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Extraction and Purification of Pullulanase from Local Mushrooms

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A R T I C L E I N F O

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A B S T R A C T

Pullulanase is defined as an extracellular carbohydrase enzyme responsible for the hydrolysis of pullulan into maltotriose. Pullulanase can be extracted from Agaricus bisporus edible mushroom and concentrated by dialysis tube using Tris-HCl buffer. This enzyme was purified by different ratios of ammonium sulphate; the optimal ratio was 30%. It can be purified by ion exchange using a DEAE-cellulose column with a final yield of 5.25 and a purification fold of 4.38. Following Sephadex G-100 gel filtration with a yield of 2.60 and purification fold of 5.0, the total activity of the enzyme reached 160IU and the specific activity was 14.5 U/mg. This study was concerned with estimating the optimum pH and temperature of pullulanase, and it recorded an optimal pH of 7, and the optimal temperature was 60°C. The enzyme was more stable at pH 8 and 70°C; the incubation period was also determined, and it appeared the most appropriate period was 30 minutes. $CaCl_2$ and $ZnCl_2$ were activator metal ions. Mercaptoethanol in different concentrations was the greatest inhibitor of pullulanase. One of the problems arising in the surrounding area is that much of the environmental pollution consists of starchy food waste. Pullulanase can degrade the glycosidic linkage of pullulan or starch present in the starch waste. The objective of this study is to produce enzymes like pullulanases to participate in minimizing environmental pollution through the degradation of starch waste as a biotechnological application. The conclusion of this study is that pullulanase can be produced from readily available, safe, and low-cost sources such as mushrooms.

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1. Introduction

Pullulan is defined as a polymer produced in high yield by A. pullulans. It is a linear D-glucan composed of maltotriose [1], Pullulanase is a carbohydrase group pullulan α-glucano- hydrolase enzyme. It is widely used as a starch debranching enzyme with different applications in the pharmaceutical industry, medical applications, and food and chemical industries [2]. These enzymes were also called debranching enzymes, and they were responsible for the hydrolysis process of α-1,6 glycosidic bonds in pullulan, amylopectin, and starch [3]. These enzymes' scarification effects indicate their importance in the food industry for the production of maltose, glucose syrup, and beer. Pullulanases have properties of α- linkage breaking making them useful in the baking industry.

Pullulanase is participating in bioethanol production, which provides a fuel source [4]. The activity of these enzymes would cause the formation of linear oligo saccharides that can be analyzed by other active enzymes, causing the production of glucose and maltose. These enzymes are widely used in detergent industries as additives in laundry detergents and dishwashing detergents to remove starch molecules as well as in the manufacturing of low-calorie beer [5]. pullulanases are also used as a control substance in the dental plaque industry [6]. They belong to a hydrolyase group of enzymes that are widely distributed in nature in different species such as bacteria, fungi, mold, plants, and animals [3]. *Agaricus bisporus (A. bisporus)* Mushrooms are important sources of pullulanases, Mushroom as a word derived from a French word refers to Fungi and Mold, it is referring to the Basidiomycetes group. Mushroom are highly valuable food because of their low calories, low in fat and carbohydrate in addition to free of cholesterol, also provides important proteins, nutrients, and fiber. So It has been reported to be beneficial in the treatment of some diseases in addition, they are considered an important source of bioactive substance, *A. bisporus* has a high value of calcium, potassium, and phosphor [7, 8]. Three types of pullulanases are in nature. Pullulanase type I is responsible for hydrolyses α-(1,6) glycosidic bonds in pullulan and polysaccharides to release maltosetriose; Pullulanase type II is responsible for hydrolyse α-(1,4) and α-(1,6) glucosidic bonds. Pullulanase I acts on the α- (1, 4) glycosidic bond of pullulan and releases panose while Pullulanase II hydrolyses α-(1,4) bonds in pullulan to release Isopanose; pullulanase III works on both α-(1,4) and α-(1,6) glycosidic bonds of starch and pullulan which release different end products consist of panose; maltotriose and maltose [9]. Pullulanase can be detected using the electrophoresis method that uses polyacrylamide gel with pullulan or starch as a substrate in addition to extracted enzyme solution, this method is simple and sensitive to low levels of pullulanase, reaching 0.001U of enzyme in a polyacrylamide gel after migration of electrons in electrophoresis [10]. Pullulanase is useful in reducing environmental pollution by hydrolyzing starch food waste after previous enzyme isolation from *Thermococcus kodakarensis* yields simple sugar that can be used in the fermentation process. Its potential role in the field of biotechnology consists of the manufacture of cyclodextrin as a substance, which has wide applications in pharmaceuticals, antioxidants, and staibilizing factors. In addition to food enhancements as an antistaling agent to improve texture, other uses of pullulanase, Simple sugars can also be released after the degradation process of starch food by enzyme, and they are used in a variety of applications, including alcohol fermentation, the beer industry, producing important substances like high fructose syrup and high maltose syrup, which have applications in the industrial and biotechnological fields. [11]. As a result, and because of the important applications of pullulanases, the present study aimed to concentrate on the extraction, purification, and characterization of pullulanase isolated from *A. bisporus*.

2. Experimental Procedure

2.1 Materials

A. bisporus, the edible mushrooms were collected from local Iraqi markets. Sephadex G-100, pullulan, soluble starch, sucrose, and other chemicals were from HiMedia Company, while DEAE Cellulose and ammonium sulphate were from Pharmacia Company.

2.2 Methods

2.2.1. Cold extraction of a Crude Pullulanase

Pullulanase enzyme was extracted from white fresh mushrooms using the cold extraction method described previously with a slight modification [12]. The mushrooms were obtained fresh from Iraqi markets during the winter season; they were used fresh, sliced, and homogenized using a blender. An extraction solution was performed using 500 mL of distilled water for 300 g of mushroom. The homogenate extract was then precipitated and centrifuged at 3000 rpm for 30 min. Then the supernatant was collected. The supernatant was used as a source of crude enzyme.

2.2.2 Dialysis Process

Dialysis is a common step that involves the process of removing unwanted small materials such as reducing agents, salts, and dyes from larger molecules such as protein molecules, polysaccharides, and DNA. In this experiment, dialysis membranes made from cellulose were used in the presence of Tris-HCl buffer 50 x 10^{-3}M and pH 7 in the refrigerator overnight. To remove unwanted materials like salts and water, dialysis was used to concentrate and minimize the total volume of the extract [13].

2.2.3 Estimation of a pullulanase Enzyme

The activity of the pullulanase enzyme was measured by determining the amount of reducing sugar liberated into the pullulan molecule using the method described by [14] with some modifications. The enzyme activity was estimated in a reaction mixture consisting of 0.4 ml of pullulan sugar 1% in 1 M potassium phosphate buffer pH 7, and 0.1 mL of crude enzyme. The enzyme-substrate mixture was incubated at 35°C for 15 min. After the incubation period, the reaction was stopped using cooling in an ice bath and the addition of a dinitrosalysalic acid reagent [15]. The pullulanase activity unit was described as the pullulanase value responsible for liberating one µmol of reducing sugar per minute under experimental conditions [16].

2.2.4 Determination of Protein Concentration

Protein concentration in this experiment was determined according to [17], depending on the dye interaction method using the standard protein of bovine serum albumin.

2.2.5 Pullulanase Precipitation with Ammonium Sulphate

The precipitation process using $(NH_4)_2SO_4$ was performed in the presence of an ice bath. Ammonium sulphate powder was weighted and added into the extract solution with a stirring process using different saturation values. Then the mixture was put into a centrifuge at 3000 rounds per minute for 40 minutes in the presence of an ice bath at 4°C. The precipitation process of pullulanase was achieved in different precipitation ratios ranging from 0-90%, and the precipitate was collected after each concentration. Then the concentration that has the highest enzyme activity was chosen and used to continue the other experiment. The precipitate was taken and dissolved in about 5ml of distilled water, then the enzyme-specific activity was estimated for each fraction [18].

2.2.6 Pullulanase Precipitation by Acetone

The crude extract was partially purified using acetone precipitation. 8 ml of acetone solution were added to a crude extract and it was precipitated at -4°C using a refrigerator for overnight. The precipitate was collected using a centrifuge at 3000 rpm for 25min., then it was dissolved in 0.2M of Tris-HCl buffer pH 8.0, and the enzyme-specific activity was determined [19].

2.2.7 Pullulanase Purification using Ion Exchange Chromatography

The enzyme sample was purified by ion exchange chromatography. It was dissolved using a 0.2MTris-HCl buffer of pH 8.0, and put into the DEAE cellulose chromatography column. Then the fraction's absorbancy was determined in a spectrophotometer at 280nm.The active fractions were collected from the DEAE column using two steps: wash and elution; then the enzyme was eluted; the enzyme activity was determined for each fraction and the fraction with the greatest activity was chosen [20].

2.2.8 Purification of pullulanase using Sephadex G-100 Column Chromatography

Ten milliliters of enzyme solution were collected from the previous step and they were loaded into a column equilibrated with sodium phosphate buffer, 0.02 M, pH 6.0. The enzyme was eluted using about 500 ml of buffer at a flow rate of about 0.5 ml/min. Fractions of 5ml were collected, then the enzyme activity was determined based on measuring the absorbance of samples at 510 nm according to the method described [21] with some modification, different enzymes from various sources were also purified using the sephadex gel filtration technique. For example, L-Glutaminase was isolated from L. Gasseri [22], and purified using Sephadex G-100. It appeared high values of enzyme activities in the presence of other optimal conditions.

2.2.9 Characterization of Enzyme

2.2.9.1 Role of Temperature and Acidity on pullulanase Action

To indicate the optimum temperature of pullulanase activity, samples were incubated at different temperatures ranging between 10 and 90°C to estimate the role of temperature on pullulanase activity for 25 min., and the enzyme activity was determined for each treatment according to the method described [23].

The effect of pH on pullulanase activity was estimated in the presence of pullulan as a substrate diluted in 100 mM of acetate buffer pH 4.0, 5.0, and 6.0, the enzyme extract pH 7.0 were incubated for 25 min and 100 mM Tris HCl buffer pH 8.0, 9.0 at 60 °C [24, 25].

2.2.9.2 Thermal and pH Stability of pullulanase

This experiment was used to estimate the first thermal stability of pullulanase. The purified enzyme was incubated at 40, 50, 60,70, 80, and 90°C for 3 hours separately. After the treatment time, the enzyme solutions were cooled and enzyme activities were determined using an enzyme assay in the presence of a control sample containing the non-treated enzyme. pH stability of pullulanase was determined by incubating the purified pullulanase in different buffers and then estimating the enzyme activity using an assay procedure. These buffers were 100 mM of acetate buffer pH 4.0, 5.0, and 6.0; the others were 100 mM of Tris-HCl buffer pH 8.0, 9.0, and 10.0, while no buffer was added in the pH7.0 treatment. According to [26], with some modifications.

2.2.9.3 Role of Incubation Time on pullulanase Activity

According to the method described previously with a slight modification [27], pullulanase was incubated in the presence of pullulan as a substrate at different times consisting of 10, 20, 30, 40, 50, 60, 70, 80, and 90 minutes at 60°C. The enzyme-specific activity was then determined to estimate the more suitable reaction time.

2.2.9.4 Effect of Metal ions on Purified pullulanase Action

The effects of different metal ions on enzyme-specific activity were studied at concentrations of 1 mM using $MgCl_2$, $CaCl_2$, $HgCl_2$, $MnCl_2$, $CuCl_2$, $ZnCl_2$, and $FeCl_2$; pullulanase activity was estimated using pullulan substrate using 1% concentration, pullulanase incubated and substrate for 30 min. at 60°C through the addition of one metal ion in each step [28].

2.2.9.5 Effect of Some Inhibitors on Purified pullulanase

Two different inhibitors were used to characterize the most effective pullulanase inhibitors using 10^{-3} and 10^{-2} M concentrations, these are mercaptoethanol and phenyl methyl sulphonyl fluoride (PMSF). The enzyme without any additive was also taken for comparison. The purified pullulanase was incubated with each inhibitor at 60°C for 30 min and the remaining activity was determined in the presence of pullulan as a substrate [12, 19].

3. Results and Discussion

3.1 Extract Preparation and pullulanase Detection

The crude extract was isolated and it was concentrated using a dialysis tube. The enzyme-specific activities were determined for each treatment, with results (Table 1). It appeared the enzyme activity of the crude extract in the presence of pullulan as a substrate was 2.9U/mg with 6156U total activity and it appeared the specific activity after the concentration step of the extract reached 5.6 U/mg with 5106U total activity.

3.2 Pullulanase Purification

The concentrated extract was precipitated using a different ratio of ammonium sulphate concentration that consisted of 0, 10, 20, 30, 40, 50, 60, 70, 80, and 90% according to the method described in [18]. Using pullulan as a substrate, the specific activities in the presence of different $(NH_4)_2SO_4$ concentrations were recorded at 5.6; 5.66; 5.8; 5.9; 5.72; 5.0; 4.2; 3.2; 3.4; 2.8 U/mg respectively as in Fig. 1 and it appeared that 30% ammonium sulphate concentration was more effective toward Pullulanase precipitation and enzyme activity. The enzyme was also concentrated and precipitated using acetone according to the method described in [19] in the presence of pullulan as a substrate, and the enzyme activity and total activity were determined according to the enzyme assay. It appeared the pullulanase-specific activity using acetone was 10.8 U/mg and the total activity was 783U. Pullulanase was partially purified using ion exchange chromatography in the presence of a DEAE-cellulose column, followed by two steps of wash and elution in the presence of Tris–HCl buffer, and the enzyme was eluted and appeared to have the maximum enzyme-specific activity of 12.7U/mg with 323 U total activity and a purification fold of 4.38 as in Table 1. In addition, gel filtration chromatography was also used in the partial purification of pullulanase in the presence of a Sephadex G_100 column prepared previously. The enzyme was eluted using sodium phosphate buffer at a flow rate of about 0.5 ml/min. Results in (Table 1) showed the enzyme-specific activity of pullulanase in gel filtration chromatography was recorded at 14.5U/mg with a total activity of 160U and a purification fold of 5.0.This result was in agreement with other experiments which purified pullulanase from *Bacillus* cereus HI.5 [29],using the chromatographic method of DEAE column and followed by Superdex gel filtration and recorded specific activity of 14.250 U/mg ,yielding 8.5% in 23.6 purification fold.

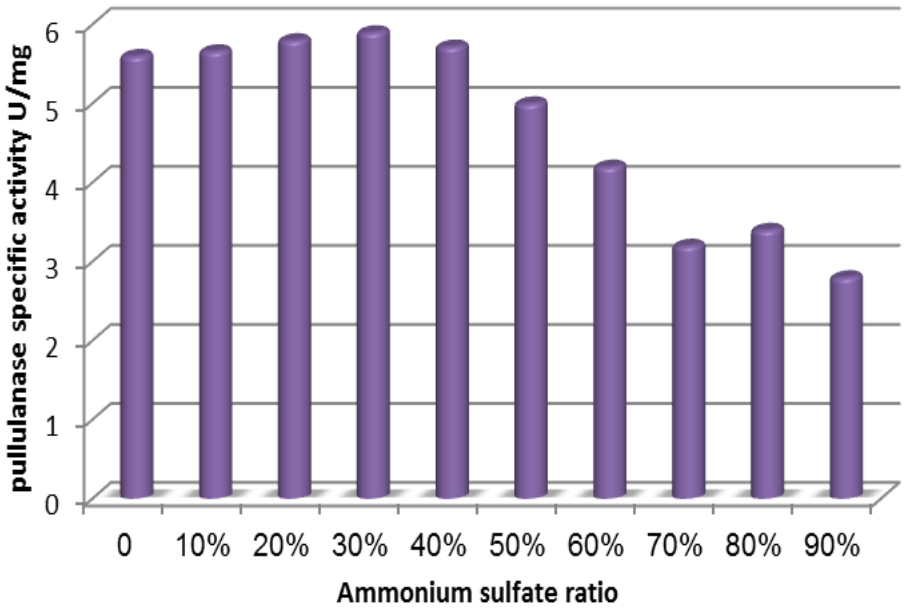

Figure 1: Pullulanase precipitation using Ammonium sulphate.

Table 1: Purification of pullulanase from300 g of edible mushroom *A. bisporus*.

Fractions	Extract volume	Activity U/mL	Protein concentration mg/mL	Enzyme Specific Activity U/mg	Total enzyme activity U	Fold of purification	%Yield
Crude extract	342	18	6.2	2.9	6156	1.00	100
Extract dialysis using sucrose	138	37	6.6	5.6	5106	1.93	82
Ammonium sulphate precipitation	112	34	5.8	5.9	3808	2.03	61.86
Concentration using sucrose	60	39	3.6	10.8	2340	3.72	38.01
Acetone purification	29	27	2.6	10.4	783	3.59	12.72
Ion exchange chromatography	17	19	1.5	12.7	323	4.38	5.25
Gel filtration chromatography	10	16	1.1	14.5	160	5.00	2.60

3.3 Enzyme Characterization

3.3.1. Effect of Temperature and acidity on Enzyme Activity

Pullulanase and enzyme-substrate were incubated separately at different temperatures ranging from 10 to 90°C to estimate the effect of different temperatures on pullulanase action. Enzyme activity was recorded at 17.8 U/mg at 60°C while other degrees 10, 20, 30, 40, 50, 70, 80, and 90°C were recorded with less enzyme activity (12.2; 14.0; 14.8; 15.2, 16.4, 15.0; 11.6; 8.8) U/mg. Previous results [30] showed that the optimum temperature of pullulanase was 40°C and gave 177 U/mg specific activity in starch debranching. The pH influences were also studied. Enzyme and substrate were incubated at different pH values which were (4, 5, 6 7,8, and 9), results in Fig. 2(b) appeared. The optimal pH of pullulanase activity was 7, and enzyme activity reached 22 U/mL while other pH values were recorded (18.1; 19.0, 19.2, 16.8, and 14.5) U/mL respectively. This result was similar to the optimal pH of purified pullulanase, which was also recorded at 7. Enzyme activity was at its maximum value [31]. This result was in agreement with previous studies; the optimal pH of L -Glutamate oxidase and the fibrinolytic enzyme was also 7 for enzymes isolated from Streptomyces species and Pseudomonas aeruginosa, respectively [32, 33].

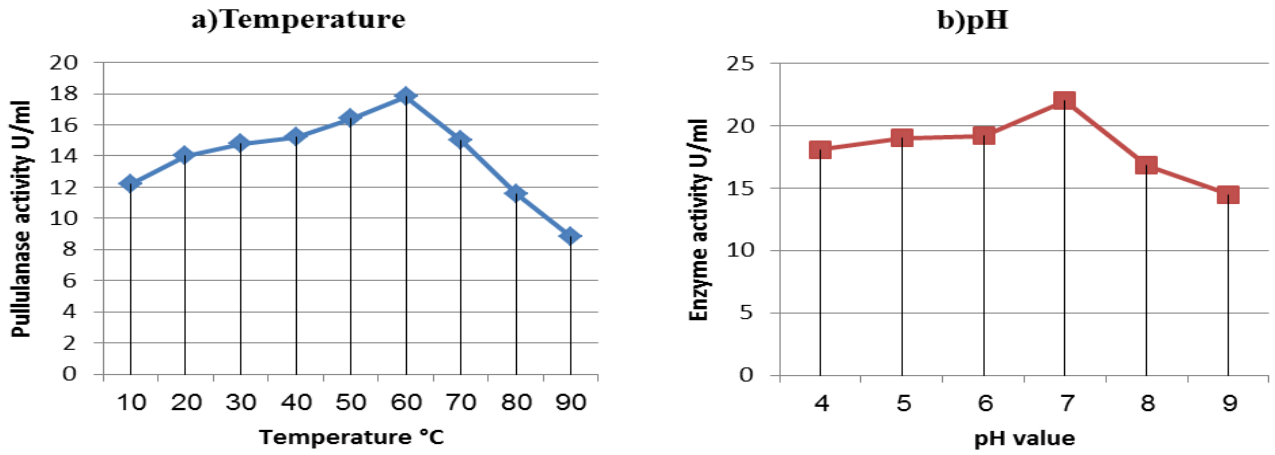

Figure 2: Effects of a) Temperature and b) acidity on pullulanase activity.

3.3.2 Effect of Temperature and pH Stability of pullulanase

To study the thermal stability of pullulanase, the purified enzyme was exposed to different temperature values separately. These values consisted of 40, 50, 60, 70, 80, and 90°C. Then the enzyme-specific activity was determined using pullulan as a substrate. The results in Fig. 3a appear to show the enzyme was stable up to 70°C. The enzyme maintained about 95% of its activity and the relative activity began to decline, enzyme maintained about 70, and 60% of its activity at 80 and 90°C respectively. In contrast, other researchers found that pullulanase retained more than 85% activity after incubation at 65 °C for 30 min [2]. On the other hand, the pH stability of pullulanase was also studied. The purified enzyme was incubated at different pH values separately consisting of (4, 5, 6, 7, 8, and 9), then the enzyme-specific activity was also determined after each treatment. The results in Fig. 3b showed that pullulanase was highly stable at a pH value of 8 and then the relative activity declined at pH 9. The enzyme maintained about 70% of its activity. Other findings indicated that pullulanase stability was detected at pH levels ranging from 3-6 [29].

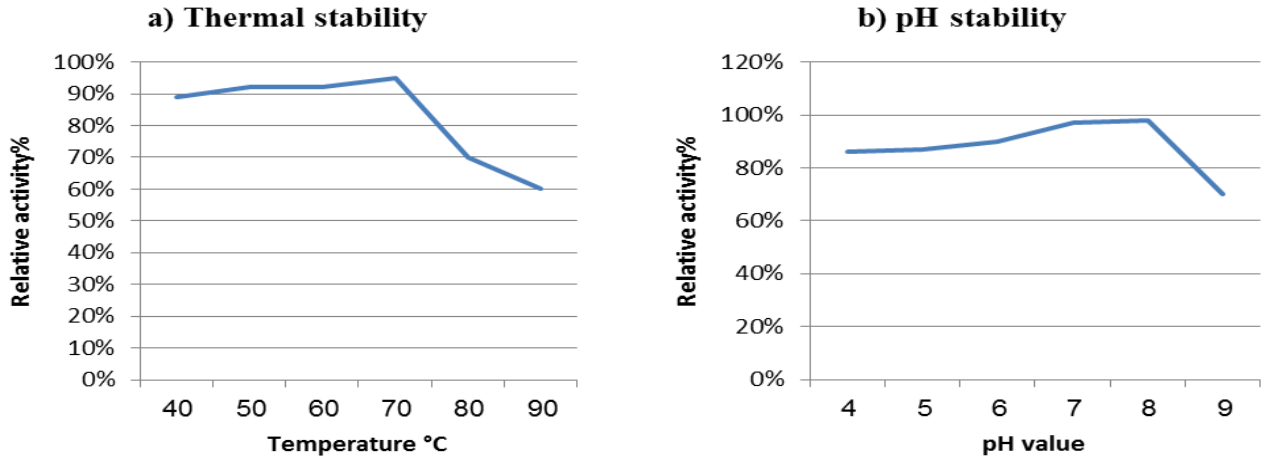

Figure 3: Effect of a) Temperature and b) pH stability on pullulanase.

3.3.3 Influence of Incubation Period on Enzyme Activity

To estimate the more suitable reaction time of the enzyme and pullulan substrate, pullulanase was incubated with pullulan in different periods while other reaction conditions were maintained constant. These are consisted of 10, 20, 30, 40, 50, 60, 70, 80, and 90 minutes, which gave pullulan-specific activity of 13, 14.4, 15.3, 15.0, 14.6, 14.2, 12.8, 10.6, and 7.8 U/mg respectively. Results in Fig. 4 appear to indicate the more suitable incubation duration was 30 minutes and recorded 15.3 U/mg pullulanase-specific activities. The results were in disagreement with

previous studies [34] which recorded that amylase enzyme from Aspergillus niger nee6-day 6-day incubation period to give the maximum enzyme-specific activity of 0.93 U/mg.

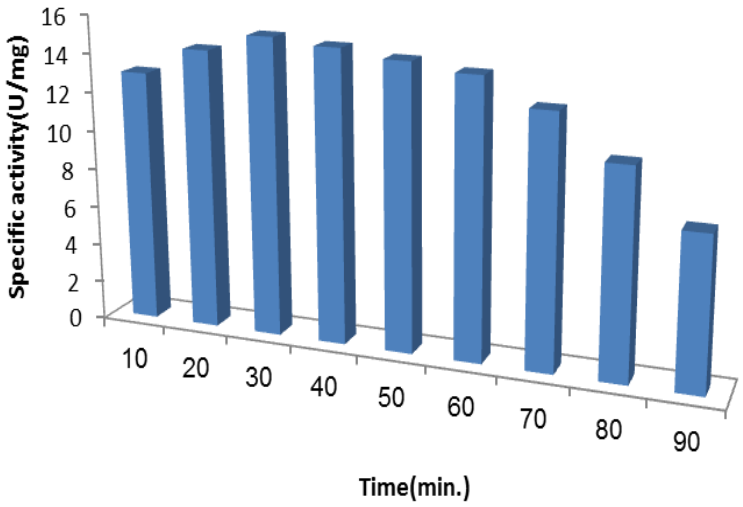

Figure 4: Influence of incubation time on Pullulanase activity.

3.3.4 Effect of Metal Ions on pullulanase

Different types of metal ions were used to record the more suitable ions for pululanase-specific activity. These consisted of $MgCl_2$, $CaCl_2$, $HgCl_2$, $MnCl_2$, $CuCl_2$, $ZnCl_2$ and $FeCl_2$, and the results are shown in Fig. 5. It appeared that $CaCl_2$ and $ZnCl_2$ were activator ions in contrast to other ions by giving 32.2 U/mg and 34.8U/mg, respectively. Other researchers studied the effect of metal ions on amylase activity and recorded that Hg^{+2} and Cd^{+2} were strong inhibitors [35]. In contrast to other research on metal ions, they mentioned that pullulanase was strongly stimulated by Ca^{+2}, which increased the pullulanase activity by around 179%, while it has been inhibited in the presence of Cu^{+2} [36].

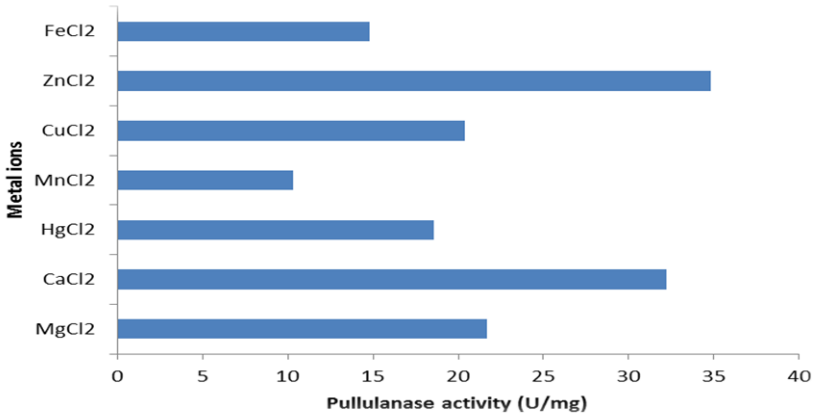

Figure 5: Effect of metal ions on pullulanase-specific activity.

3.3.5 Effect of inhibitors on purified pullulanase

Two different types of inhibitors were used in this experiment. They consisted of mercaptoethanol and phenyl methyl sulphonyl fluoride (PMSF) in different concentrations (1×10^{-3} and 1×10^{-2} M), in addition to control, results in Fig. 6. It appeared that mercaptoethanol in both concentrations was the greatest inhibitor of pullulanase. In contrast to the other inhibitors the enzyme maintained about 48 and 32.4% relative activity at $1x10^{-3}$ M and $1x10^{-2}$ M concentrations of mercaptoethanol, while phenyl methyl sulphonyl fluoride (PMSF) was less effective as an inhibitor, the enzyme maintained about 78.4 and 73.6% relative activity at 1×10^{-3} M and $1x10^{-2}$ M concentrations of phenyl methyl sulphonyl fluoride, in contrast, to control test 100% remaining activity. Researchers [37] studied

the effect of inhibitors on pullulanase isolated from Thermus caldophilus and found the activity of pullulanase was inhibited by α, β and $_{\gamma}$ cyclodextrin.

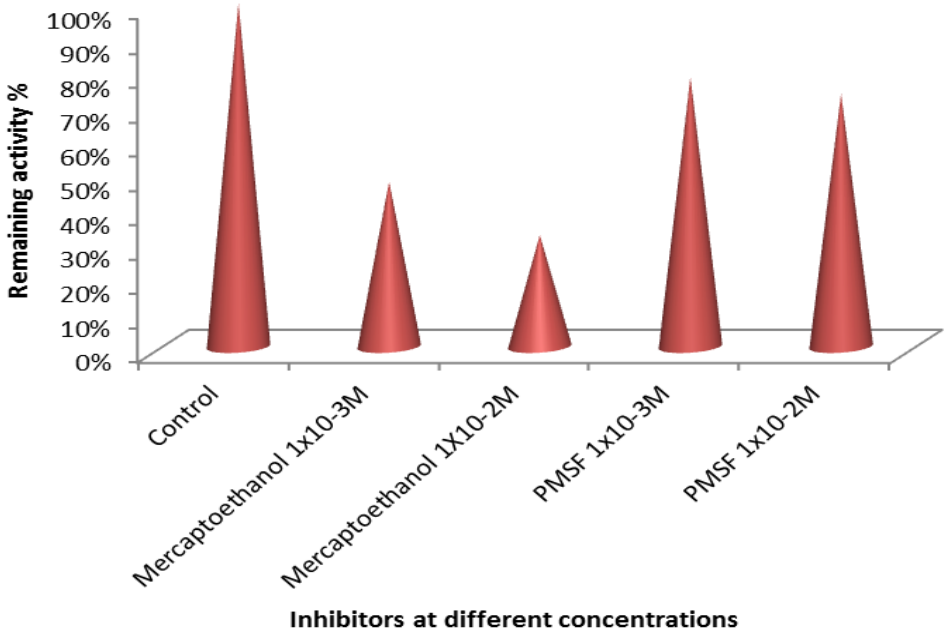

Figure 6: Effect of inhibitors on the remaining activity of Pullualnase.

4. Conclusions

It is possible to extract pullulanase enzyme from local edible mushrooms, pullulanase was capable to attack specifically α-1,4 and α-1,6 linkages of pullulan molecules and was stable at high temperatures and pH values. This work is focused on the extraction and purification of an imported enzyme, pullulanase, using simple and low-cost methods from available and safe sources such as edible mushrooms, in addition to analyzing the optimal condition of the extracted enzyme. The future applications of pullulanases consist of using these enzymes to minimize environmental pollutants like starchy food waste through the degradation of glycosidic linkages in starch or pullulan. As a result, crude simple sugars can be used in different fields of biotechnology.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] M. Abdullah and D. French, “Reverse action of pullulanase ," *Nature*, p.,200:210 ,1966.

[2] B. Pang, L. Zhou, W. Cui, Z. Liu and Z. Zhou,“Improvement of the Thermostability and Activity of Pullulanase from *Anoxybacillus sp.* WB42," *Applied Biochemistry and Biotechnology*, vol. 191, p.942–954 ,2020.

[3] X. Duan and J.Wu, “Enhancing the secretion efficiency and thermostability of *Bacillus deramificans* pullulanase mutant D437H/D503Y by N-terminal domain truncation," *Applied and Environmental Microbiology Journal*, vol. 81,no. 6, 2015.

[4] N. Prakash,S. Gupta, M.Ansari, Z. Khan and V. Suneetha, " Production of economically important products by the use of pullulanase enzyme," *International Journal of Scientific Innovations*, vol. 2, no. 2: p. 266-273, 2012.

[5] H. Olsen, P.Goddard and A. Nordisk: In Novo Nordisk A/S, 58 , 2000.

[6] M. Marotta, A. Martino, A. De Rosa, E. Farina, M. Carteni and M. De Rosa," Degradation of dental plaque glucans and prevention of glucan formation using commercial enzymes", *Process Biochemistry Journal*, vol.38, no.1, p.101-108, 2002.

[7] B. Chatterjee and T. Patel,"Edible Mushroom-A Nutritious Food Improving Human Health, " *International Journal of Clinical and Biomedical Research*,vol.2 ,no.1,p.34-37, 2016.

[8] M.N. Owaid, "Mineral elements content in two sources of *Agaricus bisporus* in Iraqi market, "*Journal of Advanced and applied sciences*, vol.3; n.2,p.46-50,2015.

[9] C. Velhal, M. Sant, S. Das and C. Kulkarni, " Production Of Pullulanase Using Novel Organic Substrates, " *International Journal of Scientific and Technology Research* ,vol. 3, Issue 3., 2014.

[10] C. H. Kim,"Specific detection of pullulanase type I in polyacrylamide gels,", *FEMS Microbiology Letters*, 116,327-332,1994.

[11] A. Shehzad, N. Ahmad, Z. Hussain et al,"Valorization of waste foods using pullulan hydrolase from *Thermococcus kodakarensis",DE GRUYTER* ,vol. 2, p.39-43, 2018.

[12] A. N. Shehata , D. A. Darwish and H. M. Masoud ,"Extraction, Purification and Characterization of Endo-Acting Pullulanase Type I from White Edible Mushrooms," *Journal of Applied Pharmaceutical Science* ,vol. 6,no.01, p.147-152, 2016.

[13] K. Haghbeen ,F. R. Jazii, A. A. Karkhane and S. S. Borojerdi,"Purification of tyrosinase from edible mushroom, " *Iranian Journal of Biotechnology* , vol. 2, no. 3, 2004.

[14] M. Waleed, A. A. Faiza and I. Nizar; "Production optimization of pullulanase enzyme produced by *Bacillus cereus* isolated from Syrian source, " *International Food Research Journal*,vol.22, no.5, p. 1824-1830 , 2015.

[15] G. Miller,"Use of dinitrosalicylic agent for determination of reducing sugars, " *Analytical Chemistry Journal*, vol.31,p. 426-428, 1959.

[16] K. Ara, K. Saeki and S. Ito, "Purification and characterization of an alkaline isoamylase from an alkalophilic strain of *BaciLLus*, ".*Journal of General Microbiology*, vol.139, p.781-786, 1993.

[17] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding". *Analytical Biochemistry Journal*.vol.72, p.248-254,1976.

[18] S. Sharma, D. Vaidya and N. Rana,"Extraction, partial purification and characterization of kiwifruit enzyme" *The Pharma Innovation Journal*; vol.7,no.1,p. 312-315,2018.

[19] R. Asha., F.N. Niyonzima and S.M. Sunil, "Purification and Properties of Pullulanase from *Bacillus halodurans*, " *International Research Journal of Biological Sciences* ,vol. 2,no.3,p. 35-43,2013.

[20] D. H. Abdul Hameed and E. H. Ali, "Extraction and Purification of Extracellular L-Glutamate Oxidase from *Streptomyces*", *Archives of Razi Institute*, vol. 76, no. 4,p. 769-779, 2021.

[21] Z. Olempska-Beer,"Pullulanase from *Bacillus deramificans* expressed in *Bacillus Licheniformis*,", *Genencor International B.V., P.O. Box 218*, 2018.

[22] B.A. Hasson, L. H. Mahdi and R. H. Essa, "Evidence of Antioxidant Activity of Novel L-Glutaminase Purified from *L. Gasseri* BRLHM, "*Journal of Applied Sciences and Nanotechnology*, vol. 1, no.4 , 2021.

[23] H.F. Lo, L.L. Lin, H.L. Chen, H.H. Hsu and C.T. Chang , "Enzymatic properties of a SDS-resistant *Bacillus sp. TS-23* a-amylase produced by recombinant *Escherichia coli," Process Biochemistry Journal*, vol.36, p.743-750, 2001.

[24] R. J. Sattar and E. H. Ali,"Purification and Characterization of lipase production from *Pseudomonas aeruginosa* and Study effect of silver nanoparticle in activity of enzyme in application of biology", *Plant Archives* ,vol. 19 ,no. 2,p. 4379-4385, 2019.

[25] A.J.R. AL-Sa'ady, M. H.A. Al-Bahrani and G. M. Aziz,"Characterization and Immobilization of Peroxidase Extracted from Horse Radish and Decolorization of Some Dyes," *International Journal of Current Microbiology and Applied Sciences,* vol. 7, no. 06, 2018.

[26] T. Nouadri, Z.Meraihi, D.Shahrazed and B.Leila, "Purification and characterization of the α-amylase isolated from *Penicillium camemberti* PL21," *African Journal of Biochemistry Research* ,vol. 4,no.6 , p. 155-162, 2010.

[27] S. P. Khabade, B. G. Lavanya., M. Mahima and J. Sandhya, "Screening, Purification and Characterization of Pullulanase from Soil Bacteria and Enhancement of Its Stability by Immobilization and Nanoforms, " *International Journal of Emerging Trends in Science and Technology*; vol.03 no.08 ,2016.

[28] S. T. Sagu, E. J. Nso, T. C. Kapseu and H. M. Rawel, "Extraction and purification of beta-amylase from stems of *Abrus precatorius* by three phase partitioning, *"Food Chemistry Journal,* vol.183, p.144–153, 2015.

[29] H. S. Ling, T. C. Ling, R. M. and A. B. Ariff, "Characterization of Pullulanase Type II from *Bacillus cereus* H1.5," *American Journal of Biochemistry and Biotechnology*, vol. 5,no.4,p.170-179, 2009.

[30] M.Thakur, N. Sharma, A. K. Rai and S. P. Singh, "A novel cold-active type I pullulanase from a hot-spring metagenome for effective debranching and production of resistant starch" , *Bioresource Technology Journal*s, vol. 320,2021.

[31] N. Prabhu1, R. U. maheswari1, M. V. P. Singh, S. Karunakaran, C. Kaliappan and T. Gaj, "Production and purification of extracellular pullulanase by *Klebsilla aerogenes* NCIM 2239," *African Journal of Biotechnology*; vol. 17,no.14, p. 486-494, 2018.

[32] D. H. Abdul Hameed and E. H. Ali," Obtaining L - Glutamate Oxidase Enzyme from *Streptomyces* and Estimation Its Toxicity on Blood Components," *Journal of Applied Sciences and Nanotechnology*, vol. 1, no. 4 ,2021.

[33] B. H. Jasim and E. H. Ali," Enhanced Production of Fibrinolytic Enzyme from *Pseudomonas aeruginosa* by Optimization Media Components," *Journal of Applied Sciences and Nanotechnology*, vol. 1, no. 2, 2021

[34] D.S. Bedan, G. M. Aziz and A.J. R. Al - Sa'ady, "Optimum conditions for α- amylase production by *Aspergillus niger* mutant isolate using solid state Fermentation," *Current Research in Microbiology and Biotechnology*, vol. 2, no. ,p. 4450-456 ,2014.

[35] W. H. Abdulaal, "Purification and characterization of α- amylase from *Trichoderma pseudokoningii," BMC Biochemistry Journals* ,vol.19,no.4, 2018.

[36] R. Malakar, A.Tiwari and S.N. Malviya,"Pullulanase:A Potential enzyme for industrial application,"*International Journal of Biomedical Research*, vol. 1 ,no.2, p.10-20. 2010.

[37] C. H. Kim, O. Nashiru and J.H. Ko; " Purification and biochemical characterization of pullulanase type I from *Thermus caldophilus* GK-24, " *FEMS Microbiology Letters* vol.138 ,p.147-152 ,1996.