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Original Article

Partial purification and some properties of α–glucosidase from *Trichoderma longibrachiatum*

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ABSTRACT: The use of hydrolase enzyme plays an important role in the industrial production of α-D-glucose from carbohydrate sources. This study investigated partial purification and characterization of α-glucosidase from *Trichoderma longibrachiatum* with a view to enhancing its potentials in biotechnological processes. Strains of *Trichoderma longibrachiatum* were cultured on rice bran medium at 30°C for 96 hour for the production of α–glucosidase. The enzyme was partially purified by eluting the ammonium sulphate (70%) saturation precipitated sample on Sephadex G-75 and Sephadex G-25. Enzyme assay was carried out using p-nitrophenyl-α-D-glucopyranoside (PNP- α-G) as the substrate and protein concentration was determined. Kinetic parameters, molecular weight, pH effect, temperature and thermostability were also determined. The activity of enzyme in the presence of arylglucosides and different cations were monitored. The partially purified protein migrated as a single band in 10% SDS-Polyacrylamide gel-electrophoresis. The enzyme presented a relative molecular weight of about 58KDa as estimated by PAGE. The extracellular α–glucosidase showed typical α–glucosidase activity, hydrolyzing p-nitrophenyl-α-D-glucopyranoside and exhibited optimum catalytic activity (4.89µmol/ml/min) at 40°C and pH 4.5. The enzyme was stable at 40°C for 150 minutes. Carboxymethylcellulose was also hydrolyzed by this enzyme. The K_m and V_{max} with p-nitrophenyl- α -D-glucopyranoside were 33.33mM and 20.00 µmol/min/mg protein respectively. This study therefore revealed the presence of α–glucosidase in *Trichoderma longibrachiatum* which could serve as alternative species for the production of α–glucosidase enzyme.

KEYWORDS: Enzyme purification, characterization, α-glucosidase, *Trichoderma longibrachiatum*

INTRODUCTION

α-Glucosidases (α-D-glucoside glucohydrolase; EC 3.2.1.20) are a widespread group of enzymes that catalyze the hydrolysis of the α-glucosidic bond from the non-reducing end of a chain as well as the α-glucosidic bond of free disaccharides[1,2]. They also catalyze other aryl- and alkyl- α-glucopyranoside (3). Many known α-glucosidases seem to prefer the α-1,4 bonds of maltose or maltooligosaccharides [1]. Thus, the enzymes are classified on the basis of the type of glycosidic bond they cleave. For instance, α-glucosidase shows specificity for α-glucoside, thus:

α-D-Glucoside + H_2O ⟶ D-Glucose +Alcohol

Glucosidase enzymes are involved in several biological processes such as the intestinal digestion, the biosynthesis of glycoproteins and the lysosomal catabolism of the glycoconjugates (Homonojirimycin isomers and N-alkylated homonojirimycins) [4]. It has been discovered that many organisms that produce extracellular amylolytic enzymes also produce an intracellular α-glucosidase. In this instance, α-glucosidase is the final enzyme involved in the metabolism of starch, or perhaps other carbohydrates, to glucose. Intestinal α-glucosidases are involved in the final step of the carbohydrate digestion to convert these into monosaccharides which are absorbed from the intestine.

Alpha-glucosidase has potential uses in biotechnological processes such as the production of glucose syrup and in brewing industry [3]. In view of the fact that there are several cellulosic wastes that are still remain untapped because of the unavailability of degrading enzymes, this study was therefore carried out with the aim of producing, purifying and characterize alpha-glucosidase enzyme from Trichoderma longibrachiatum which could ultimately be of industrial importance.

MATERIALS AND METHODS

Materials

Materials used include soluble starch, D-glucose, p-nitrophenyl- α-D-glucopyranoside (PNP α-G), bovine serum albumin (BSA), 66,000; egg albumin, 45,000; glyceraldehyde-3-

phosphate dehydrogenase (G3PG), 36,000; and bovine carbonic anhydrase (BCA), 29,000 were purchased from Sigma Chemicals Company, St. Louis, MO, U.S.A. Sephadex G-25 and Sephadex G-75 sodium dodecyl sulphate (SDS), acrylamide gel and methylene bisacrylamide were purchased from Pharmacia Fine Chemicals, Upsalla, Sweden. Other reagents used were of analytical grade and were obtained from BDH Chemicals, Poole, U.K.

Culture condition/organism

Stock culture of *Trichoderma longibrachiatum* was obtained at the Department of Microbiology, University of Agriculture, Abeokuta, Nigeria. The organism was maintained on potato dextrose agar and kept at 4°C until needed.

Crude enzyme extraction from rice bran

Extraction of crude enzyme from the rice bran was carried out following the procedure described by Iwata et al., [5]. Briefly, 1g of rice bran was suspended in 10ml of 10 mM acetate buffer, pH 5.0, containing 5 mM DTT and 90 mM NaCl and maintained at 4°C for 12 h. The mixture was centrifuged at 20°C (10,000 x g) for 20 min. After filtration through a 0.45 µm membrane filter (Gelman Sciences, Ann Arbor, USA), the supernatant was used for further investigation as a crude enzyme extract.

Production of crude α-glucosidase

Trichoderma longibrachiatum production was carried out on a solid state medium containing rice bran, soybean flour and cassava starch mixed in ratio (10:3:1, w/w) according to Akpan and Adelaja, [6]. The mixture in petri dish was moistened with sterile distilled water to 55% moisture content. The pH was adjusted to 4.5 with 0.1M HCl and sterilized at 121°C for 15 minutes. The sterilized medium was inoculated with a loopful of spores of a 24 hour old culture of *Trichoderma longibrachiatum* and incubated at 30°C for 72 hours.

Isolation and purification of α-glucosidase: Ammonium sulphate precipitation

All steps were carried out at 20°C unless stated. The crude enzyme was precipitated with ammonium sulphate (70% saturation) at room temperature, stirred for 2 hour and then centrifuged at 15,000 x g for 30 minutes. The pellet was suspended in 20mM Tris-HCl buffer, pH 8.5, and dialyzed against this buffer for 4 hours.

Gel-filtration on Sephadex G-75 Column

The ammonium sulphate precipitated α-glucosidase (1.0mg/ml) was carefully layered on Sephadex G-75 column bed surface (2.6 x 90cm) that had been previously calibrated (using Blue Dextran G-2000) and equilibrated with about 3 litres of 0.05M sodium acetate buffer, pH 5.0, containing 1mM EDTA and was then allowed to drain into the bed. The sample was washed into the bed with additional buffer. Enough buffers was then added to the column and then connected to the reservoir. Elution was performed at a flow rate of 10ml/hr and fractions of 5ml each were collected. Absorbance of fractions was measured at 280 nm and assay carried out on the fractions to determine the α-glucosidase activity. The fractions containing α-glucosidase activity were pooled and concentrated.

Sephadex G-25 chromatography

The concentrated precipitated samples containing ammonium sulphate were desalted by using Sephadex G-25 (2.0 x 30cm). 2g/20ml solution of Sephadex G-25 in distilled water was swollen for an hour. After boiling it was allowed to cool at room temperature and then pack into the small fractionating column using sodium acetate buffer. The sample was eluted and assays carried out on the fractions collected (as previously described) in order to determine the α-glucosidase activity.

Enzyme Assay

The activity of α-glucosidase was determined as described by Constantino *et al.*,[3]. A typical reaction mixture consisted of, in final concentration, 2.0mM PNP α-G (dissolved in 10mM sodium acetate buffer, pH 5.0 containing 1mM EDTA) and an appropriate amount of enzyme solution. The total reaction mixture was 3ml and was incubated at 40°C for 30 minutes. After incubation, an aliquot of 0.5ml was added to 5ml of 0.1M sodium carbonate (to stop the reaction) and the absorbance of the mixture was read at 400 nm. The enzyme activity was expressed as the number of micromole of p-nitrophenol formed at 40°C per minute using the extinction coefficient of $18.8x10^{-3}$ M^{-1} cm^{-1}.

Determination of protein concentration

Protein concentration of the reaction mixture was determined according to the method of Bradford [7] using bovine serum albumin (BSA) as standard.

Determination of kinetic parameters

The kinetic parameters (K_m and V_{max}) were determined for the partially purified α-glucosidase by measuring α-glucosidase activity at various concentrations of p-nitrophenyl- α-D-glucopyranoside (PNP α-G) from 0.0005M to 0.05M. The graph of reciprocal of the activity was plotted against the reciprocal of the substrate concentration. (i.e 1/v vs 1/[S]) and K_m and V_{max} estimated as described by Lineweaver and Burk [8].

Determination of relative molecular weight

The relative molecular weight of the partially purified α-glucosidase was determined on polyacrylamide gel electrophoresis (PAGE) according to method of Weber and Osborn [9] in 10% gel.

Effect of temperature on enzyme activity

A solution of enzyme at a concentration of 0.02mg/ml was assayed by the standard assay procedure at temperatures of 30, 40, 50, 60 and 70°C to investigate the effect of temperature on the rate of hydrolysis of p-nitrophenyl- α-D-glucopyranoside (PNPαG). 0.1ml of enzyme solution was incubated at 40°C for 30 minutes, and the reaction was stopped with 1ml of 0.1M Na_2CO_3. The absorbance (A_{400nm}) was measured against a blank. 1 U of α-glucosidase activity was defined as the amount of enzyme that produced 1µmol of p-nitrophenol per minutes under the conditions used.

Thermostability of α-glucosidase

Partially purified α-glucosidase was diluted 1:10 in 0.1M sodium phosphate (pH 6.5) and incubated for various length of time (30, 60 ,90, 120 and 150 minutes) in a water bath maintained at 40°Cwhile remaining α-glucosidase activity was measured as earlier described. 0.1ml of enzyme solution was incubated at 40°C, portions were taken at various time points, and the reaction was stopped with 1ml of 0.1M Na_2CO_3. The absorbance (A_{400nm}) was measured against a blank by using 0.1ml of distilled water instead of enzyme solution. 1U of α-glucosidase activity was defined as the amount of enzyme that produced 1µmol of p-nitrophenol per minutes under the conditions used.

Effect of pH on enzyme activity

The effect of pH on α-glucosidase activity was determined by carrying out the assay of the enzyme using the following three buffers at different pH values: 0.05M citrate buffer (pH 2.0 – 6.0) and 0.05M phosphate buffer (pH 7.0 – 8.0).

0.1ml of enzyme solution was incubated at 40 for 30 minutes, and the reaction was stopped with 1ml of 0.1M Na_2CO_3. The absorbance (A_{400nm}) was measured against a blank by using 0.1ml of distilled water instead of enzyme solution. 1U of α-glucosidase activity was defined as the amount of enzyme that produced 1µmol of p-nitrophenol per minutes under the conditions used.

Effect of substrates

Alpha glucosidase activity was determined using carboxymethyl cellulose (CMC) and maltose as substrates. The enzyme solution, 50µl was incubated with 0.05M acetate buffer pH 5.0 at 40°Cfor 30 minutes. After incubation, 0.25ml of the reaction mixture was pipetted and added to 2.5ml 0.1M Na_2CO_3 (ice cold) to stop the reaction. 1U of α-glucosidase activity was defined as the amount of enzyme that produced 1µmol of p-nitrophenol per minutes under the conditions used.

RESULTS

Activity profile/elution

The elution profile of the crude enzyme extract of the *Trichoderma longibrachiatum* on Sephadex G-75 column is shown in Fig. 1. The regions of high activities were pooled and solid ammonium sulphate added to 80% saturation, this was then desalted on Sephadex G-25 column. Summary of purification of α-glucosidase was also shown in Table 1. The ammonium sulphate precipitate crude sample was applied to a Sephadex G-75 column (2.6 x 90cm) which was previously equilibrated with 0.05M sodium acetate buffer pH 5.0. The flow rate was 10ml/hr and 5ml fractions were collected.

Determination of molecular weight

The relative molecular weight of the pure enzyme was determined by polyacrylamide gel electrophoresis accordance with the procedure of Weber and Osborn (9) on a 10% rod gel. Standard proteins were as contained in Sigma Molecular Weight Markers Calibration Kit for SDS polyacrylamide gel electrophoresis (Daltons Mark VII-L, Molecular Weight Marker Range 14,000-70,000). Polyacrylamide gel electrophoresis on 10% gel gave a single protein band for α-glucosidase, Fig 2, with relative molecular weight of 58,000 KDa, as intrapolated from the standard plot of log relative molecular weight (Rw) versus relative mobility (Rs) of each marker protein.

Kinetic parameters

The Lineweaver-Burk plot for the determination of kinetic parameters (K_m and V_{max}) for the partially purified α-glucosidase is shown in Fig. 3. The apparent Km and Vmax values estimated were 33.33mM and 20µmol/min/mg protein respectively for p-nitrophenyl-α-D-glucopyranoside(PNPG) as substrate.

Effect of temperature

The effect of temperature on the activity of α-glucosidase is shown in Fig. 4. The maximum enzyme activity (4.89 µmol/ml/min) was observed at 40°C.

Thermostability of α-Glucosidase

Fig. 5 shows the thermostability of *Trichoderma longibrachiatum* α-glucosidase. Partially purified α-glucosidase from *Trichoderma longibrachiatum* was stable for about 150 minutes following incubation at 40°C.

Effects of pH on α-Glucosidase activity

The effect of pH on the activity of α-glucosidase is shown in Fig. 6. The optimum pH range of α-glucosidase from *Trichoderma longibrachiatum* at 40°C was found to be between 4.0-4.5.

Effect of substrates

The extent of hydrolysis of two other substrates namely: maltose and carboxymethyl cellulose by this enzyme is presented in Table 2.

DISCUSSION

The existence of α-glucosidase isolated from fungi *Trichorderma species* had previously been reported (10). Since α-glucosidase is a glycoside hydrolase (amylolytic) enzyme, it is not surprising that it can be grown in carbohydrate media such as rice bran [11]. This study revealed the existence of α-glucosidase enzyme in a fungus *Trichoderma longibrachiatum*. This enzyme was partially purified by a procedure that consisted ammonium sulphate precipitation (70% (w/v)) and gel filtration on sephadex G-75. The relative molecular weight of 58,000 daltons obtained for α-glucosidase from *Trichoderma longibrachiatum* agreed fairly well with 59,000 daltons obtained for the α-glucosidase from protein extracts of *Rhizobium species* by Karine and Francis [12]. The molecular weight was also similar to that of intermediate species of *Aspergillus niveus*, isoenzyme with subunit molecular weight of 56,000 and 52,000 daltons as reported by Da Silva et al.,[13]. Kato et al., [14] reported the existence of heterodimeric forms of the α-glucosidase (AgdB) enzyme with subunit molecular weight of 74,000 and 55,000daltons from *Aspergillus nidulans.*

TABLE 1 Purification of **α**-glucosidase from *Trichoderma longibrachiatum*

Sample	Volume (ml)	Protein (mg/ml)	Total Amount (mg)	Activity (U/ml)	Specific Activity (U/mg)	Total Amt. (U)	Yield (%)	PF
Crude Extract	50	41.5	2075	7.20	0.174	360	100	1.00
$(NH_4)_2SO_4$ Precipitation	50	25.0	1250	6.82	0.273	341	95	1.6
Pooled Sephadex G-75	30	4.2	126	6.0	1.429	180	550	8.2
Pooled Sepahdex G-25	25	2.25	56.25	4.89	2.173	122.25	34	12.49

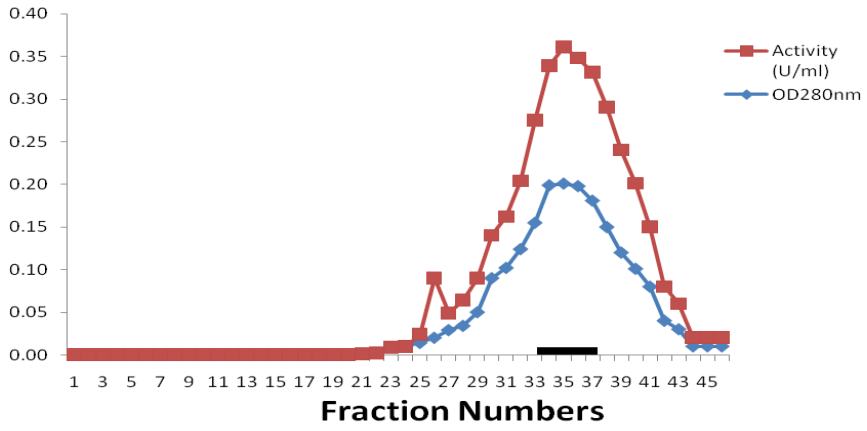

FIGURE 1 Elution profile of **α**-glucosidase on Sephadex G-75 column

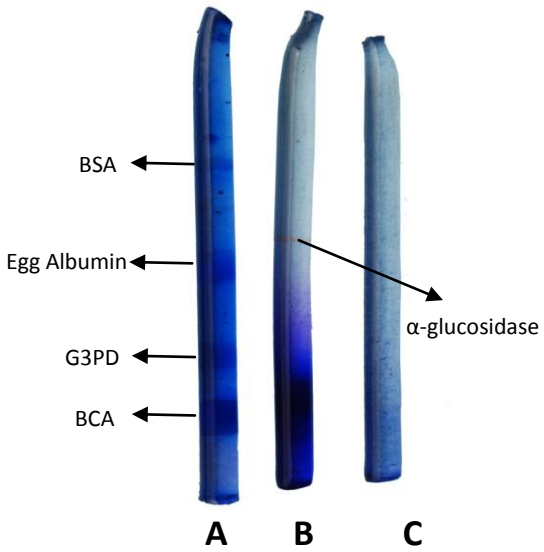

FIGURE 2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of **α**-glucosidase from *Trichoderma longibrachiatum*. The separating gel was 10% gel. The running buffer was 0.1 M phosphate, pH 7.2. The standard proteins were as contained in Sigma Molecular Weight Markers Calibration Kit consisting of BSA, 66,000; egg albumin, 45,000; glyceraldehydes-3-phosphate dehydrogenase(G3PD), 36,000; and bovine carbonic anhydrase (BCA) 29,000. A: The standard proteins. B: **α**-glucosidase. C: Control

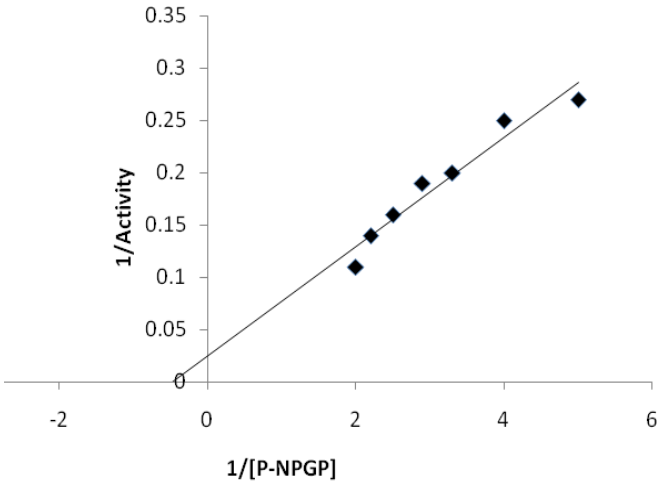

FIGURE 3 Lineweaver-Burk plot for the determination of the kinetic parameters (K_m and V_{max}) for partially purified **α**-glucosidase. Plot of 1/v vs 1/[PNPG]; 1/Activity (µmole of p-nitrophenol formed per ml per min); 1/ [PNPG] (mM^{-1}).

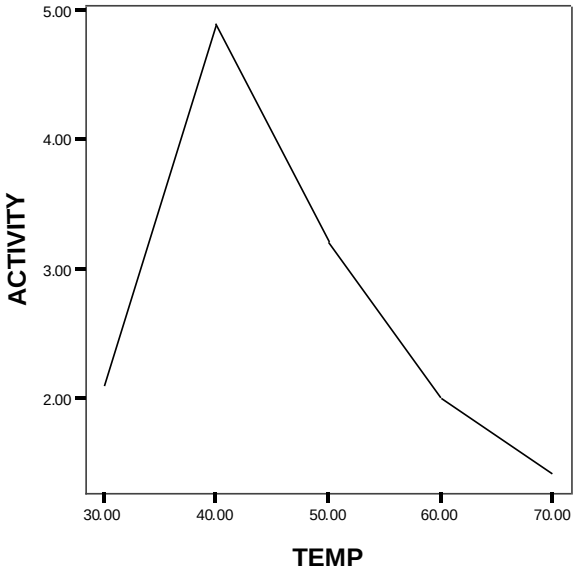

FIGURE 4 Effect of temperature on activity of **α**-glucosidase enzyme from *T. longibrachiatum*. The residual **α**-glucosidase activity of the partially purified enzyme was determined at various temperatures.

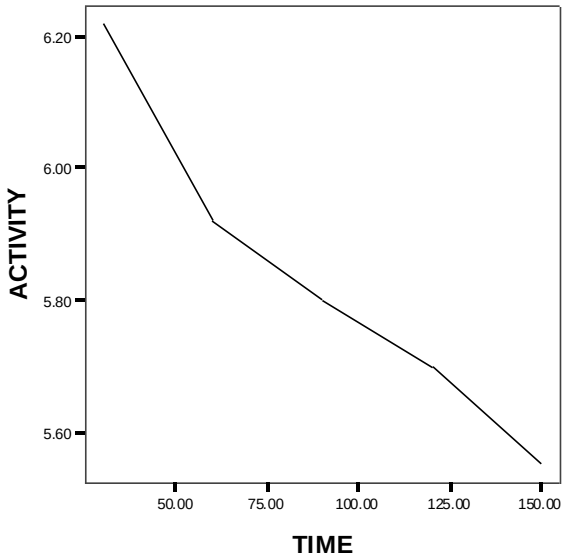

FIGURE 5 Thermostability of *T. longibrachiatum* **α**-glucosidase. The enzyme was incubated at 400C at various time intervals.

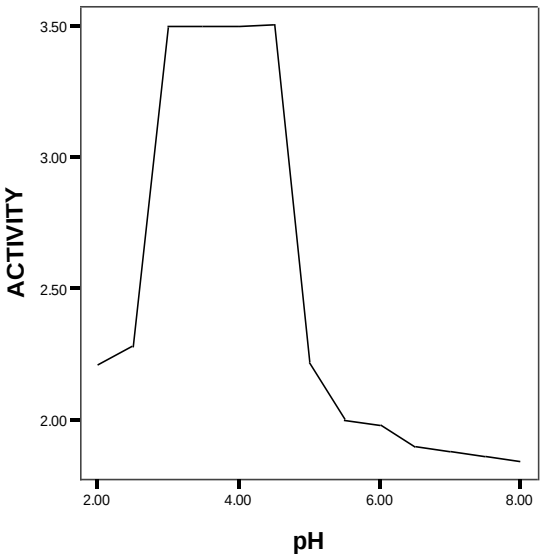

FIGURE 6 Effect of pH on *T. longibrachiatum* α-glucosidase activity. The relative α-glucosidase activity of the partially purified enzyme was determined at various pH values.

However, in the present study, we cannot say precisely whether *Trichorderma longibranchiatum* α-glucosidase is a monomeric, dimeric or heteromeric protein until the purified sample is subjected to sodium dodecylsulphate- polyacrylamide gel electrophoresis (SDS-PAGE).

The kinetic studies show that the α-glucosidase from *Trichoderma longibrachiatum* has a K_m and V_{max} values and 33.33 µmol/min/mg protein using p-nitrophenyl- α-D-glucopyranoside as substrate. Constantino et al., [3] reported that the substrate specificity of the purified enzyme was examined by kinetic analysis on various glycosides at 108°C. *Pyrococcus furiosus* α-glucosidase had a strong affinity for the α-enantioner. He remarked that the K_m values obtained for the alkyl- and aryl-glucopyranosides, PNPG, methyl- α-D-glucopyranoside were relatively low compared with the values determined for the dissacharides. This result is also similar to the reports of Suzuki et al. [15] in respect of other α-glucosidases.

TABLE 2 **Effect of substrate on α-glucosidase activity**

Substrates	Final concentration (mM)	Activity (µmol/ml/min)
Carboxymethyl cellulose(CMC)	5.0	10.67
Maltose	5.0	6.87
p-Nitrophenyl-α-D-glucopyranoside (PNPG)	5.0	4.89

Optimum temperature was found to be 40°C. On the basis of this, the enzyme was considered to be similar to extracellular α-glucosidase obtained from *Brettanomyces lambicus* with optimum temperature of 40°C [16]. The partially purified enzyme was found to be unstable at high temperatures of 60°C and 70°C probably as a result of irreversible denaturation of the enzyme. Most α-glucosidase has an optimum temperature below 40°C. The enzymes from *Trichoderma longibranchiatum* could best be compared with the α-glucosidase found in animals, plants, bacteria, fungi and yeast, (EC. 3.2.20), which also catalyzes glucotransferase reactions. Optimal activity was observed at pH 4.5. However, the buffers used were very important, suggesting that different ions had cooperative effects. Enzyme activity was optimal with citrate buffers, contrast to Tris HCl buffers, which had an inhibitory effect on activity. A similar result was reported by Karine and Francis, [12] except that the optimal pH buffer was optimal with sodium phosphate buffer, pH 7.2. The result was similar to many α-glucosidases from other sources [3].

The enzyme α-glucosidase from *Trichoderma longibrachiatum* exhibited stability with a half-life of about two and half hours (150min) at 40°C and pH 6.5. In general, α-glucosidases were known and reported to be only moderately thermostable [17]. For instance, the extracellular α-glucosidase from *Bacillus thermoglucosidus* (KP 1006), although stable at 60°C for 2hours with no loss of activity, was highly unstable at temperature above 72°C. At temperature above this, the enzyme exhibited a half-life of only 10 minutes or less [15]. Similarly, the exo- α-1, 4-glucosidase enzyme from *Bacillus stearothermophilus* ATTC 12016 [15a, b], which was optimally active at 70°C, retained only 71% of initial activities when incubated for 10 minutes at this temperature. The α-glucosidase from *Bacillus caldovelox* DSM 411 demonstrated greater thermostability, with an half life of about 1 hour at 70°C [18],

The study also revealed that the enzyme could hydrolyse aryl glycosides and disaccharides i.e carboxymethyl cellulose and maltose. The spilting of PNPG and lower oligosaccharides, including maltose, indicated that the enzyme exhibited glucosidase activities (16). Karine and Francis, [12] also reported that maltose, trehalose and sucrose were also hydrolyzed by α-glucosidase but at a lower rate. Moreover, Shantha Kumara et al., [16] reported that during hydrolysis, maltotriose give maltose and glucose, maltopentaose yielded maltotetraose and glucose, hexaose yielded pentaose, tetraose and glucose. However, with oligosaccharides larger that maltotriose, no maltotriose was detected. Thus, with maltotetraose only glucose was found. This might indicate that the enzyme rapidly transforms the maltotriose and maltose formed from the higher oligosaccharides.

In conclusion, comparism of α-glucosidase activities from *Trichoderma longibranchiatum* and other *Trichoderma* species showed that the α-glucosidase obtained from former exhibited lower α-glucosidase activity than the later. Meanwhile, to the best of our knowledge, this is the first reported case of the partial purification of α-glucosidase from *Trichoderma longibranchiatum*, therefore further work is still in progress in respect of the probing into other purification approaches/strategies/scheme and mechanism of action of this enzyme from this organism.

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