Purification, characterization of thermostable Amylopullulanase from *Bacillus licheniformis* (BS18) by using solid state fermentation (SSF)

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Abstract:

An amylopullulanase enzyme has a wide range of applications in the food processing and distillery industries, including the conversion of starch to sugers and the production of conversion syrups (maltose and fructose syrups). Amylopullulanase was purified by three stepsincluded precipitation with 40% saturation ammonium sulfate, ion exchange chromatography by DEAE- cellulose column, and gel filtration by Sephacryl S-200 column. The characteristics of purified amylopullulanase were studied. The optimum pH of enzyme activity was 7.0. It was more stable at pH 7, andthe maximum enzyme activity was observed at 70°C. The optimum thermal stability for the enzyme was at (37-50°C). The effect of some metal ions on amylopullulanase activity was determined. It was observed that 10 mM of Ca^{++} and Mg^{++} enhanced enzymes activity. Different levels of inhibition revealed when enzyme was treated with Fe^{++}, Cu^{++}, Zn^{++}, and Hg^{++} at 5 and 10 mM. An effect of some chemical agents on purified enzyme activity was investigated. Results obtained that a slight effect the amylopullulanase activity occurred after incubation with 5, 10 of EDTA and 10mM of PMSF on amylase activity. Hence, amylopullulanase activity have increased effect after incubation with 5, 10mM of 2-mercaptoethanol, while amylopullulanase lost most of its activity after incubation with 5M of urea.

Key words: Amylopullulanase ; solid state fermentation ; *Bacillus licheniformis* ; Thermostable.

Introduction:

Amylopullulanase (AP) (EC 3.2.1.1/41) is a pullulanase type II enzyme which attacks α-1,6 and α-1,4 glycosidic linkages in branched and linear polysaccharides, producing glucose, maltose and maltotriose as degradation products (Tonkova 2006). In recent years, AP has been used as an alternative enzyme to replace α-amylase during starch liquefaction for the production of glucose and conversion syrups (maltose and fructose syrups) (Roy and Gupta 2004). The production of AP has been mostly reported from bacteria, including *Lactobacillus amylophilus* GV6 (Vishnu et al. 2006), *Bacillus* sp. DSM405 (Brunswick et al. 1999), *Bacillus cereus* (Bakshi et al. 1992), *Geobacillus thermoleovorans* NP33 (Satyanarayana et al. 2004),*Clostridium thermosulfurogenes* SV9 (Swamy and Seenayya 1996)and *B. .subtilis* (Yuji, *et al*. 1996).. There is as yet no report on the production of Amylopullulanase byan actinomycetes species.We have isolated and identified as a strain of *Bacillus licheniformis* and studying the optimum conditions under (SSF),The process parameter, i.e. culture filtrate (2ml), pH (6), solid substrate (corn bran+ rice bran), hydration ratio (1:2), hydration solution (phosphate buffer), incubation period (48 h), incubation temperature (37°C). (Khalaf and Aldeen 2013). Solid-state fermentation (SSF)

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processes can be defined as the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water (Cannel and Young, 1980).These processes have been used for the production of food, animal feed, and both pharmaceutical and agricultural products (Young *et al.*, 1983). Solid state fermentation (SSF) even though conventional but is still extensively employed due to less energy requirements, high product yield, less catabolic repression and end product inhibition, low capital investment, and better product recovery (Regulapati *et al.*, 2007).*Bacillus licheniformis* is rod shaped, Gram positive, endospore forming and they are common in most soils and dominate in nutrient-poor soils such as moorlands and deserts. (Da Silva *et al*, 2005). Also they are common in foods including natural agricultural products such as cereals which it presumably colonizes from wind-blown dust and soil particles (Da Silva *et al*, 2005). *B. licheniformis* is considered as one of the examples of thermophilic bacteria belong to *Bacillus* genus, it is optimal growth temperature which is around 50-55°C (Burgess *et al*., 2010).Among amylopullulanase are interesting in starch processing industry, moderately thermostable (50°C) due to the specific debranching capacity of hydrolyzing either α-1,6 and α-1,4 glycosidic linkages.the aim of this study was, therefore, to explore the possibility of purification and characterization of a thermostable amylopullulanase from *Bacillus licheniformis* in solid state fermentation (SSF).

Materials and methods:

Microorganism

Bacillus licheniformis isolated from soil and identified according to the morphological and microscopic examination (Khalaf and Aldeen 2013).

Determination of amylopullulanase production

Two methods were used for production of enzymes were assayed in (Khalaf and Aldeen 2013).

Effect of fermentation parameters on enzyme production in (SSF)

Five grams of rice bran, corn bran, respectively .was hydrated with 1:1.5 (w/v) phosphate buffer containing 1% (w/v): 1 starch, 0.5 yeast extract, 0.03 K_2HPO_4, 0.02 $MgSO_4$.7H2O, 0.1 $(NH_4)_2SO_4$, 0.02 $CaCl_2.{}_2$H20, 0.001 $FeSO_4.7H_2O$,$MnCl_2.4H_2O$, in Roasting bags , autoclaved at 121Cº for 20 min , and inoculated with (2ml) of 24hr activated bacterial suspension (OD=0.8 at 600nm) and incubated at 37Cº for 48hr. the enzyme was extracted by added (50ml) of tap water to the solid substrate culture and well mixed by agitation for (5 min), then filtered through a cloth ,the filtered was centrifuged at (8000rpm) for (20min), the supernatant was used as crude enzyme, the activity , protein concentration , and specific activity were assayed (Khalaf and Aldeen 2013).

Assay of amylopullulanase activity

The activities of enzymes were assayed by measuring the amount of reducing sugar released from pullulan and starch Amount of reaction solution 0.5 ml was added to 0.5 ml of crude enzyme source in 2 ml of phosphate buffer (0.02 M, pH 6) then 0.5 ml of $CaCl_2$ was added to the reaction solution. Tubes were incubated at 37 °C for 20 min. The reaction was stopped by cooling the tubes in an ice bath and reducing sugar released by enzymatic hydrolysis of pullulan and starch were determined by addition of 1 mL of 3, 5-dinitrosalicyclic acid reagent, then incubated in a boiling water bath for 5 minutes, and measured the enzyme activity at (540nm). One unit of

pullulanase is defined as the amount of enzyme which released one micro mole of reducing sugars as glucose per min under standard assay conditions.

Determination the optimum conditions of enzymes production

The effect of solid substrate by using (wheat bran, rice bran, corn bran and soya bean), temperature (37-45-50-60), incubation period (24-48-72 h), pH (4-9), hydration ratio (1:1.5-1:2-1:2.5), and hydration solution (tap water-phosphate buffer- PYEmedium), inoculum size(0.5-1-1.5-2-2.5 ml) on enzyme production by *Bacillus licheniformis* was investigated by (Khalaf and Aldeen 2013).

Extraction of enzyme

The enzyme was extracted from *Bacillus licheniformis* cultured under optimum conditions of SSF medium as described in (Khalaf and Aldeen 2013).

Purification of enzyme

The enzymewas purified by more than one step using different purification techniques, as mentioned bellow:

Precipitation of enzyme with ammonium sulfate

The first step of enzyme purification was achieved by precipitation with ammonium sulfate. Ammonium sulfate was added to the supernatant (crude enzyme) with gradual saturation ratios ranging between 30% to 90%. The mixture was mixed gently on magnetic stirrer at 4°C for 30 minutes. The precipitated proteins were separated by centrifugation at 8000 rpm for 20 min, and dissolved in a small volume of 0.02M phosphate bufferat pH 6. pullulanase, amylase activity, protein concentration and specific activity were estimated before and after ammonium sulfate precipitation.

Dialysis of enzyme

The solution (crude enzyme precipitated) was dialyzed against phosphate buffer at 4°C for 24 hrs with three increments of substitutions, and concentrated by sucrose. pullulanase activity and amylase activity, protein concentration and specific activity were estimated.

Ion exchange chromatography by DEAE-Cellulose

DEAE-Cellulose column was prepared according to (Whitaker and Bernard, 1972).Three ml of enzyme concentrated was then applied to ion exchange chromatography column packed with DEAE-Cellulose equilibrated previously with small volume of phosphate buffer (0.02M, pH 6). Then column was washed with an equal volume of the same buffer to displace unbinding protein (wash), while attached proteins were stepwise eluted phosphate buffer with gradual concentrations of sodium chloride (0.1-0.4 M respectively). Fractions were eluted at a flow rate 3ml/fraction and the absorbance of each fraction was measured at 280 nm using UV- VIS spectrophotometer. Pullulanase and amylase activity for each fraction was determined. Fractions representing enzymes activity were pooled together and concentrated for the last step of purification by gel filtration chromatography.

Gel filtration chromatography by Sephacryl S-200

It was prepared according to Pharmacia Fine Chemicals Company.Three ml of concentrated enzyme solution obtained from the elution fraction of ion exchange chromatography step was added gently to Sephacryl S-200 column in dimensions of (1.5cm x 70 cm), equilibrated previously with 0.02M phosphate buffer pH6. And eluted using the same buffer with flow rate of 30 ml / hour (5 ml for each fraction).Absorbance of each fraction was measured at 280 nm. Enzyme activity and protein concentration was also determined in each fraction. Fractions represents enzyme activity

were pooled and kept at 4°C for further studies.

Characterization of purified enzyme

Determination of the optimum pH for enzyme activity

The reaction solution for pullulanase activity and reaction solution for amylase activitywere prepared at different pH values (4-10) by adjusting with 0.1 M acetate buffer pH 4, 0.02 M phosphate buffer pH 6, and 0.1 M glycine buffer pH 10. 0.5 ml of purified enzyme was mixed with 0.5 ml of reaction solutions, incubated at 37°C for 20 min, the reaction was stopped by incubation in an ice bath and the activity of pullulanase and amylase were measured at 540 nm.

Determination of enzyme stability at different pHs

The purified enzyme solution (0.5 ml) was mixed with equal volume of buffer solutions at different pHs (4-10) and incubated at 37°C for 30 min, then cooled in an ice bath. 0.5 ml of the reaction solution of pullulan and starch were added to each mixture separately and incubated at 37°C for 20 min. the reaction was stopped by using an ice bath and the remaining activity of pullulanase and amylase were measured.

The effect of temperature on enzyme activity

The purified enzyme solution (0.5 ml) was added to 0.5 ml of reaction solution of pullulan and starch at pH 7.0 and incubated at different temperatures ranged from (37-90°C) for 20 min, the reaction was stopped by using an ice bath and the activity of pullulanase and amylasewere assayed at 540 nm.

Determination of enzyme stability at different temperatures

Half ml of purified enzyme solution was incubated in a water bath at different temperatures (37-90°C) for 30 min, then immediately transferred into an ice bath, then 0.5 ml of reaction solution of pullulan and starch were added to each tubes separately at pH 7 and incubated at 70°C for 20min, the reaction was stopped and the remaining activity of pullulanase and amylase were estimated.

The effect of metal ions on the enzyme activity

The purified enzyme solution (0.5 ml) was incubated with and without metal ion solution (FeSO4, $MgSO_4$, $CuSO_4$, $ZnSO_4$, $HgCl_2$, $NaCl_2$, KCl_2, $CaCl_2$) in a final concentrations of 5 and 10 mMat ratio of 1:1 at 37°C for 30 min, then 0.5 ml of the reaction solution pullulan and starch were added at pH 7 and incubated at 70°C for 20 min, the remaining activity of pullulanase and amylase were assayed.

The effect of 2- mercaptoethanol, EDTA and PMSF on enzyme activity

Half ml of the purified enzyme solution was incubated with and without 2-mercaptoethanol, EDTA and PMSF solutions in a final concentrations of 5 and 10 mM at ratio of 1:1 at 37°C for 30 min, then 0.5 ml of the reaction solution of pullulan and starch were added at pH 7 and incubated at 70°C for 20 min, the remaining activity of the pullulanase and amylase were assayed.

The effect of urea solution on enzyme activity

Half ml of the purified enzyme solution was incubated with and without (urea solution) ina final concentration of 5 M at ratio of 1:1 at 37°C for 30 min, then 0.5 ml of the reaction solution of pullulan andstarch were added at pH 7 and incubated at 70°C for 20 min, the remaining activity of the pullulanase and amylase were assayed.

Results and discussion:

Purification of Amylopullulanase enzyme

After selected the best optimum conditions for the production of

amylopullulanase by *Bacillus licheniformis*Bs18 (corn bran+ rice bran as solid substrate, inoculated with 2 ml and incubated for 48 hrs at pH 6 hydrated with phosphate buffer at ratio 1:2) it was purified.Concentration of enzymes is one of the most widely used techniques in enzyme purification. One of these techniques depends on the precipitation by ammonium sulfate salts (salting out) it was widely used for the fractionation of protein. It was rather used as an expensive way for concentrating a protein extract (Clive, 2002).Results showed that the maximum specific activity 31.8 U/mg for pullulanase and 34.8 U/mg for amylase revealed at 40% saturation with 1.01fold of purification and 53.5 recovery for pullulanase; and 1.06 fold of purification with 55.8 recovery for amylase; whereas the higher ammonium sulfate saturation ratios decreased the enzyme specific activity.Shekufeh *et al.* (2010) revealed that 1.0 fold of purification and 100% yield reported of amylopullulanase from *Geobacillus* sp. after ammonium sulfate precipitation with a saturation ratio of 75%.(Asoodeh and Lagzian, 2012) observed that 1 fold of purification and 92.3% was obtained of glucoamylopullulanase from *Bacillus subtilis* after ammonium sulfate precipitation with a saturation ratio of 85%.Enzyme extracted from *B.lichenoformis* BS18 was purified by ion exchange chromatography, using DEAE-cellulose an anionic excharger. This mixture was used for purification because it has high capacity for bio separation, easily to prepare, multiple use, in addition simplicity to separate different bio molecules (Karlsson *et al*., 1998).Results indicated in figure (1) showed that there are two protein peaks that appeared after elution by the gradient concentrations of sodium chloride. With one peak contained pullulanase and amylase activities located in the first protein peak between the fractions 35-46. All these protein peaks were detected by measuring the absorbance at 280 nm.The activity increased in this step from 22.3 U/ml to 32.1 U/ml for pullulanase and 24.4 U/ml to 36.3 U/ml for amylase, fold of purification were 2.05 for pullulanase and 2.22 for amylase with 42.8% and 46.1% recovery respectively (Table 1). (Reddy *et al*., 1998) illustrated that purification of pullulanase and amylase from thermostable *Clostridium thermosulfurogenes*, using precipitation with $(NH_4)_2SO_4$ at 60%, then ion exchange chromatography by DEAE cellulose gave 22.4 fold for pullulanase and 17.2 fold of purification for amylase with yield 17.4% for pullulanase and 20% for amylase, and the specific activity 3.93 U/mg protein for pullulanase and 2.44 U/mg protein for amylase.

Sephacryl S-200 has a separation limits ranging between (5000-300000 Dalton) which allow the ability of separation with high degree of purification (Sivasankar, 2005). Results in table (1) finally Gel filtration by using Sephacryl S-200 revealed that there is an increase in the activity of purified enzyme (47U/ml) for pullulanase and (51 U/ml) for amylase with purification fold (7.92) for pullulanase and (8.2) for amylase with slight decrease in the yield of pullulanase (37.6%) and amylase (38.9%).Roy *et al*. (2003) demonstrated that purification of pullulanase produced by *Bacillus* sp. by gel filtration (Bio-Gel P-2 column), gave a maximum specific activity 8.8 U/mg with 2.6 fold of purification and only 16 % Yield. Ara *et al*. (1992) observed that pullulanase from alkalophilic *Bacillus* sp. was purified by gel filtration using Sephacryl S-200 and obtained 0.71(mg) total protein,

181(U) total activity, 255 specific activity (U/mg), with16.2(%) Yield, and 40.5 fold of purification.According to the purification steps are summarized in table (1), illustrated that the enzyme is amylopullulanase because after purification the two enzymes was continuous activities. In the end step, the enzyme had a specific activity of pullulanase (247.3 U/mg) and amylase of (268.4 U/mg).

Characterization of purified enzyme

Optimum pH for enzyme activity

Optimum pH for enzyme activity was determined by incubation the purified amylopullulanase with its reaction solution pullulan and reaction solution (starch) at different pH values. Results indicates in figure (3) that amylopullulanase was active over a wide range of pHs (4-9), but it is more active at pH 7 than other pHs activity for pullulanase was 20 U/ml and 29.7 U/ml for amylase.

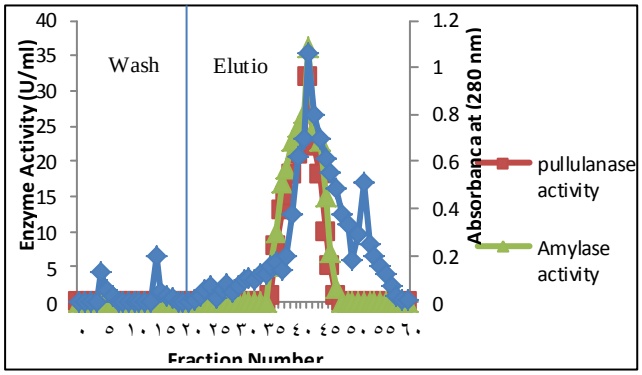

Fig. (1): Ion exchange chromatography of enzyme produced from *B.lichenoformis* BS18 isolate by DEAE-Cellulose column (2x23 cm), then was washed and eluted with an equal volume of (0.02 M) phosphate buffer, the fractions were collected with 3 ml tube at a flow rate 30 ml/ hour and eluted with gradient (0.1-0.4M) of sodium chloride solutions.

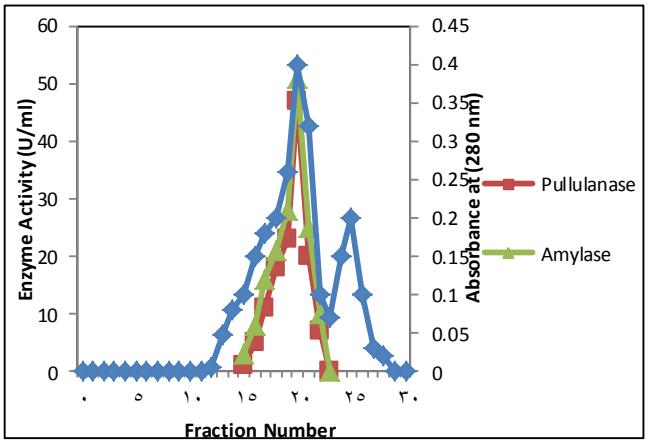

Fig. (2) Gel filtration chromatography of amylopullulanase produced by *B.lichenoformis* BS18 isolate using Sephacryl- S-200 column (1.5cm x70cm) equilibrated with phosphate buffer pH 6, fraction volume was 5 ml at flow rate 30ml/ hour.

Table (1) purification steps for enzymes of *B.lichenoformis* BS18.

purification step	Type of enzyme	Volume (ml)	Enzyme Activity (U/ml)	Protein Concentr-ation (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification (Folds)	Yield (%)
Crude enzyme	Pullulanase Amylase	60	12.5 13.1	0.4	31.2 32.7	750 786	1	100
Ammonium sulfate precipitation (40%)	Pullulanase Amylase	18	22.3 24.4	0.7	31.8 34.8	4014 439.2	1.01 1.06	53.5 55.8
Ion exchange by DEAE-cellulose	Pullulanase Amylase	10	32.1 36.3	0.5	64.2 72.7	321 363	2.05 2.22	42.8 46.1
Gel filtration by Sephacryl S- 200	Pullulanase Amylase	6	47 51	0.19	247.3 268.4	282 306	7.92 8.2	37.6 38.9

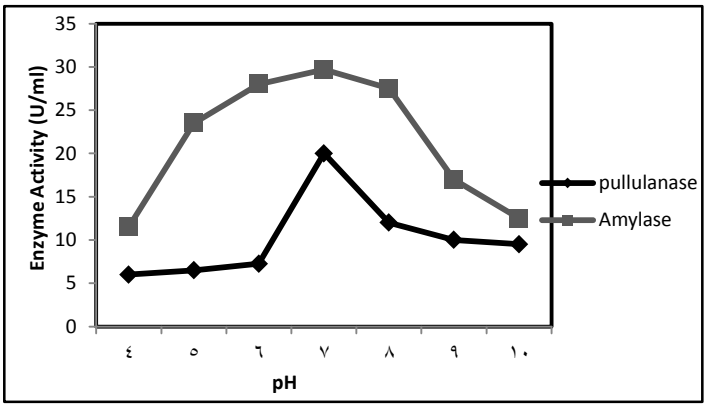

Fig. (3) Effect of pH on purified enzyme activity produced by *B.lichenoformis* BS18

The effect of pH on the rate of hydrolysis of substrate is due to the effect of enzyme on the velocity of enzyme- substrate complex formation and breakdown, in addition to its effect on the ionic state of the active site of enzyme, and the change in hydrogen ion concentration may affect the ionization of side chains located in the active site of enzyme (Bezkorvainy and Rafelson, 2007).

Kim *et al*. (2009) obtained that the maximum activity of amylopullulanase from *Lactobacillus plantarum*were is around pH 4.0–4.5 in 0.1M acetate buffer.Other studies revealed that the optimum activity of amylopullulanase from *Steptomyces erumpens* was at pH 7 (Shaktimay*et al*., 2011).

Optimum pH for enzyme stability

To determine the optimum pH for enzyme stability, purified amylopullulanase was incubated at different pH values. The remaining activity then determined after assaying enzyme activity. Results indicated in figure (4) showed that amylopullulanase was more stable at pH 7 because at this pH pullulanase gain maximum remaining activity (95%) and amylase gave (108%), while the remaining activity was decreased at pH less or more than optimum pH.

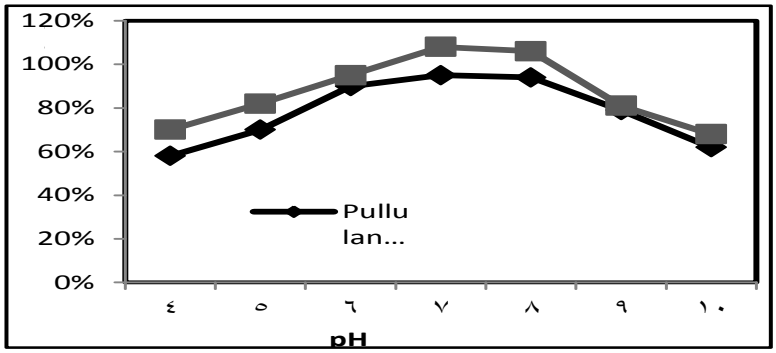

Fig. (4) Effect of pH on purified enzyme stability produced by *B.lichenoformis* BS18.

Enzymes like other proteins are stable over only a limited range of pH. outside this range, changes in the charges or ionisable residues result in modifications of the tertiary structure of the protein and eventually cause denaturation. (Zubay, 1993).

Gomes *et al.* (2003) observed that both amylase and pullulanase from *Rhodothermus marinus* exhibited a good stability over a broad pH-range (5–9), retaining 100% activity at pH 6, at higher or lower pH values pullulanase showed slightly more stability than amylase.

Effect of temperature for enzyme activity

Temperature is an important factor that affects enzyme activity. The favorable temperature for enzyme activity may differ with diverse amylopullulanase sources.

The results indicated in figure (5) showed that enzyme activity increased with increasing of temperature and reached its maximum value 24 U/ml for pullulanase and 31 U/ml for amylase at 70°C, and then enzyme activity was decreased at higher than 70°C.

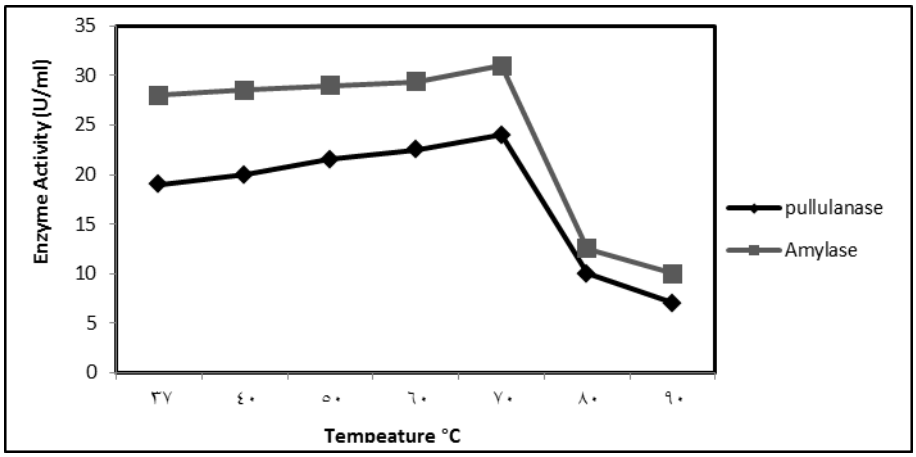

Fig. (5) Effect of temperature on purified enzyme activity produced by *B.lichenoformis* BS18

(Asoodeh and Lagzian, 2012) showed that the optimum temperature of glucoamylopullulanase from *Bacillus subtilis* was determined at 70°C. Kim *et al.*(2009) showed that Maximum temperature activities of amylopullulanase from *Lactobacillus plantarum*were detected at around 40–45°C.

Optimum temperature for enzyme stability

Results indicated in figure (6) obtained that amylopullulanase was more stable at 37-50C° because at these temperature amylopullulanase gain maximum remaining activity 98% for pullulanase and 110% for amylase, then enzyme began to lose its activity after incubation at temperature 60°C and above.The decrease in the enzyme activity with the increase of the temperature may lead to the denaturation of enzyme by destruction the three dimensional structure of protein and that cause a change in the active site which leads to inactivation of enzyme at high temperature (Prescott *et al*., 2005).

Reddy *et al*. (1998) showed that the optimum amylase and pullulanase stability from *C. thermosulfurogenes* was reported at 70C° for amylase and 75C° for pullulanase and the remaining activity was 48% for amylase and 68% for pullulanase.

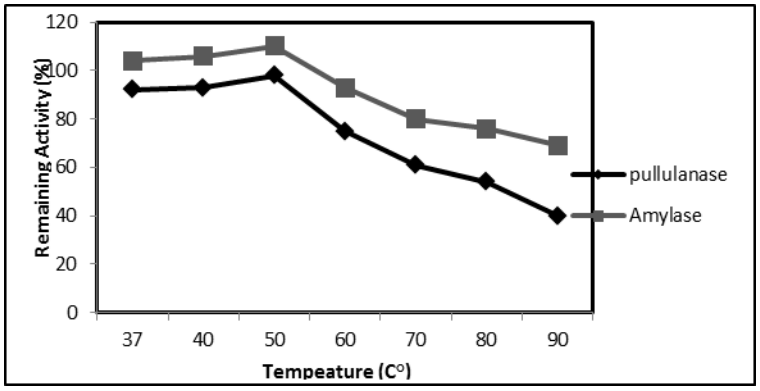

Fig. (6) Effect of temperature on purified enzyme stability produced by *B.lichenoformis* BS18 incubated at different temperatures.

Effect of heavy metals on enzyme activity

Purified amylopullulanase of *Bacillus licheniformis* Bs18 was treated with different metals. Results in table (2) showed that mercury, zinc, copper and ferric ions have an inhibitory effect on amylopullulanase activity and Hg+2 had the highest inhibition effect since it decreased the activity to 2% for pullulanase and 1% for amylase at concentration 10mM.Other ions such as (Na^{+2} and K^{+}) slightly decreased the enzyme activity. From these results, it can be concluded that the metal ions vary in their effect on amylopullulanase depending on concentration and this may give some knowledge about the activation or inhibition of *B.lichenoformis* BS18 amylopullulanase by monovalent and divalent cations.Results showed in table (2) indicated that amylopullulanase activity increased when the enzyme was treated with Ca^{+2}, and Mg^{+2} at concentration 10 mM, the maximum remaining activity was 154% for pullulanase and 144% for amylase, this means that amylopullulanase needs these metal ions as a cofactor. Shekufeh *et al*. (2010) reported that amylopullulanase from *Geobacillus* sp. showed thermal stability of the enzyme at 75C° for 30 min in the presence of 2 and 5 mM Ca^{+2} ion and increasing the enzyme activity to 80%.

Vishnu *et al.* (2006) reported that various metals (Zn^{+2}, Ca^{+2}, Cu^{+2}, Fe^{+2}, Ni^{+2}, Cd^{+2} and Ba^{+2}) inhibited amylase and pullulanase activities completely from *L. amylophilus* at 2.5mM.

Table (2) Effect of metal ions on enzyme activity from *B.lichenoformis* BS18.

Metal ion	Concentration (mM)	Remaining activity (%)	
		Pullulanase	Amylase
Ca^{++}	5	124	125
	10	154	144
Na^{++}	5	90	89
	10	95	93
K^{+}	5	94	91
	10	91	85
Mg^{++}	5	115	113
	10	140	135
Fe^{++}	5	45	40
	10	38	37
Cu^{++}	5	20	18
	10	10	11
Zn^{++}	5	18	14
	10	15	12
Hg^{++}	5	6	7
	10	2	1

Effect of Reducing and chelating agents

Results in table (3) indicated that pullulanase activity did not affect after treatment with 10mM EDTA; slight effect in amylase activity was detected after incubation with 10mM of EDTA, these results indicates that amylopullulanase produced by *B.lichenoformis* BS18 is not belong to metaloenzymes. Which require metal ion for activity, and removal of metal ions from enzyme structure leads to entire loss in enzyme activity (Price and Steven, 1982). The reducing agent 2- mercaptoethanol enhanced the activity of both activities (pullulanase and amylase) at 5mM, and no effect was observed when incubated at 10mM, It means that amylopullulanase is not thiol enzyme sulfhydryl groups are not involved in the active site of the enzyme, (Scopes, 1987). PMSF also increased the enzyme activity at 10mM indicated that no effected on the activity of enzyme, it mean that amylopullulanase is not serine enzyme, (Scopes, 1987). Urea was the inhibitory agent for enzyme, decreased the activity to 20% for pullulanase and to 9% for amylase, the reason for that could be attributed to the urea acts as denaturation agent for proteins, by breaking intramolecular hydrogen bonds (Kauzmann *et al*, 1954). Other studies showed that amylopullulanase activity from *L. amylophilus* was affected after incubation of 5 and 10Mm with EDTA, (Vishnu *et al*., 2006).

Table (3): Effect of reducing and chelating agents on enzyme activity, purified from *B.lichenoformis* BS18

Materials	Concentration(mM)	Remaining activity (%)	
		pullulanase	Amylase
EDTA	5	100	86
	10	85	73
2-Mercaptoethanol	5	154	104
	10	131	100
PMSF	5	124	98
	10	139	86
Urea	5M	20	9

Kim *et al*., (2009) observed that amylopullulanase from *Lactobacillus plantarum* lost most of its activity after incubation with 5M urea. (Sodhi *et al.*, 2005) recorded similar results for amylase preparation from *Bacillus* sp. PS-7 which exhibited very little improvement in the presence of 2-mercaptoethanol, meaning that amylopullulanase is not a thiol enzyme which is not involved sulfer group in the active site of the enzyme.

Conclusion:

Solid state fermentation technique (SSF) is more efficient in pullulanase production from *B. licheniformis* Bs 18. The best production of enzyme by SSF is observed when incubated with a mixture of rice and corn bran instead of one substrate. Ammonium sulfate, at 40% saturation, was used to precipitate the enzyme. The enzyme has two activities pullulanase and amylase

activity and the affinity of enzyme to starch is high from pullulan. The net charge of enzyme is negative. The enzyme is produce with a broad range of pH and temperatures and the production of enzyme in high temperatures leading to advantage in the industries this indicates enzyme is thermoactive and thermostable. And *B. licheniformis* Bs18 of Amylopullulanase was neither metallo nor thiol or serine enzyme. Finally enzyme loss its activity when treated with urea at concentration of 5M.

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تنقية وخصائص انزيم الاميلوبليونيز الثابت حرارياً من بكتريا *Bacillus licheniformis* Bs 18 باستخدام تخمرات الحالة الصلبة

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الخلاصة :
تضمنت الظروف المثلى لانتاج الانزيم amylopullulanase باستخدام وسط التخمر الصلبSSF بزرع 2 مل من العالق البكتيري المنشط على الوسط الحاوي على مخلفات الرز ومخلفات الذرة والمرطبة بمحلول الفوسفات الدارئ بنسبة ترطيب 1:2 (وزن/حجم) عندالرقم الهيدروجيني 6 لمدة 48 ساعة وحضنت في درجة حرارة 37 درجة مئوية وبلغت الفعالية النوعية لانزيم البليولنيز 33.8 وحدة /ملغم بروتين وبلغت 36.9 وحدة /ملغم للاميليز تمت تنقية الانزيم amylopullulanaseالمنتج من العزلة Bs 18 بثلاث خطوات شملت الترسيب بكبريتات الامونيوم بنسبة اشباع 40% ثم كروموتوغرافيا التبادل الايوني باستخدام عمود DEAE celluloseوالترشيح الهلامي في عمودSephacryl S-200 . حيث بلغت الفعالية النوعية للبليولنيز 47 وحدة/ ملغم بروتين والاميليز 51 وحدة / ملغم بروتين بعدد مرات تنقية 7.92 للبيلولنيز و8.2 للاميليز وبحصيلة انزيمية بلغت 37.6% للبليولنيز و38.9% للاميليز .
تمت دراسة بعض صفات الانزيم المنقى وكانت قيمة الرقم الهيدروجيني الامثل لفعالية الانزيم 7 وكان الانزيم اكثر ثباتا في ارقام هيدروجينية تراوحت بين(7-8) وظهرت اقصى فعالية للانزيم عند درجة حرارة 70 درجة مئوية وكان الثبات الحراري الامثل للانزيم بين (37-50) درجة مئوية واحتفظ الانزيم باكثر من 50%من فعالية البليولنيز و80% للاميليز عند حضنه بدرجة 80 درجة مئوية لمدة 30 دقيقة .
درس تاثير بعض الايونات الفلزية في فعالية الانزيم amylopullulanase ولوحظ زيادة في فعالية الانزيم عند حضنه مع ايونات الكالسيوم والمغنيسيوم بتركيز 10 ملي مولار وبلغت الفعالية الانزيمية للبليولنيز154، 140% ولانزيم الاميليز144، 135%على التوالي ، كما لوحظ نسب مختلفة من التثبيط تراوحت بين (1%- 38%) عند معاملته بايونات الحديد والنحاس والخارصين والزئبق.
لم تظهر كل من العوامل الكلابية (العامل المخلبي EDTA) بتركيز 10 ملي مولار والعوامل المختزلة (2-مركبتوايثاناول) و PMSF تاثيرا واضحا على فعالية الانزيم وهذا يعطي مؤشرا على ان الانزيم هو ليس من الانزيمات الفلزية ولا الكبريتية ولا السيرينية بينما اظهرت اليوريا بتركيز 5 مولار تاثيرا مثبطا للانزيم .