Journal of Applied Pharmaceutical Science Vol. 6 (01), pp. 147-152, January, 2016
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2016.600123
ISSN 2231-3354

Extraction, Purification and Characterization of Endo-Acting Pullulanase Type I from White Edible Mushrooms

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ARTICLE INFO

Article history:
Received on: 09/10/2015
Revised on: 07/11/2015
Accepted on: 22/11/2015
Available online: 26/01/2016

Key words:
Pullulanase, White edible mushrooms, Purification, Characterization, HPTLC plate.

ABSTRACT

Pullulanase (EC 3.2.1.41) has been isolated and purified from white edible mushrooms by ammonium sulphate precipitation (20-70%) followed by ion exchange chromatography (DEAE-cellulose) and gel filtration (Sephadex G 75-120), with final yield (20%) and purification fold (17.8). The molecular mass of pullulanase enzyme was 112 kDa as estimated by SDS-PAGE and the *p*I value was 6.2. The apparent K_m and V_{max} values for purified pullulanse on pulluan were 0.27 mM and 0.74 µM min^{-1} respectively. The activity was optimum at 40°C and pH 6. Pullulanase showed moderate thermo-stability. A relative substrate specificity for hydrolysis of soluble starch, amylopectin and glycogen was 80, 60 and 30% respectively. Enzyme activity was highly activated by Fe^{+2}, Mn^{+2} and Ca^{+2} ions, while the activity was inhibited by Hg^{+2} and Ag^{+} ions. Ethylenediaminetetraacetic acid (EDTA) and Dithiothreitol (DTT) were activated the enzyme activity. On contarary iodoacetate and sodium fluoride were inhibited the activity. HPTLC (High Performance Thin Layer Chromatography) plate showed that the purified pullulanase caused the complete hydrolysis of pullulan to maltotriose.

INTRODUCTION

Pullulan is a polymer synthesized by the yeast-like fungus *Aureobasidium pullulans*. It is a linear α-D-glucan built of maltotriose subunits (Abdullah and French 1966). Pullulanase, It belongs to the α-amylase family, which is identified as glycoside hydrolase and break α-1,6 linkages in pullulan, starch, amylopectin and related oligosaccharides (Duan and Wu, 2015). Pullulanases are classified into five types based on the substrate specificity (Roy *et al.,* 2003; Haki and Rakshit, 2003). (1) the glucoamylase type is an exo-acting carbohydrase (Reilly, 1979), which hydrolyzes pullulan from non-reducing ends to produce glucose; (2) the pullulanase type I specifically hydrolyses α-1,6 glycosidic linkages in pullulan or branched substrates such as amylopectin forming maltotriose (3) the isopullulanase type from *Aspergillus niger*, which hydrolyzes α-1,4 linkages of pullulan to produce isopanose; and (4) Pullulan hydrolase type I (neopullulanase) from *Bacillus stearothermophilus* (Ecel *et al.,* 2015) which hydrolyzesα-1,4 linkages of pullulan to produce panose. (5) pullulanase type II (amylopullulanase) attacks α-1,6 linkages in pullulan and branched substrates in addition to α-1,4 links in polysaccharides other than pullulan (Bertoldo and Antranikian, 2002; Duffner *et al.,* 2002). Pullulanases belong to group of glycosylhydrolases that are widely distributed in nature and are produced by an extremely wide variety of species such as animals, plants and microorganisms (Hyun and Zeikus, 1985; Zhang *et al.,* 2013; Duan and Wu, 2015). Pulluanases have gained important in current saccharification processes as starch-debranching enzymes. In the starch processing industry for the production of maltose syrups and high purity glucose and fructose (Jensen and Norman 1984). This occurs when pullulanase is used in combination with glucoamylase or α-amylase, respectively in the saccharification process. Pullulanases that specifically attack the branching points of amylopectin are of special interest. The action of such enzymes would lead to the formation of linear oligosaccharides that can be attacked efficiently by other amylolytic enzymes, leading to high levels of glucose or maltose. Pullulanases are used in detergent industry as effective additives in dish washing and laundry detergents for the removal of starches under alkaline conditions (Van and Willem 1990) and in the manufacturing of low caloric beer (Olsen *et al.,* 2000). It is also possible to use pullulanase as a dental plaque control agent (Marotta *et al.,* 2002). Because of the various importance uses of pullulanase, it has been the subject of various applications so, the present study was conducted to extract, purify and characterize a highly active pullulanase from white edible mushrooms.

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MATERIALS AND METHODS

Materials

White edible mushrooms were purchased from local market. Diethylaminoethyl-cellulose (DEAE-cellulose) and molecular weight marker kits were purchased from Sigma Company while Sephadex 75-120, pullulan, maltose, amylopectin, glycogen, dextrin, soluble starch, EDTA, DTT and malto-oligosaccharides (G_6-G_3) from Fluka chemical Co., all other chemicals were of analytical grade.

Methods

Preparation and extraction of crude enzyme

Mushrooms were extracted by distilled water at 9°C with continuous shaking over a period of 2 h. This extract was then centrifuged at 3000 rpm for 15 min, and the supernatant was collected, dialyzed against distilled water and then used as the crude enzyme preparation.

Purification of pullulanase enzyme

1) *Ammonium sulfate precipitation*

The crude extract was brought to 20-70% saturation by gradually adding solid $(NH_4)_2SO_4$ and stirred for 30 min at 4°C. The pellet was obtained by centrifugation at 12000 *x g* for 30 min and dissolved in 0.02M sodium phosphate buffer pH 6 and dialyzed extensively against the same buffer.

2) *DEAE-cellulose column chromatography*

The dialyzed sample was chromatographed on a DEAE-cellulose column (12 x 2.4 cm i.d.) previously equilibrated with 0.02M sodium phosphate buffer pH 6. The adsorbed proteins were eluted firstly with sodium phosphate buffer (0.02M-pH 6) then with a stepwise NaCl gradient ranging from 0.05 to 0.5 M prepared in the equilibration buffer at a flow rate of 60 ml/hour. 5 ml fractions were collected and the fractions containing pullulanase activity were pooled and lyophilized.

3) *Sephadex G 75-120 column Chromatography*

The concentrated solution containing the pullulanase activity was applied onto a Sephadex G75-120 column (40 cm x 1.8 cm i.d.). The column was equilibrated and developed with 0.02 M sodium phosphate buffer pH 6 at a flow rate of 30 ml/hour and 2 ml fractions were collected.

Enzyme assay

Pullulanase activity was assayed by measuring the reducing sugar released from pullulan. The reaction mixture (1ml) containing pullulan (1% w/v) in sodium phosphate buffer 0.02 M, pH 6 and enzyme was incubated at 40 °C for 30 min. The reducing sugar was measured by the dinitrosalicylic acid method (Millar, 1959). One unit of pullulanase activity is defined as the amount of enzyme which produces 1µmol of reducing sugar with glucose as the standard per min under the optimum conditions.

Electrophoretic analysis and isoelectric point

Native gel electrophoresis was carried out with 7% PAGE (Smith, 1969). In order to examine the subunit composition of the pullulanase, protein samples were also analyzed by 12% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli, 1970 after the samples had been heated at 100 °C for 5 min. Low-molecular weight marker proteins were used as standards. Following native PAGE and SDS-PAGE the proteins were stained with Coomassie blue (Weber and Osborn, 1969).

The isoelectric point of the pullulanase was determined by isoelectric focusing under native conditions. Electro-focusing was performed according to O'Farrell, (1975) and the isoelectric point (*p*I) value was calculated from a calibration curve (Ubuka *et al.*, 1987). The proteins were stained with 0.25% Coomassie brilliant blue R-250.

Protein determination

Protein was determined by the dye binding assay method of Bradford (1976) using Bovine serum albumin (BSA) as a standard protein.

Biochemical characterization of the purified enzyme

Effect of temperature on enzymatic activity and thermal stability

To determine the temperature effect on the enzyme activity the reaction mixture containing pullulan and purified pullulanase were incubated at different temperatures for 30 min and the activity was assayed. Thermal stability was determined by incubating the enzyme samples in different temperatures at different time intervals.

Effect of pH on pullulanase activity

To 0.5 ml of 1% (w/v) of pullulan in various pH's (4-8) at 0.02 M concentration, 0.5 ml of the purified pullulanase was added for 30 min at 40°C.

Substrate specificity

The ability of the purified enzyme to hydrolyze various carbohydrates was examined at 40 °C and pH 6 in 0.02 sodium phosphate buffer. The carbohydrates tested were pullulan, amylopectin, soluble starch, glycogen and dextrin at a concentration 1% (w/v).

Effect of metal ions and chelating agents on the activity of pullulanase

The purified pullulanase was preincubated with various metal ions at 0.1 M concentration and chelating agents at 1M concentration.

The purified treated enzyme was incubated at 40°C for 30 min in 0.02 M sodium phosphate buffer, pH 6. The enzyme sample without any additives was considered as control.

Kinetic determination

Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of pullulanase were estimated according to Lineweaver and Bark, 1934 using different concentration of pullulan.

End product analysis

Purified pullulanase samples were added to pullulan at a concentration of 1% (w/v) in 0.02 M sodium phosphate buffer, at pH 6. The reaction mixtures were incubated at 40°C for 1, 2 and 24 hours. The products were analyzed by HPTLC plate with malto-oligosaccharide (G_3–G_6), maltose and glucose used as standards. Chromatography was carried out using the solvent system, Butanol: acetic acid: water (40:10:50 v/v/v). Carbohydrates were detected by staining with aniline-diphenylamine phosphoric acid reagent. The enzymatic products was visualized as blue spots after incubating the plate at 70°C for 5 min (Asha *et al.*, 2013).

RESULT AND DISCUSSION

Purification of pullulanase enzyme from mushroom.

A typical purification scheme of pullulanase from the mushroom is presented in Table (1). The purification procedure was carried out by ammonium sulfate precipitation (20-70%) followed by ion exchange chromatography on DEAE-cellulose column and gel filtration chromatography on Sephadex G75-120 column. The specific activity of the pullulanase of mushrooms crude extract was found to be 10 units/mg protein. Similar purification procedure of pullulanase was reported from the fungus *Hypocrea jecorina* (Orhan *et al.*, 2014).

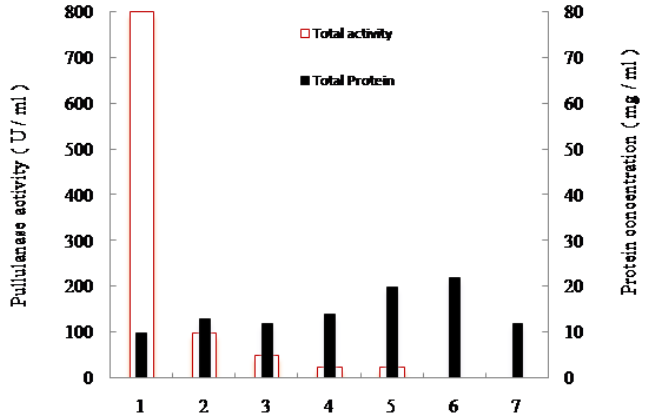

Fig. 1: Measurment of pullulanase activity and protein content that eluated from DEAE-cellulose using 1-)0.02M sodium phosphate buffer and (2-7) 0.05-0.5M NaCl in sodium phospate (pH6.0, 0.02M).

The DEAE-cellulose elution profile (Figure 1) revealed the presence of one peak containing pullulanase activity eluted with 0.02 M sodium phosphate buffer pH 6. The DEAE-cellulose fraction of this peak were pooled, concentrated by lyophilization and applied onto a Sephadex G75-120 column (Figure 2) which revealed the presence of one peak of pullulanase enzyme activity. The pullulanase was purified with a specific activity of 97 units/ mg protein and 17.8 purification fold with 20% yield (Table 1). Different purification fold and recovery percent values of pullulanase were reported, pullulanase from the fungus *Hypocrea jecorina* was purified 11 folds with 10.1% yield (Orhan *et al.*, 2014). Purification fold of pullulanase from *Bacillus subtilis* was 24 with 75% yield (Asoodeha and Lagziana 2012).

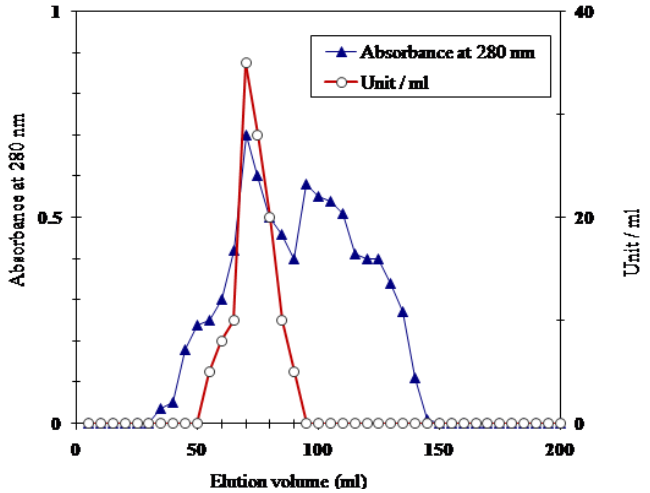

Fig. 2 A typical elution profile for the chromatography of mushroom DEAE-cellulose fraction on Sephadex G-75-120 column (40 cm x 1.8 cm i.d.) previously equilibrated with 0.02 M sodium phosphate buffer pH 6.0.

Table 1: Purification table of mushroom pullulanase.

Steps of purifacation	Total activity (U)	Total protein (mg)	Specific activity (U/gm)	Recovery (%)	Purification feld
Crude Extract	1600	160	100	100	1
200-70%(NH4)2SO4	1272	30	42.4	79.5	4.24
DEAE-cellulose ion-exchange chromatography	800	10	80	50	8
Sephadex G-75-120 gel filteration chromatography	175	108	97	20	17.8

Electrophoretic analysis and isoelectric point of the purified pullulanase

The purity of pullulanase was investigated by electrophoretic analysis. Samples from crude extract, DEAE-cellulose and Sephadex G75-120 fractions of pullulanase were analyzed electrophoretically on 7% native PAGE (Figure 3).

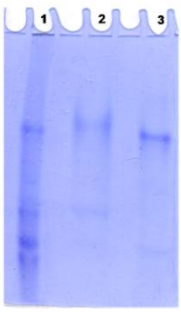

Fig. 3: Electrophoretic analysis of pulluamase on 7% native PAGE: (1) Mushroom crude extract (2) DEAE-cellulose fraction, (3) Sephadex G-75-120 fraction.

Single protein band of the purified pullulanase was obtained indicating the tentative purity of the preparation. The molecular mass of the purified pullulanase enzyme was determined by SDS-PAGE to be 112 kDa (Figure 4). Different molecular masses were reported; pullulanase from *Bacillus* sp. has a molecular mass of 106 kDa in consistent with our preparation (Kunamneni and Singh, 2006). Pullulanase from the fungus *Hypocrea jecorina* has a molecular mass of 136 kDa (Orhan *et al.*, 2014). Pullulanase from bacterium *Fervidobacterium pennavorans* has a molecular mass of 240 kDa (Koch *et al.*, 1997). The native protein exhibited *p*I of 6.2 (Figure 5). This is consistent with the isoelectric point (*p*I) value of pullulanase from *Bacillus subtilis* which was around pH 6 (Asoodeha and Lagziana, 2012).

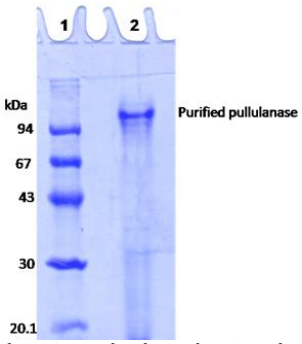

Fig. 4: Molecular weight determination by electrophoretic analysis analysis on 12% SDS-PAGE: (1) molecular weight marker proteins and (2) purified pullulanase.

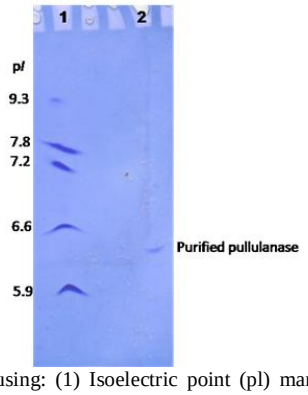

Fig. 5 Isoelectro focusing: (1) Isoelectric point (pl) marker proteins, (2) the pufified pullulanase.

Biochemical characterization of the purified enzyme

Effect of temperature on the activity and thermal stability of purified pullulanase

The maximum activity was observed at 40°C (Figure 6). Thermal stability was studied at different temperatures at different time intervals, purified pullulanase showed moderate stability as the residual activity after 30 min incubation at 60°C was 50%, while the residual activity was 20% after incubation for 45 min at 70°C (Figure7). Asha *et al*. (2013) showed that the maximum activity of pullulanase from *Bacillus halodurans* was observed at 50°C. Thermal stability was studied at 50°C for an extended range of incubation period. Up to 45 min. incubation had less effect on enzyme activity. At 1st hour, 60% residual activity was observed, 2nd hour 46% residual activity and at overnight incubation, 17% residual activity was observed.

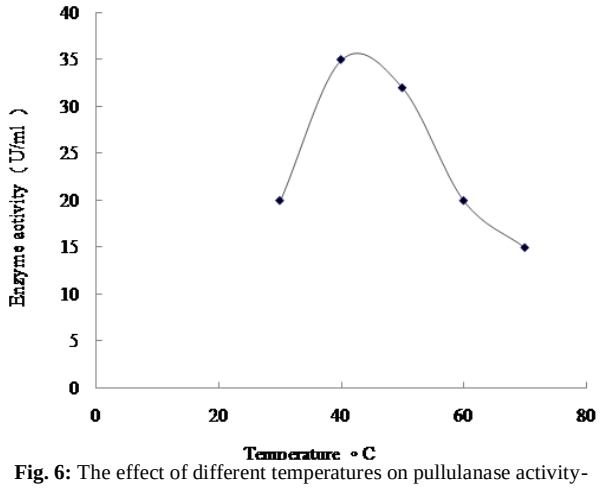

Fig. 6: The effect of different temperatures on pullulanase activity-

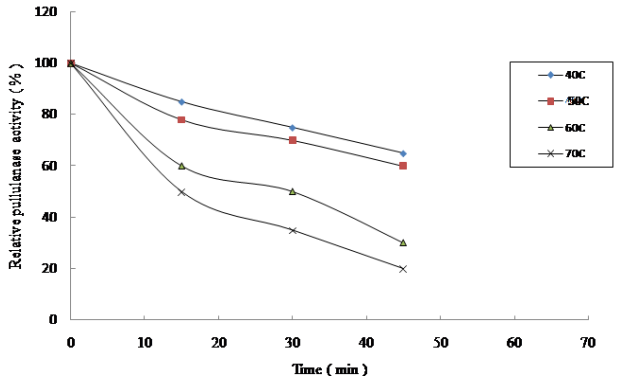

Fig. 7: Thermostability of the pullulanase. The purified enzyme was incubated at different temperatures at different time intervals.

Effect of pH on pullulanase activity

As shown in Figure (8) the pH value at which the pullulanase showed maximum activity was observed at pH 6. The same result was reported by Ling *et al.*, 2009, while Ara *et al.*, 1995 showed that maximum activity was observed at pH 9.5.

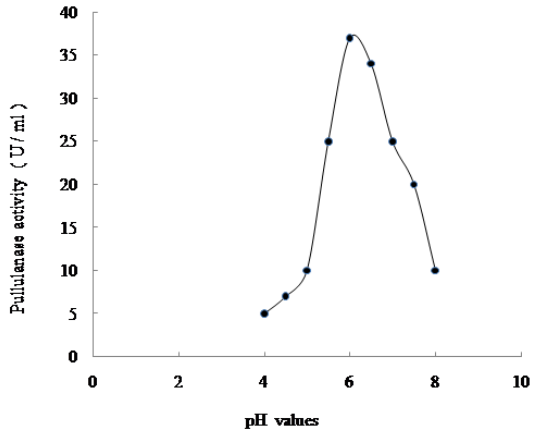

Fig. 8: The effect of different pH's on pullulanase activity at 0.02M concentration of the tested buffers.

Substrate specificity

Among all different substrates soluble starch gave high relative enzyme activity (80%) compared to others, glycogen (30%) and dextrin (10%) but also there is considerable good activity for amylopectin (60%) (Table 2). Similarly a pullulanase from *Bacillus halodurans* showed also a broad substrate specificity with high relative activity towards soluble starch (90%) and amylopectin (75%).

On contrary, Ling *et al*. (2009) reported that starch, amylopectin and dextran gave low relative enzyme activity 28, 20 and 0.2% respectively.

Table 2: Substrate specificity of pullulanae from edible mushrooms.

Substrate	Relative activity
Pullulan	100
Soluble starch	80
Amylopectim	60
Glycogen (bovine muscle)	30
dextrin	10

Effect of metal ions and chelating agents on the activity of pullulanase

Hg^{2+}, Ag+ and Co^{2+} showed strong inhibition on pullulanase activity, while Mg^{2+} slightly inhibited the enzyme. Almost Ni^{2+}, Na^{+} and Cu^{2+} have no effect on the activity. Fe^{2+}, Mn^{2+} and Ca^{2+} ions have very strong effect on stimulating the activity of pullulanase (Table 3).

Ara *et al*., 1995 reported that pullulanase activity of the enzyme was strongly inhibited by Hg^{2+} and Mn^{2+}. While Co^{2+} ions slightly stimulated the pullulanase activity.

Table 3: Effect of metals on pullulanase activity.

Metals	Concentration(M)	Relative activity(%)
$NiCl_2$	0.1	105
$CoCl_2$	0.1	40
$MnCl_2$	0.1	150
$MgCl_2$	0.1	70
Nacl	0.1	100
$FeCl_2$	0.1	160
$CuCl_2$	0.1	100
$CuCl_2$	0.1	140
$AgNO_3$	0.1	50
$HgCl_2$	0.1	30

Table 4: Effect of chelating agents on pullulanase activity.

Activators and inhibitor	Concentration(M)	Relative activity(%)
EDTA	1	130
Mercaptoethanol	1	100
Iodoacetate	1	90
DTT	1	110
Sodium fluoride	1	90

Table (4) Showed that EDTA and DTT stimulate the activity of pullulanase while iodoacetate and sodium floride slightly inhibited the enzyme activity, while mercaptoethanol has no effect. Ara *et al*. (1995) showed that monoiodoacetate had moderately inhibitory effect on the enzymatic activity, while Asha *et al*. (2013) reported that EDTA and DTT did not obviously inhibit the pullulanase activity.

Kinetic properties of pullulanase

The activity of pullulanase from mushrooms on pullulan as a substrate showed Michaelis-Menten Kinetics. The apparent Michaelis-Menten constant (K_m) value for pullulan was 0.27 mM, while the value of V_{max} was 0.74 µmol min^{-1} (Fig. 9). The K_m of pullulanase type II from *Bacillus cereus* H 1.5 was 1.1 mg mL^{-1}, while the value of V_{max} was 0.275 µmol min^{-1} (ling *et al.*, 2009).

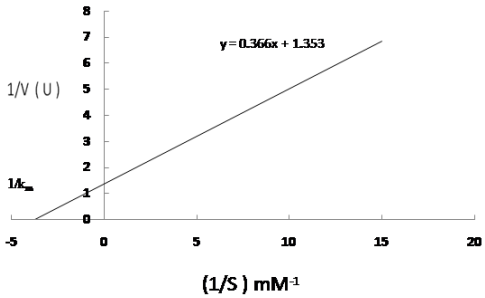

Fig. 9: Lineweaver- burk plot for pullulam hydrolysis by mushroom pullulanase. The assay was conducted in 0.02 M phosphate buffer at 40°C.

End products of pullulanase action

Maltotriose was the only trimeric (degree of polymerization, DP3), product of pullulan hydrolysis after incubation at 1, 2 and 24 hours (Figure 10), indicating that the enzyme hydrolysis is specific for α-1-6 glucosidic linkage of pullulan and the enzyme is endo – acting enzyme and belong to type I pullulanase.

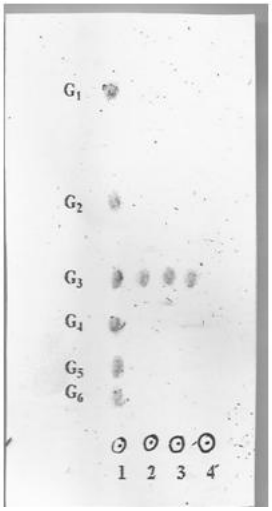

Fig. 10: Analysis of end products of pullulanase with pullulam by HPTLC technique. Lane 1 represent malto-oligosacharide standards (G_6-G_3) maltose (G_2) and glucose (G_1) lane (2-4) represent the end products at 1,2 and 24 hours incubation respectively.

CONCLUSION

Pullulanase type I produced by edible white mushrooms was capable to attack specifically α-1,6 linkages in pullulan to generate maltotriose as the major end product. Pullulanase is moderately stable at the high temperature range. Current research work is focused on the extraction, purification and characterization

of an industrial enzyme such as pullulanase by very simple and inexpensive methods from white edible mushrooms which are considered very safe source in food industry. Pullulanases have wide scale application in pullulan processing industry on account of their thermo-stability and ability to degrade row pullulan. The high substrate specificity of pullulanase together with its thermal stability makes this enzyme a good selection in the starch-processing and detergent industries and other biotechnological applications.

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How to cite this article:

Shehata AN, Darwish DA, Masoud HMM. Extraction, Purification and Characterization of Endo-Acting Pullulanase Type I from White Edible Mushrooms. J App Pharm Sci, 2016; 6 (01): 147-152.