy point to highlight here, the ITS rRNA gene sequence in most fungi species is usually conserved within the organisms and therefore, this information can be used to accurately identify fungal species. Based on the result of BLASTn, the nucleotide sequence of β-glucosidase T12 was highly identical to β-glucosidases from other Trichoderma species *viz. Trichoderma harzianum* strain IOC-3844 (99%) followed by *Trichoderma gamsii* and *Trichoderma virens* bgl1 (86 %), *Trichoderma reesei* strain SJVTR and *Trichoderma viride* strain AS 3.3711 (84 %). However, these hyperproducing enzymes have been mainly investigated for biomass i.e. cellulose degradation (GOYARI et al., 2014), soil nutrient cycling (CONTRERAS-CORNEJO et al., 2009), regulating the growth and development of Arabidopsis (*Arabidopsis thaliana*) seedlings (LI et al., 2011) and bioconversion of cellulose into glucose (GAUTAM et al., 2010), and not as a bioactive component for fungicide. Table 3 show BLASTn search results with significant alignments with the top 5 entries species in the NCBI database.

Table 3. Comparison of the 16s RNA nucleotide sequence of β-glucosidase T12 from *T. harzianum* T12 with other β-glucosidases.

Microorganisms Description	Accession Number	Sequence Identity
Trichoderma harzianum strain T12 β-glucosidase (bgl)	KY199423	-
Trichoderma harzianum strain IOC 3844 β-glucosidase (bgl2)	KU201604.1	99 %
Trichoderma gamsii β-glucosidase	XM018806048.1	68 %
Trichoderma virens bgl1 (BGL1) β-glucosidase	KU535892.1	86 %
Trichoderma reesei strain SJVTR β-glucosidase	KF979307.1	84 %
Trichoderma viride strain AS 3.3711 β-glucosidase 2 (bgl2)	AY343988.1	84 %

CONCLUSIONS

A purified β-glucosidase T12 harvested from the culture supernatant of *T. harzianum* showed good inhibition against the causal agent of charcoal rot disease i.e. *M. phaseolina*.

The optimum condition that gave the highest β-glucosidase activity is as follows: 45˚C, pH 7, inoculum size of 10 % (w/v), supplementation with metal ions, Zn^{2+} and Mn^{2+} ions and Tween 80, using wheat bran and $(NH_4)_2SO_4$ as the carbon and nitrogen sources, respectively.

BLASTn results showed the nucleotide sequence of β-glucosidase T12 was highly identical to β-glucosidases from *Trichoderma harzianum* strain IOC-3844 (99%) followed by *Trichoderma gamsii* and *Trichoderma virens* bgl1 (86 %), *Trichoderma reesei* strain SJVTR and *Trichoderma viride* strain AS 3.3711 (84 %).

Kinetic assessments tested under optimized conditions revealed the β-glucosidase T12 has a relatively high activity with values of K_m, V_{max} and k_{cat} corresponding to 0.79 mM, 8.45 mM min^{-1} mg^{-1} protein and 10.69 s^{-1}, respectively.

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RESUMO: Devido à toxicidade e ineficiência dos fungicidas químicos para controlar a infestação de Macrophomina phaseolina (Tassi) Goid que causa o apodrecimento das plantas, uma abordagem biotecnológica usando β-glicosidase (EC.3.2.1) como o ingrediente bioativo alternativo do fungicida é por este meio, proposto. A enzima extracelular foi isolada de um antagonista fúngico altamente eficiente, o Trichoderma harzianum T12. As massas moleculares altamente similares obtidas usando SDS-PAGE (96 kDa) e espectrometria de massa MALDI-TOF (98,3 kDa) afirmaram que a β-glicosidase foi purificada até a homogeneidade. Consequentemente, os parâmetros catalíticos ótimos que apresentaram a maior atividade enzimática foram: 45˚C, pH 7, tamanho do inóculo de 10% (p / v), suplementação com íons de metais Zn2+ e Mn2+, e Tween 80. Adição de farelo de trigo e (NH4) 2SO4 como fontes de carbono e nitrogênio também melhoraram a atividade enzimática. O BLASTn mostrou que a sequência da β-glicosidase T12 era altamente idêntica a outras β-glicosidase viz. A estirpe T. harzianum IOC-3844 (99%), T. gamsii e T. virens bgl1 (86%) assim como a estirpe T. reesei SJVTR e a estirpe T. viride AS 3.3711 (84%). A avaliação cinética mostrou que β-glicosidase T12 catalisa a actividade hidrolítica caracterizada por um Km de 0,79 mM e Vmax de 8,45 mM min-1 mg-1 de proteína, com um correspondente kcat de 10,69 s-1.

PALAVRAS-CHAVE: β-glicosidase. Trichoderma harzianum. Avaliação cinética. Macrophomina phaseolina.

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