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Purification and characterization of a novel pullulanase enzyme from *Bacillus thuringiensis* for detergent industry

Purificación y caracterización de una nueva enzima pululanasa de *Bacillus thuringiensis* para la industria de detergentes

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Abstract

In the present study purification and characterization of a novel extracellular pullulanase enzyme from *Bacillus thuringiensis* was carried out for use in detergents. Maximum production of pullulanase (5.71 U/mL) was obtained in the medium containing tryptone as carbon and energy sources. The pH of media was 6.0 when it was inoculated with 3% overnight grown inoculum and incubated at 37°C for 24 h. Optimal conditions for pullulanase enzyme activity were also determined and maximum activity of enzyme (8.584 U/mL) was found with 4% pullulan as a substrate in phosphate buffer of pH 7.0 at 50°C after 20 min of incubation. Purification of pullulanase enzyme was achieved to homogeneity by ammonium sulphate precipitation as well as by ion exchange chromatography and a distinct band of 97 kDa was analyzed by SDS-PAGE. Purification fold of purified enzyme was calculated as 16.83 with 37% yield and 45.45 U/mg specific activity. The enzyme was stable up to 90°C and pH 4.0-8.0. In the presence of Ca^{+2} ions, the activity of the enzyme was increased, whereas EDTA found to reduce the pullulanase enzyme activity. The addition of 1% Tween 80 and Tween 20 did not show considerable effects but SDS and DMSO slow down the pullulanase enzyme activity. No significant effect of organic solvents (ethanol, methanol, acetone, isopropanol, and *n*-butanol) was detected on enzyme's residual activity. Purified pullulanase enzyme showed great stability with laundry detergents and wash performance was increased in the presence of pullulanase along with amylase against starchy stain.

Keywords: Pullulanase; *B. thuringensis*; Purification, Characterization; Detergent industry.

Resumen

En el presente estudio, se llevó a cabo la purificación y caracterización de una nueva enzima pululanasa extracelular de *Bacillus thuringiensis* para su uso en detergentes. La producción máxima de enzima pululanasa (5.71 U/ mL) se obtuvo en el medio que contenía triptona como fuente de carbono y energía con un pH de 6,0 cuando se inoculó con un inóculo al 3 % cultivado durante la noche y se incubó a 37 °C durante 24 horas. También se determinaron las condiciones óptimas para la actividad de la enzima pulalansa y se encontró la actividad máxima de la enzima (8.584 U/mL) con 4% de pululano como sustrato en tampón fosfato de pH 7,0 a 50°C después de 20 minutos de incubación. La purificación de la enzima pululanasa se logró hasta la homogeneidad mediante precipitación con sulfato de amonio así como mediante cromatografía de intercambio iónico y se analizó una banda distinta de 97 kDa mediante SDS-PAGE. La cantidad de purificación de la enzima purificada se calculó como 16,83 con un rendimiento del 37 % y una actividad específica de 45,45 U/mg. La enzima fue estable hasta 90°C y pH 4,0-8,0. En presencia de iones Ca^{+2}, la actividad de la enzima aumentó, mientras que el EDTA redujo la actividad de la enzima pululanasa. La adición de Tween 80 y Tween 20 al 1% no mostró efectos considerables, pero SDS y DMSO inhiben la actividad de la enzima pululanasa. No se detectó ningún efecto significativo de los disolventes orgánicos (etanol, metanol, acetona, isopropanol y *n*-butanol) sobre la actividad residual de la enzima. La enzima pululanasa purificada mostró una gran estabilidad con detergentes para ropa y el rendimiento del lavado aumentó en presencia de pululanasa junto con amilasa contra las manchas de almidón.

Palabras clave: pululanasa; *B. thuringensis*; Purificación, Caracterización; Industria de detergentes.

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1 Introduction

Starch is one of the most abundantly found reservoirs of carbohydrate on earth which is naturally present in wheat, potatoes, corn, and cassava. Major components of starch are amylose that are high molecular weight linear copolymers containing alpha-1,4 glucopyranose units and amylopectin, having alpha-1,4 glucopyranose as well as alpha-1,6 glycosidic bonds at every 17 to 26 residues (Satyanarayana *et al*., 2004; Tester *et al*., 2004). Plants store starch in the form of semi-crystalline granules in fruits, seeds, rhizomes, and tubers. Depending upon the origin, the ratio of amylose and amylopectin varies among starch and their physical properties also differ significantly (Swinkels, 1985; Nakamura *et al*., 1995). Other factors involved in the variation of the structure and composition of starch are plant variety, organ, age, species, and growth conditions. All these factors influence the crystalline arrangement of starch in the granules and in turn affect the enzymatic hydrolysis of starch in the starch processing industries (Martin & Smith, 1995).

Amylolytic enzymes hydrolyze starch in different ways. These enzymes are; alpha amylase, having family13 of glycosyl hydrolase, and beta-amylase; having family14 and glucoamylase belongs to family15 (Antranikian, 1990). These enzymes are useful for the hydrolysis of 1,4-glycosidic bonds of amylose and amylopectin due to which maltose, glucose, and maltotriose produced in higher amount which can be utilized in various industrial applications. For complete hydrolysis of starch in its monomeric units, debranching amylolytic enzymes also play very important role which cleave 1,6-glycosidic bonds present in amylose and amylopectin. Pullulanases comprise the important group of debranching amylolytic enzymes which specifically act upon 1,6-glucosidic bonds present in amylopectin and pullulan (Ara *et al*., 1995). These enzymes are widely distributed in several species of animals (Lee & Forstner, 1990; Witmer & Martínez, 2001), plants (Francisco *et al*., 1998; Renz *et al*., 1998), fungi (Aquino *et al*., 2001; Nguyen *et al*., 2000), yeasts (Büttner *et al*., 1987; Wartmann *et al*., 1995)and gram-positive or gram-negative bacteria (Albertson *et al*., 1997; Nakamura *et al*., 1975).

Pullulanases are classified into two types based on the products as well as their substrate specificities. Pullulanases type I hydrolyze the α-(1, 6) glycosidic bonds present in pullulan and other branched polysaccharides (Messaoud *et al*., 2002) while pullulanases type II cleave both α-(1, 6) glycosidic bonds as well as α-(1, 4) glucosidic linkages present in the starch. Both types of pullulanases produce maltotriose units (Duffner *et al*., 2000). Most of the starch used in starch hydrolysing industries contains almost 80% amylopectin and branch point occurs at every 20-25 residues. α-(1, 6) glycosidic linkages present in the amylopectin molecules hinder in the activity of most of the starch degrading enzymes (Jensen & Norman, 1984). These α-(1, 6) glycosidic linkages are cleaved by both types of pullulanase and thus enhance the efficiency of other enzyme to hydrolyze the starch into simple sugars.

Although pullulanases are distributed among large variety of organisms, but microbial sources are preferred for most of the industrial applications due to the ease of production, their specific mode of action and rapid microbial growth. To date, many pullulanases have been derived from various bacterial sources such as *B. deramificans* (Uhlig, 1998), *B. acidopullulyticus* (Waleed *et al*., 2015), *B. cereus* FDTA-13 (Brumm & Teague, 1988), thermophilic *Bacillus*. sp. AN-7 (Nair *et al*., 2007), Geo*Bacillus stearothermophilus* (Kunamneni & Singh, 2006), *B. stearothermophilus*, and *B. subtilis* (Kuriki *et al*., 1988), *B. flavocaldarius* KP 1228 (Zareian *et al*., 2010), *Clostridium thermohydrosulfuricum* (Suzuki *et al*., 1991) and *Fervidobacterium pennavorans* (Hyun & Zeikus, 1995) etc. However, pullulanase from *B. thuringensis* not been reported yet.

B. thuringensis is a facultative anaerobic, Gram-positive soil bacterium. It has great industrial potential for its production of insecticidal proteins (Koch *et al*., 1997). The present study concerned with the production and the purification of pullulanase from *B. thuringensis* and to our knowledge this is the first report regarding pullulanase production from *B. thuringensis*.

2 Materials and methods

2.1 Selection of microorganism and its maintenance

For the production of pullulanase, a *Bacillus* strain, *B. thuringiensis* which was obtained from University of the Punjab, Lahore, Pakistan was selected and maintained on Luria Bertani (LB) medium (1%

Tryptone, 0.5% NaCl, 0.5% Yeast Extract, 1% Agar) in its purified and viable form (Bertani, 1952).

2.2 Inoculum preparation

Seed culture of *B. thuringensis* for pullulanase production was prepared in LB broth under sterile conditions. Freshly grown single colony of *B. thuringensis* was transferred into 50 mL of LB broth with the help of inoculating needle under aseptic conditions. Overnight incubation was carried out at 37°C in a shaking incubator.

2.3 Enzyme production

Pullulanase production was carried out by using submerged fermentation technique. Freshly prepared inoculum was diluted to 1% in the same LB medium as mentioned above and incubated again at 37°C for 24 h. After 24 h fermented broth was withdrawn from incubator and centrifuged at 7500 g for 10 min. Supernatant was separated from the cell pellet in a clean sterilized falcon tube. Tris-HCl buffer (50 mM, pH: 7.5) was used for the resuspension of the cell pellet and subjected to the sonication for cell disruption in order to find out the intracellular enzyme production. Cell lysate was obtained by the centrifugation of these sonicated cells at 15,000 g for 10 min. Both extracellular and intracellular fractions were checked for the enzyme activity.

2.4 Enzyme assay

Pullulanase activity was determined for its production of reducing sugar against pullulan hydrolysis by using Dinitrosalicylicacid (DNS) method given by Miller, 1959. Both, intracellular and extracellular enzyme fractions (300 μL) were incubated with 1% pullulan (300 μL) at 50°C for 20 min. After incubation, 400 μL of DNS was added in both samples which served as reaction stopper. Enzyme was added after incubation in blank. All tubes were placed in boiling water for 5 min after addition of DNS. After boiling, 1 mL of distilled water was added in each tube and absorbance was measured at 540 nm in a double beam spectrophotometer. Pullulanase unit is defined as "the amount of enzyme required to produce 1 μM of reducing sugar from substrate in unit time".

2.5 Protein assay

The total protein contents were calculated by using Bradford method (Bradford, 1976). A 200 μL of cell lysate along 500 μL of citrate phosphate buffer and 5 mL of Bradford reagent was prepared. A blank containing only 1 mL of phosphate buffer was run in parallel. In each tube 5 mL of Bradford reagent was added and shaken well and the absorbances were taken in a spectrophotometer at 595 nm.

2.6 Optimization of pullulanase production

Various parameters (incubation temperature, pH of medium, time of incubation, inoculum size, and media composition) were optimized in order to get the maximum enzyme production. Effect of incubation temperature on enzyme production was determined by incubating the freshly inoculated LB medium (1%) with *B. thuringiensis* cells overnight at different temperatures (22, 30, 37, 42, 50 and 60°C). Effect of the medium pH on pullulanase production was observed by maintaining the initial pH of the media at various levels (3, 4, 5, 6, 7 and 8) before the sterilization and then these media were used for enzyme production. To analyze the maximum amount of enzyme production, enzyme assay was performed after 24 h. To analyze the effect of incubation time on enzyme production, culture was grown for different time periods (16, 24, 32, 40, 48, 56, 64, and 72 h) after inoculation with freshly prepared inoculum and enzyme the assay was performed for each time period after its completion under specific conditions in order to find out the optimal time of incubation for maximum enzyme production. Inoculum concentration of different ranges as (0.5, 1, 1.5, 2 and 2.5 %) was used to analyze the effect of inoculum size on pullulanase production. Enzyme assay was performed after incubation of cultures for 24 h. to analyze the maximum amount of enzyme production. Pullulanase was produce by using different media e.g. LB medium (yeast extract 0.5 g, tryptone 1 g, NaCl 0.5 g), LB-Miller (peptone 1 g, yeast extract 0.5 g, NaCl 0.5 g and agar 1 g), LB-Lennox (peptone 1 g, yeast extract 0.5 g and NaCl 0.5 g). Enzyme assay was performed after incubation of cultures for 24 h. to analyze the maximum amount of enzyme production.

2.7 Optimization of pullulanase activity

Optimal conditions are required for pullulanase activity to attain the maximum enzyme potential. Several conditions (temperature, pH, time of incubation, substrate concentration, and enzyme specificity towards its substrate) were optimized. To

optimize the enzyme-substrate incubation temperature 300 μL of enzyme fraction was mixed with 300 μL of freshly prepared 1% pullulan and incubated at a range of temperatures (30, 40, 50, 60, and 70°C) for 10 min. and then the enzyme activity was checked by using standard method as mentioned above. Optimum pH for pullulanase activity was discovered by using buffer of different pH ranges (3.0 to 8.0). Incubation of enzyme-substrate complex was carried out for diverse time periods ranging from 10 to 30 min in order to determine the effect of incubation time on pullulanase activity. Different concentrations of pullulan such as (0.5, 1.0, 1.5, 2.0, and 2.5%) were used to observe the effect of substrate concentration on activity of enzyme. Different types of substrates were used to observe the substrate specificity of pullulanase. These substrates were pullulan, starch amylopectin and malto oligosaccharide.

2.8 Enzyme purification

2.8.1 Partial purification

Ammonium sulphate precipitation technique was used to partially purify the pullulanase. Under optimized conditions pullulanase was produced from *B. thuringiensis* in a 500 mL flask. After specific time duration, supernatant was separated from bacterial cell by centrifugation at 9000 g for 10 min. Different concentrations (10% to 80%) of the ammonium salt were added to the crude enzyme pinch by pinch with constant stirring at 4°C until saturation achieved. Centrifugation of all samples were carried out at 15,000 g and 4°C for 10 min and checked for the enzyme activity. Fractions with maximum enzyme activity were pooled and subjected to dialysis using dialysis membrane in citrate phosphate buffer. After overnight dialysis, sample was drawn from the dialysis membrane and used for further analysis.

2.8.2 Ion exchange chromatography

Pullulanase from *B. thuringiensis* was further purified by using ion exchange chromatography. Bio-scale Mini Unosphere anion column was used for purification. Tris-HCl buffer (pH: 7.6, 50 mM) was used as binding buffer. For elusion, same buffer along with 1M NaCl was utilized. Column was equilibrated with binding buffer after washing with deionized water and then 1 mL lyophilized protein (pullulanase) sample (40 mg/ mL) was loaded on column. Unbound proteins were removed by using washing buffer. The concentration gradient was used of 0-1M NaCl for elution of bound protein at 1.5 mL /min flow rate with collection of mL fraction per Eppendorf. Fractions with peaks were collected and analyzed for the enzyme activity and purity.

2.9 Characterization of purified pullulanase

2.9.1 Effect of temperature and pH

Temperature and pH stability of purified pullulanase was determined in order to utilize it in industrial processes. In order to find out the thermo stability of purified pullulanase, pre-incubation of the enzyme was carried out at a range of temperatures (40°C to 90°C) for 4 h. and then the enzyme residual activity was determined under optimal conditions. pH stability of purified pullulanase was observed after incubating the enzyme at room temperature with different pH-buffers phosphate (0.05 M), citrate (0.05 M) and glycine-NaOH (0.05 M) ranging in pH from 4.0-9.0 for 1-3 h. and the residual activity was measured under standard enzyme assay conditions.

2.9.2 Effect of metal ions

Effect of different divalent ions such as Ca^{2+}, Mg^{2+}, Ni^{2+}, EDTA, Zn^{2+}, Na^{+}, and K^{+} on enzyme was examined by pre-incubating purified pullulanase with these metal ions (1-10 mM). All metal ions were pre-incubated with enzyme for 1h at ambient temperature. Remaining activity was determined under optimal conditions.

2.9.3 Effect of organic solvents and various inhibitors

The effect of organic solvents (ethanol, methanol, acetone, isopropanol, and *n*-butanol) was examined on the activity of purified pullulanase after incubation with these solvents with 10-30% (v/v) for 1 h at room temperature. Standard conditions of enzyme assay were used to calculate the residual activity. Effect of different inhibitors like DMSO, Tween-20, DTT, SDS, and Tween-80 on purified pullulanase was detected. The enzyme was pre-incubated with various concentrations (1-3%) of these inhibitors at room temperature for 1 h. Standard conditions of the enzyme assay were used to calculate the residual activity.

2.10 Potential of purified pullulanase in starch stain wash

To check the efficiency of pullulanase in starch stain wash, purified pullulanase was utilized in combination with commercially available amylase enzyme and different laundry detergents (SurfExel, Bright, and Ariel). To check the compatibility of pullulanase with detergents, 8 mg/mL dilutions of all detergents were heated at 90°C for 60 min to inactive all endogenous amylolytic enzymes. Cooled dilutions of each detergent were supplemented with 200 units of purified pullulanase and incubated at 50°C for 1 h. Residual enzyme activity was determined under standard assay conditions along with control which was without detergent.

Wash potential of pullulanase was checked against starch stain in the presence of detergent with and without commercially available amylase enzyme. Six white cotton cloth pieces (5cm x 5cm) were stained with starch containing solution were heated at 80°C for 30 min to ensure the binding of starch present in the solution to the cloth. Wash performance was determined by incubating the stained cloth with various combinations (simple distilled water, distilled water + 8 mg/mL detergent, distilled water + 200U pullulanase, distilled water + 200U pullulanase + 50U amylase enzyme, distilled water + 8 mg/mL detergent + 200U pullulanase + 50U amylase enzyme) at 50°C for 30 min in a shaking incubator. Reducing sugar was determined after incubation by DNS method using a control which contained distilled water instead of sample liquid.

3 Results

3.1 Pullulanase production

Production of pullulanase from *B. thuringiensis* was carried out by using submerged fermentation technique. In order to get the maximum enzyme production from *B. thuringiensis*, various growth parameters were optimized which are as following:

3.1.1 Effect of pH on enzyme production

Initial pH of LB medium is very important factor affecting the enzyme production. To determine the effect of initial pH, a range (3.0-8.0) of pH was used. Maximum enzyme production (3.224 U/mL) was achieved in a medium with pH 6.0 with total protein contents of 1.121 mg/mL. At other pH like, at 3.0 pH the observed enzyme production was 1.516 U/mL, 2.245 U/mL at 4.0 pH, 2.857 U/mL at 5.0 pH, 2.547 U/mL at 7.0 pH and 1.824 U/mL at 8.0 pH with total protein contents as 0.214 mg/mL, 0.523 mg/mL, 0.947 mg/mL, 0.624 mg/mL and 0.347 mg/mL, respectively, as shown in Figure 1A.

3.1.2 Effect of temperature on enzyme production

The effect of temperature on pullulanase production by *B. thuringiensis* was studied by incubating the culture at different temperature ranges as 22, 30, 37, 42, 50, and 60°C. The pullulanase production was maximum (3.457 U/mL) at 37°C with 1.45 mg/mL. At other temperatures like 22, 30, 42, 50, and 60°C, the observed enzyme activities are 0.879, 2.147, 1.757, 0.945, and 0.241 U/mL, respectively with total protein contents 0.124, 0.987, 0.741, 0.117, and 0.012 mg/mL, respectively as shown in Figure 1B.

3.1.3 Effect of incubation time on enzyme production

To determine the optimum incubation period for maximum production of pullulanase, different incubation times ranging from 16 to 72 h. were used. It was observed that *B. thuringensis* produced maximum pullulanase (4.52 U/mL) after 24 h of incubation with 2.241 mg/mL total protein contents as shown in Figure 1C. After 16, 32, 40, 48, 56, 64, and 72 h the production of enzyme was found as 3.421, 4.487, 4.442, 4.448, 4.412, 4.3997, and 4.4287 U/mL, respectively. Total protein contents after 16, 32, 40, 48, 56, 64, and 72 h of incubation were 1.42, 2.2157, 2.224, 2.1987, 2.219, 2.204, and 2.1874 mg/mL, respectively as shown in Figure 1C.

3.1.4 Effect of inoculum size on enzyme production

The effect of size of inoculum on production of pullulanase was analyzed by using a range of inoculum size 1% to 5%. Maximum production of enzyme (5.71 U/mL) with 2.874 mg/mL total proteins was found when inoculation was carried out with 3% freshly prepared inoculum as shown in Figure 1D. With other inoculum sizes like at 1% the production of pullulanase was found as 3.42 U/mL, 4.61 U/mL at 2%, and 5.67 U/mL at 4% and 5.612 U/mL at 5% with total proteins 1.74, 2.175, 2.971, and 3.157 mg/mL, respectively as shown in Figure 1D.

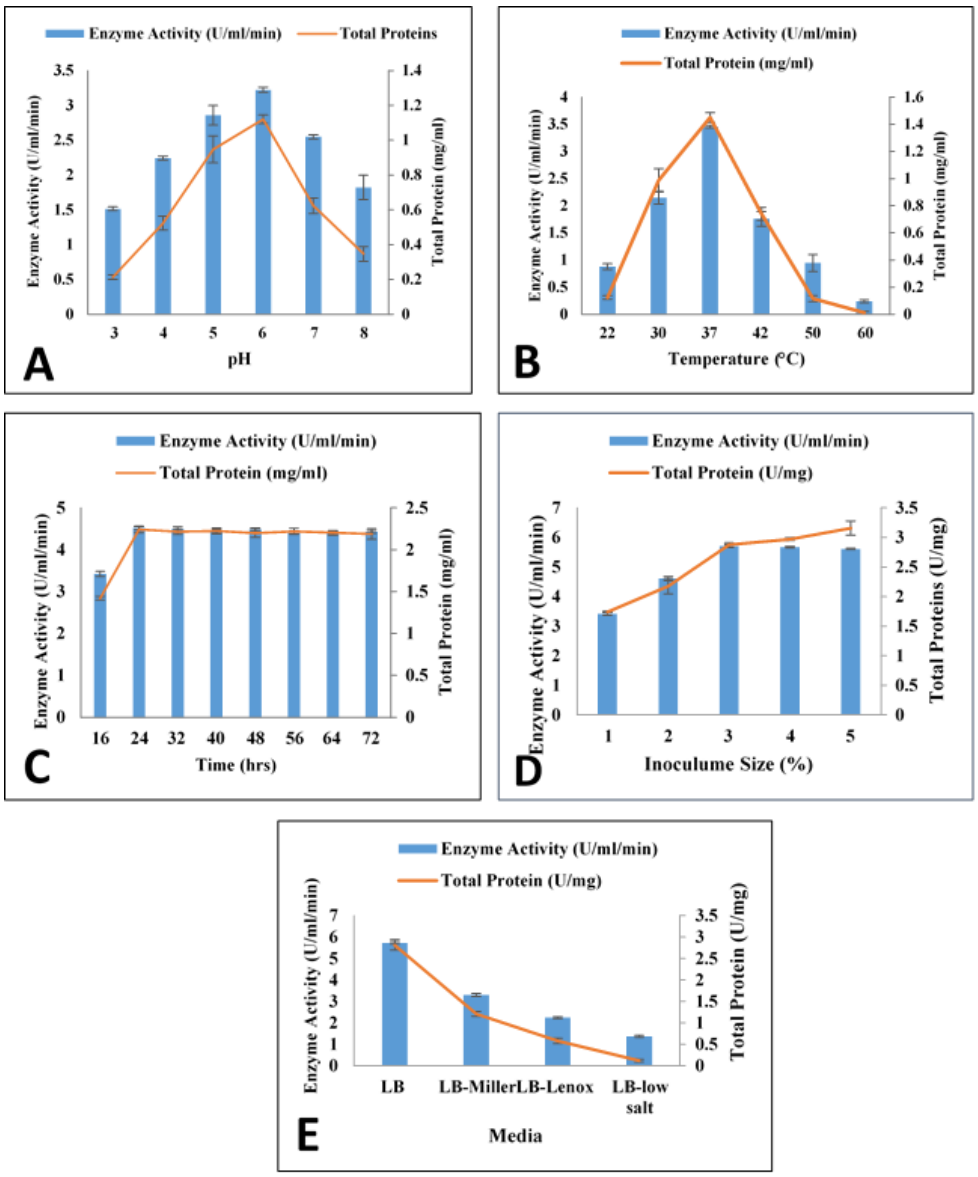

Figure 1. Optimization of pullulanase production from B. thuringiensis: (A) Effect of initial pH of medium (B) Effect of incubation temperature (C) Effect of incubation time (D) Effect of inoculum size (E) Effect of media composition.

3.1.5 Effect of media composition on enzyme production

Different media such as (LB-media, LB-Lenox, LB-Miller, LB-low salt) were used to determine the supreme production of enzyme. Best results of the enzyme production (5.724 U/mL) were obtained in LB medium with 2.814 mg/mL total proteins as shown in Figure 1E. In LB-Lenox, LB-Miller and LB-low salt media the production of enzyme was calculated as 2.24, 3.30, and 1.37 U/mL with total protein contents as 0.578, 1.21, and 0.12 mg/mL, respectively, as shown in Figure 1E.

3.2 Optimization of pullulanase activity

Following parameters were used to govern the optimized condition for the pullulanase activity:

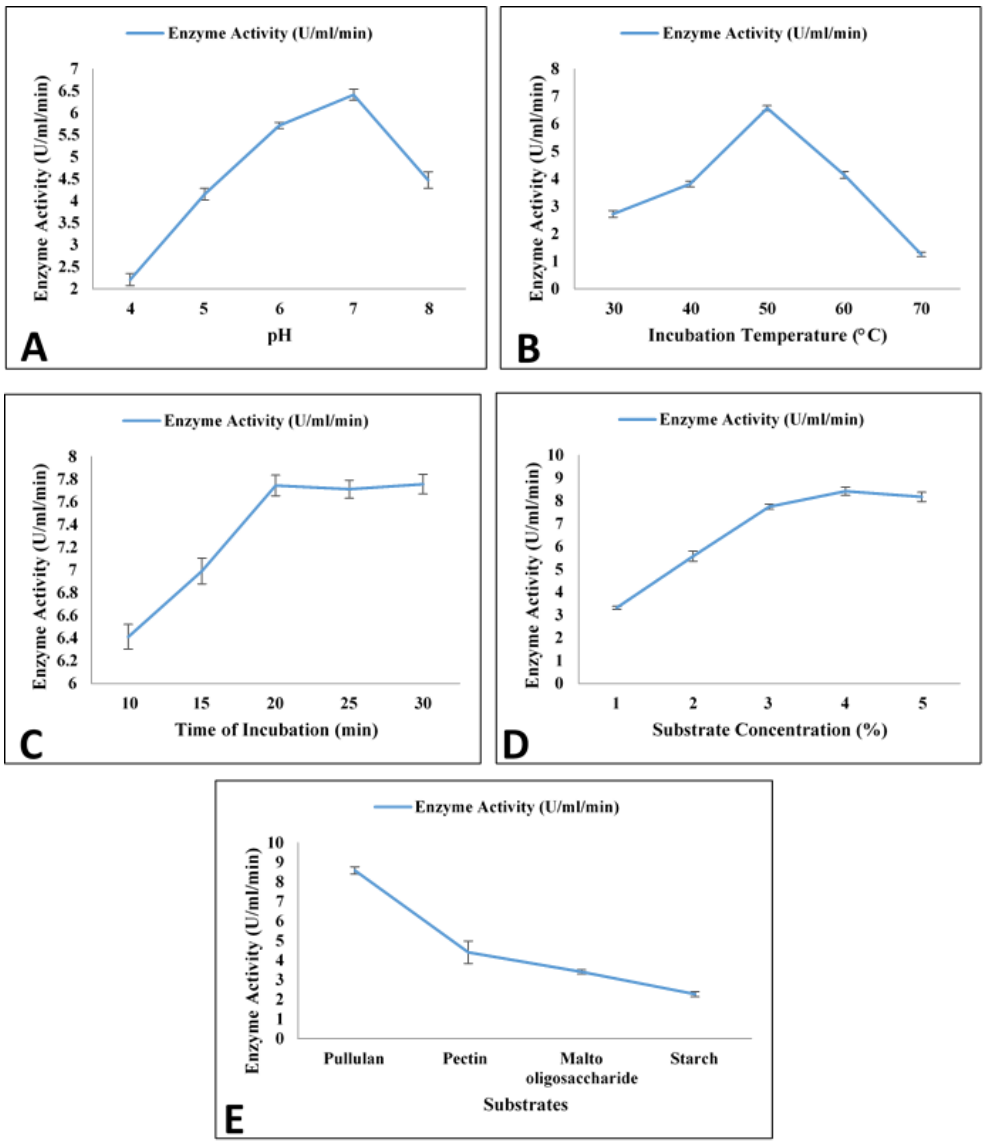

Figure 2. Optimization of pullulanase activity: (A) Effect of pH on pullulanase activity (B) Effect of incubation temperature on pullulanase activity (C) Effect of time of incubation on pullulanase activity (D) Effect of substrate concentration on pullulanase activity (E) Substrate specificity of pullulanase.

3.2.1 Effect of pH on enzyme activity

The optimal pH for the maximum activity of pullulanase was calculated using various buffers with different pH ranges from 3 to 8. Enzyme showed maximum activity (6.414 U/mL) with potassium phosphate buffer of pH 7.0. At different pH like 4.0 pH the enzyme activity was found as 2.206 U/mL, 4.151 U/mL at 5.0 pH, 5.714 U/mL at 6.0 pH and 4.471 U/mL at 8.0 pH, as shown in Figure 2A.

3.2.2 Effect of temperature on enzyme activity

To determine the optimum temperature for pullulanase activity, enzyme was incubated along with its substrate at different temperatures (30, 40, 50, 60, and 70°C). Enzyme showed maximum activity (6.571 U/mL) when enzyme substrate complex was incubated at 50°C as shown in Figure 2B. Activity of enzyme was found to be reduced by deviation in temperature from 50°C. The enzyme activity at 30°C was observed as

2.721 U/mL, at 40°C 3.814 U/mL, at 60°C 4.142 U/mL and 1.247 U/mL at 70°C, as shown in Figure 2B.

3.2.3 Effect of incubation period on enzyme activity

Activity of pullulanase also affected by the incubation time which is determine by incubating the enzyme with substrate complex at 50°C in potassium phosphate buffer of pH 7.0 for different time periods (10, 15, 20, 25, and 30 min). Optimum activity of pullulanase (7.745 U/mL) was observed after 20 minutes of incubation. At other time intervals like 10, 15, 25, and 30 min the activity of pullulanase was 6.41, 6.991, 7.712 and 7.757 U/mL as shown in Figure 2C.

3.2.4 Effect of substrate concentration on enzyme activity

In order to optimize the substrate concentration for maximum enzyme activity, enzyme was incubated with different concentrations of pullulan (1.0, 2.0, 3.0, 4.0, and 5.0%) at 50°C in a buffer of pH 7.0 for 20 min. Pullulanase was found to be maximally active (8.417 U/mL) with 4% pullulan. With other concentrations of pullulan such as 1.0, 2.0, 3.0 and 5.0%, the enzyme activity was 3.314, 5.574, 7.745, and 8.172 U/mL as shown in Figure 2D.

3.2.5 Substrate specificity of pullulanase

Enzyme's specificity for its substrate was analyzed by incubating 4% of various substrates (pullulan, starch, pectin and malto oligosaccharide) with enzyme at 50°C, pH 7.0 for 20 minutes. Maximum activity of pullulanase (8.584 U/mL) was of *B. thuringiensis* was observed with 4% pullulan as shown in Figure 2E. With other substrates used the enzyme activity was 2.266 U/mL with starch, 4.403 U/mL with pectin and 3.41 U/mL with malto oligosaccharide.

3.3 Enzyme purification

The purified pullulanase along with partially purified fraction and crude enzyme are subjected to the SDS-PAGE analysis. A known molecular weight marker was also run in parallel. A clear distinct band of almost 97 kDa was observed after ions exchange chromatography as shown in lane 4 of Figure 3. In the first lane protein marker is present while in lane 2 and 3, crude and partially purified fractions of pullulanase are present. Purification summary of pullulanase is presented in table 1.

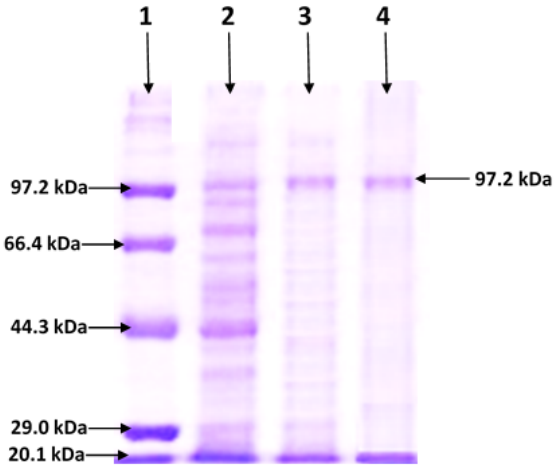

Figure 3: Pullulanase purification. In lane 1 protein ladder is present while in lane 2, 3, and 4, crude extracellular pullulanase, partially purified pullulanase and purified pullulanase from B. thuringensis are present, respectively.

Enzyme was purified with 16.83 purification fold with 45.45 U/mg specific activity and 37% yield as presented in table 1.

Table 1: Purification summary of pullulanase enzyme from *B. thuringiensis*.

Sr. No.	Purification steps	Enzyme Activity (U/ml/min)	Total Protein (mg/ml)	Specific Activity (U/mg)	Percentage yield (%)	Purification fold
1	Crude enzyme	405	150	2.7	100	1
2	Ammonium Sulphate Precipitation	245	55	4.45	60	1.6
3	Ion Exchange Chromatography	150	3.3	45.45	37	16.83

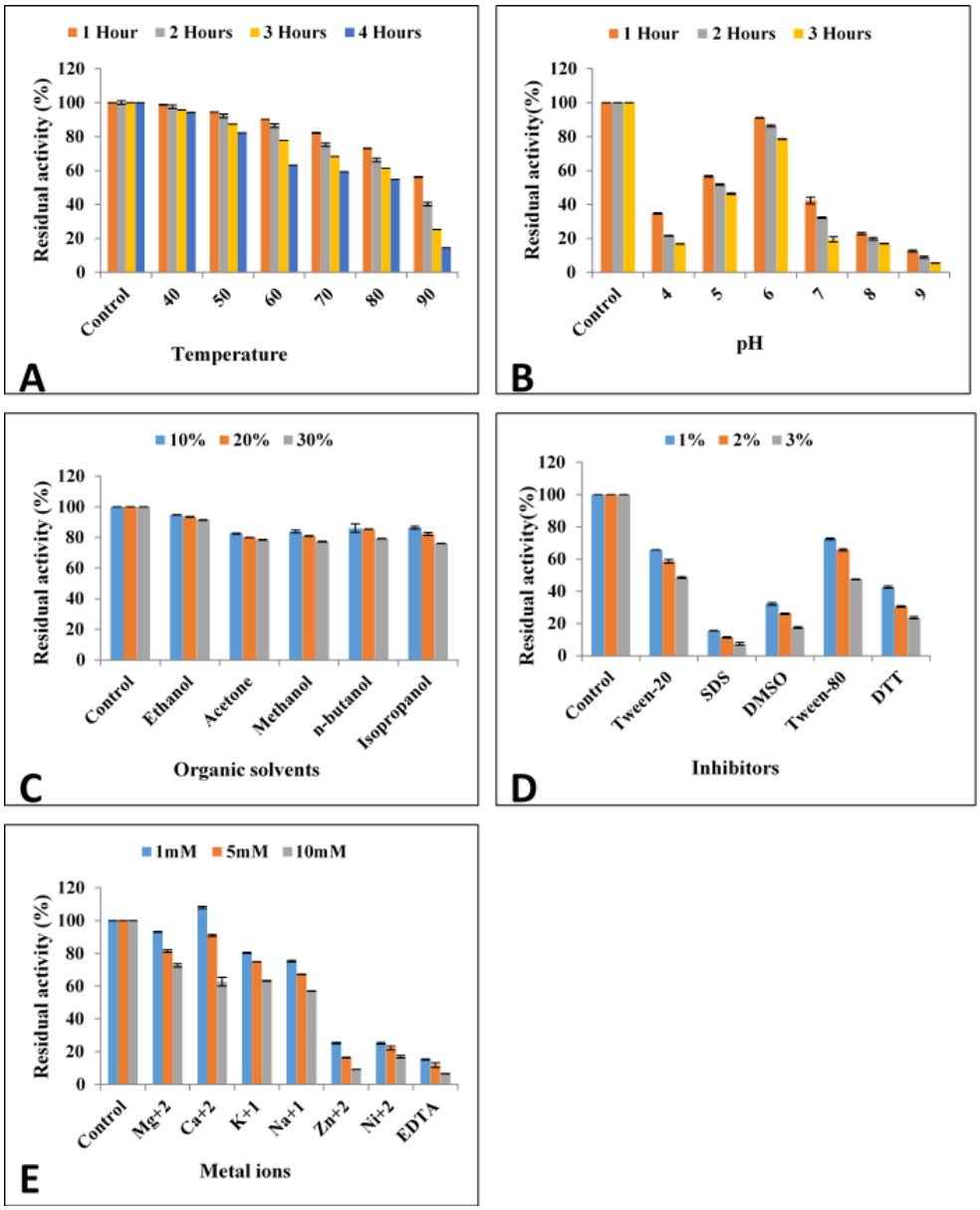

Figure 4: Characterization of purified pullulanase: (A) Thermostability of pullulanase (B) pH stability of purified pullulanase (C) Effect of organic solvent on pullulanase activity and stability (D) Effect of inhibitors on pullulanase activity and stability.

3.4 Enzyme characterization

3.4.1 Thermostability

After pre-incubation of purified pullulanase at 40-90°C, residual activity of enzyme was measured. The results represented that the purified pullulanase showed 56% thermostability after 4 h of incubation at 90°C. Enzyme retained the maximum activity (up to 73%) at 80°C after 1 h. of incubation. At other temperatures like 40°C, 50°C, 60°C and 70°C the residual activity of enzyme after 4 h of incubation was 94, 82, 63, and 59%, respectively as shown in Figure 4A. At 80°C, the enzyme activity was measured as 54% after 4 h. of incubation. However, the enzyme activity was greatly reduced (up to 14%) after 4 h of incubation at 90°C as shown in Figure 4A.

3.4.2 pH stability

Residual activity of pullulanase was determined by pre-incubation of enzyme with various pH buffers (citrate, glycine-NaOH and phosphate) at room temperature for 1-3 h. Enzyme activity was found to be stable after 1 h at pH 6.0 but at pH 4.0 and 5.0, residual activity of purified pullulanase was highly reduced respectively to 34% and 56% after 1 h of incubation. Likewise, the purified pullulanase showed 78% residual activity when incubated at pH 6.0 for 3 h (Figure 4B). However, at pH 7.0 purified pullulanase was declined to 42, 32, and 19% after incubation of 1, 2 and 3 h respectively. The purified pullulanase revealed a fall in the stability at pH 9.0 with a residual activity of 12% after incubation of 1 h.

3.4.3 Effect of organic solvents

The effect of different organic solvents (ethanol, methanol, *n*-butanol, acetone, isopropanol) on purified pullulanase was determined by pre-incubating the enzyme with different concentrations (10-30%). It was observed that organic solvents play important role in the stability of enzyme with 10% concentration. If concentrations of organic solvents become higher than 10%, activity of enzyme was inhibited as shown in Figure 4C). Some organic solvents such as 30% acetone decrease the activity up to 78%, 30% methanol decrease 77% and 30% isopropanol decrease activity 76%.

3.4.4 Effect of inhibitors

Effect of various concentrations (1-3%) of inhibitors (Tween-80, DMSO, SDS, Tween-20 and DTT) was determined after the purified pullulanase incubated with inhibitors at ambient temperature for 1 h. The residual activities of purified pullulanase were 65% and 72% in the presence of 1% Tween-20 and Tween-80, respectively (Figure 4D). However, the residual activity of purified pullulanase after incubation with 3% concentration of Tween-20, SDS, DMSO, DTT, and Tween-80 was calculated as 48, 7, 17, 23, and 47% respectively.

3.4.5 Effect of metal ions

To determine the effect of metal ions and EDTA on purified pullulanase, different concentrations (1-10 mM) of metal ions (Ca^{2+}, Mg^{2+}, Ni^{2+}, EDTA, Zn^{2+}, Na^{+}, and K^{+}) were used. It was determined that the residual activity of the purified pullulanase was enhanced by 8% in the presence of 1 mM CaCl2 as compared to control (Figure 4E). 10 mM EDTA solution was incubated with purified pullulanase and it caused a fall in the residual activity of the enzyme (94%). Incubation of purified pullulanase with high concentrations of EDTA (5 mM and 10 mM) caused an inhibition in the residual activity of the enzyme.

3.5 Application of purified pullulanase in detergent industry

Purified pullulanase showed the good compatibility and stability with all of three types of detergents used in this research. Results showed that pullulanase was highly compatible and stable in the presence of SurfExel and Ariel and showed 87% and 82% residual activities after 60 min of incubation at 50°C as shown in table 2. However, a slight decrease in residual activity (68%) was observed along with third tested detergent (Bright).

The wash performance of pullulanase was tested along with amylase enzyme and detergent (SurfExel). It was determined that combination of distilled water, pullulanase, amylase and detergent resulted in enhanced efficiency towards the removal of starch stain from cotton cloth. Enzyme activity in term of reducing sugar released was 85U/mL when pullulanase was used along with amylase and detergent as shown in Figure 5. However, less enzyme activity like 68, 21, 39, and 55 U/mL were observed with detergent + water, pullulanase + water, amylase enzyme + water, and pullulanase + amylase + water, respectively as shown in Figure 5.

Table 2: Effect of different detergents on stability and activity of pullulanase enzyme.

Sr. no.	Supplements	Residual Activity (%)
1	Control	100
2	SurfExcel	87
3	Arial	82
4	Bright	68

4 Discussion

Pullulanase is an important debranching enzyme that hydrolyzes α-1,6) glycosidic linkages present in starch and is necessary for complete hydrolysis of starch into simple sugars along with other starch degrading enzyme. It is used in many industries including starch, detergent and sugar industries.

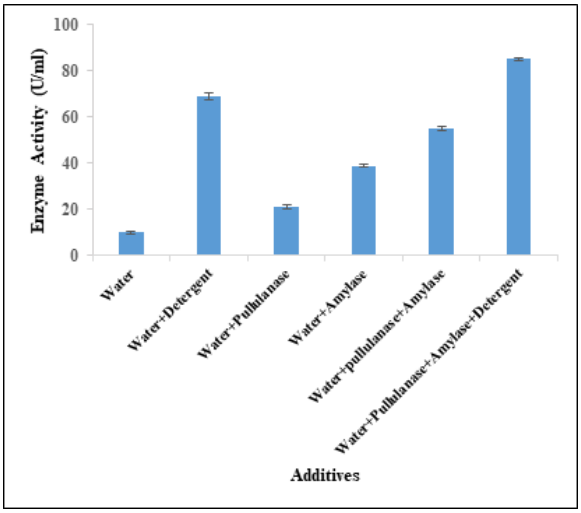

Figure 5: Wash potential of purified pullulanase to remove starch stain from cotton cloth.

Production of pullulanase has been reported from different bacterial strains, but there is no report so far on *B. thuringiensis*. The main purpose of this study was the production of pullulanase, its purification, optimization and characterization from soil bacterium *B. thuringiensis* for the utilization in detergent industry.

The production of bacterial enzymes is affected by various factors like pH, temperature, inoculum size, composition of media, and incubation time. pH of the medium is very necessary for the growth of microorganism and production of enzyme or other metabolites from that microorganism. Change in pH of the growth medium resulted in reduction of metabolic activities of the microorganisms due to the less availability of the specific nutrients to the organism because of ionization change (Willey, 2008). In the present study maximum production of pullulanase (3.224 U/mL) from *B. thuringiensis* was obtained at pH 6.0. Waleed *et al*. (2015) reported the production of pullulanase from *B. cereus* at pH 7.5 while Wei *et al*. (2015) obtained the maximum production of pullulanase at pH 6.0. Another crucial factor which influences the growth of microorganisms, is the growth temperature. Maximum production of pullulanase (3.457 U/mL) from *B. thuringiensis* was achieved at 37°C. These results are in accordance with the previous studies (Waleed *et al*., 2015; Wei *et al*., 2015) of pullulanase production from *Bacillus* species. However, the production of recombinant pullulanase in *Escherichia coli* in various studies (Chen *et al*., 2014; Zhang *et al*., 2013) was observed at 25°C. These variations in the optimal temperature of pullulanase production might be due to the different bacterial strains.

In this study maximum production of pullulanase (4.52 U/mL) was obtained after 24 h of incubation as shown in Figure 1C. Further increase in the incubation temperature did not considerably affect the production of enzyme which might be due to the limitation of important nutrients in the medium. Nair *et al*. (2007) observed that when *B. cereus* was incubated for 48 h, maximum quantity of pullulanase was produced. Ara *et al*. (1998) reported that *B. halodurans* produced maximum pullulanase when it was incubated for 5 days. Inoculum size and composition of medium also seem to affect the production of pullulanase from *B. thuringiensis* in this study. Best production of enzyme (5.71 U/mL) was achieved by inoculation with 3% freshly prepared inoculum. While LB-medium was proven as the best medium for the maximum production of pullulanase (5.724 U/mL). Increased inoculum size did not show considerable increase in the enzyme production. Swaamy and Seenaya. (1996) reported that 2.5% inoculum of *B. cereus* was required for higher production of pullulanase.

Various parameters could affect the activity of pullulanase. These parameters are pH, substrate concentration, substrate specificity, incubation time of substrate and temperature. Maximum activity of pullulanase (6.414 U/mL) in this study was found in potassium phosphate buffer of pH 7.0. Further deviation in pH resulted in the reduction of enzyme activity which might be due to the enzyme denaturation at high and low pH. Waleed *et al*. (2015) reported pH 6.0 as optimal activity of pullulanase from *B. cereus* while Asha *et al*. (2013) found the maximum activity of pullulanase from *B. halodurans* at pH 10.0. Best activity of pullulanase (6.571 U/mL) was observed when it was incubated at 50°C along with its substrate. Enzyme activity was found to be gradually decreased beyond this temperature. These results are in accordance to the previous studies from other *Bacillus* species (Waleed *et al*., 2015; Asha *et al*., 2013).

The activity of pullulanase from *B. thuringiensis* was also seemed to be affected by incubation time and substrate concentration. Maximum activity of enzyme (8.417 U/mL) was achieved after 20 min of enzyme substrate incubation with 4% pullulan. Wei *et al*. (2015) also obtained the maximum activity of pullulanase from *Paeni Bacillus polymyxa* after 20 min of incubation with substrates while Qiao *et al*. (2015) obtained the maximum activity of pullulanase from *Exiguobacterium acetylicum* after 10 min. The

hydrolytic potential of pullulanase was checked by using various substrates like, pullulan, starch, pectin and malto oligosaccharide and maximum activity of enzyme (8.584 U/mL) was achieved with pullulan. These results are in accordance with various previous studies (Duffner *et al*., 2000, Hyun & Zeikus, 1985, Wei *et al*., 2015, Asha *et al*., 2013, Qiao *et al*., 2015 and Cheong, 1996). Purification of pullulanase was carried out by ion exchange chromatography and a single well distinct band of 97 kDa was observed by SDS-PAGE analysis as presented in Figure 3. Purification fold of purified enzyme was 16.83 with 37% yield and 45.45 U/mg specific activities which are much higher than previous reports. Asha *et al*. (2015) reported a purified pullulanase of 37 kDa with 11.26 purification fold and 8.83% recovery. Similarly, Orhan *et al*. (2014) purifiified a pullulanase from *Hypocrea jecorina* with 10.12% recovery and 11.0 purification fold.

Effect of temperature on stability of purified pullulanase resulted more than 70% residual activity at 80°C after 1 h of incubation (Figure 4A). Thermostability of the enzymes is mainly determined by their primary structures but external factors like co-enzymes, substrates, cations etc. often influence upon the thermostability of the enzymes (Ward, 1988). High temperature changes the enzyme structure and turns into more flexible form to depict the active sites for maximum binding to the substrate. The high thermostability of pullulanase is attributed to the reassociation of debranched free glucan chains into further firm and compact structures with improved crystallinity through retrogradation (Miao *et al*., 2009). Pang *et al*. (2019) characterized the thermostability of pullulanase from deep sea *Pyrococcus yayanosii* and stated that this enzyme retained more than 50% activity at 95°C after incubation of 10 h. Iqbal *et al*. (2020) described the thermostability of an enzyme at the range of 50-90°C. pH stability is another important factor for the utilization of enzyme in industrial applications. The enzyme under study was found to be stable over a wide pH range (4.0-9.0) and showed maximum stability (78%) at pH 6.0 after 3 h of incubation at room temperature as shown in Figure 4B. Erden-Karaoglan *et al*. (2019) stated the pH stability of pullulanase about 94% at pH 6. Yang *et al*. (2020) characterize the pH stability of pullulanase from *Fervidobacterium nodosum* at pH of 5.0-5.5. Wu & Chen, (2014) also reported the stability of the pullulanase from *Thermus thermophilus* HB27 in a pH range from 6.0 to 8.0.

At industrial level most of the enzymatic reactions are carried out in organic solvent due to the efficient solubility of non-polar compounds. In the current study, purified pullulanase was found to be stable in high concentrations (30%) of various organic solvents. Thermostable enzymes are known to be resistant to organic solvents (Klibanov, 2001) Mesbah *et al*. (2018) reported that the amylopullulanase was resistant to chemical reagents, organic solvents like methanol. In another study, Siroosi *et al*. (2014) also reported that the pullulanase showed stability in the presence of various polar and non-polar organic solvents. Purified pullulanase was found to be stable (more than 60%) in the presence of 1% Tween-20 and Tween-80. Similarity was present between purified form of pullulanase in this study and reported detergent-resistant type I pullulanases from *B. pseudofirmus* 703 (Rajaei *et al*., 2015) and *Paeni Bacillus. polymyxa* Nws-pp2 (Wei *et al*., 2015). Huang *et al*. (2020) also observed the pullulanase from *Alkalibacterium* sp. SL3 that was resistant to Tween-20 and Tween-80. The useful application of pullulanase in its purified form is its use as biocatalyst in industrial bioprocesses that involve in addition of detergent in the enzymatic reaction.

Effect of metal ions is very important in enzyme characterization as they act as a co-factor in some enzymes. The stability of pullulanase and other amylolytic enzymes is affected by various metal ions (Yang *et al*., 2020). The presence of various divalent of metal ions poorly affected the stability of purified pullulanase while Ca^{+2} make a significant increase in the residual activity, it increases the activity up to 8% as shown in Figure 4E. Enzymes have metal ions which are chelated by chelating reagent such as EDTA and act as an inhibitory modulator, indicating metal to be an important factor to influence the conformational stability of the purified pullulanase. Ca^{+2} after binding to calcium sites could confer a stable confirmation to the purified pullulanase protein as Qoura *et al*. (2014) also described the role of Ca^{+2}. Thakur *et al*. (2021) also conducted a study on the novel-cold active type I pullulanase and stated that enzyme was stable in the presence of calcium and magnesium ion. Pullulanase hydrolyze the pullulan to maltotriose, whereas the conversion rates for amylopectin and soluble starch are very less and no activity for amylose. Hamid *et al*. (2019) also detected the role of Ca^{+2} in increasing the residual activity of esterase enzyme up to 15% in presence of 1 mM CaCl2. Kang *et al*. (2011) also detected the hydrolytic activities of pullulanse enzyme with amylopectin, starch, and glycogen. Li *et al*. (2018) also observed the higher substrate specificity

of pullulanase from *Geobacillus kaustophilus* towards pullulan as compared to other substrates. Wei *et al*. (2014) also reported the specific activity of purified Pul_{BC} was higher with pullulan as a substrate as compared to the soluble starch.

Above mentioned wide range of pH and temperature for the activity and stability of pullulanase offer an advantage to this enzyme to be utilized in detergent industry. In addition, stability of this enzyme in the presence of high concentrations of detergents and organic solvents as well as metal ions confers it as a suitable candidate for the removal of starch stains along with commercial detergents which are environmentally friendly (Souza, 2010). Good compatibility of this enzyme with commercial detergent (87%) is also an important parameter for its utilization in detergent manufacturing (Lahmar *et al*., 2017).

To date, no report is available on the collective utilization of pullulanase and amylase in detergent industry for the removal of starchy stains from clothes. However, several reports on amylase utilization for starch degradation in textile and detergent industry are present (Zafar *et al*., 2019, Simair *et al*., 2017 and Bhange *et al*., 2016). Present study revealed that utilization of pullulanase along with amylase enzyme and detergent enhances the removal of starch stains from cotton cloths in contrast to the individual amylase enzyme or detergent used. This is a great attribution towards the utilization of microbial enzymes in detergent industries which in turn could be beneficial for human skin as well as ecofriendly.

Conclusions

Currently, the research is paying attention on discovery of new suitable enzymes to be used in particular environments such as low-temperature, automatic washing detergents due to the requirement to save energy and to adapt to new fragile clothing materials. pullulanase from *B. thuringiensis* fulfilled all the necessities for its application in those detergent: high stability in a wide temperature range (30-70ºC), high activity in pH (7.0), stability of enzyme in the presence of organic solvents, inhibitors and metal ions showed it very good compatibility along with other enzymes to the commercial detergent additives.

By last, this work reports a new pullulanase from *B. thuringiensis* with potential application to the laundry detergent industry. In this sense, we believe that this research work has novelty and as well, the enzyme is very simple and cheap.

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