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A Ca^{+2} INDEPENDENT PULLULANASE FROM *Bacillus licheniformis* AND ITS APPLICATION IN THE SYNTHESIS OF RESISTANT STARCH

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Polysaccharides including resistant starch can be categorized as a part of dietary fiber and used as an important prebiotic. Like soluble fibers, resistant starch also has a number of physiological effects which have been proved to be beneficial for health. Enzymatically modified starch with resistant to digestion may find unique applications. Starch hydrolyzing enzymes display essential roles in the production of resistant starch, and the most important is pullulanase. In current study, an industrially important pullulanase from *Bacillus licheniformis* has been produced and purified for its potential use in synthesis of resistant starch type III. *B. licheniformis* is an efficient producer of pullulanase when checked on pullulan containing media. Different organic substrates were evaluated for production of pullulanase in fermentation media and ascribe that corn bran was an excellent source for pullulanase production (16 U/mg). The pullulanase was purified to homogeneity with a molecular mass of about 75 kDa on SDS-PAGE and specific activity of 191 U/mg. Pullulanase was optimally active at pH 5 and 50°C. In addition, Ca^{+2} and EDTA have no noticeable effect on pullulanase activity. Substrate specificity and thin layer chromatography results specify pullulanase as pullulanase type I. Purified pullulanase were used for the preparation of resistant starch from maize flour. Addition of pullulanase into gelatinized maize starches significantly increased the yield of resistant starch III. Final yields of resistant starch III with pullulanase treated maize starches were increased (14.32%) as compare to untreated (1.34%) and heat treated maize starch (4.25%). These results provided new information that may shed light on the further development of method to modify starches with enzymatic treatment.

Keywords: *Bacillus licheniformis;* pullulanase; organic substrate, corn bran, resistant starch.

INTRODUCTION

Starch is the most abundant biopolymer after cellulose and has become an important raw material for industrial production, ranging from food, pharmaceutical, and biofuel to textile and detergent industries (Bharathiraja *et al.*, 2017). Most starch contains 70–95% amylopectin, which is composed of α-1, 4 linked glucose residues ramified with α-1, 6 branch point links, depending on its botanical source (Hii *et al.*, 2012). Starch-hydrolyzing enzymes, including α-amylases and glucoamylases, are unable to efficiently hydrolyze branch points, containing α-1, 6 glycosidic linkages in starch (Nisha and Satyanarayana, 2013). Therefore, complete hydrolysis of starch can only be achieved in the presence of a debranching enzyme (Zhang *et al.*, 2020). Pullulanase (EC 3.2.1.41) is endo-acting debranching enzyme also known as pullulan 6-glucanohydrolase and responsible for the hydrolysis of α -1, 6 glucosidic bonding in pullulan, amylopectin and other polymers and producing maltotriose as an end product. Pullulanase belong to a family of 13 glycosylhydrolases, also known as α-amylase family which is an extracellular carbohydrase. Pullulanase hydrolyze pullulan which consists of repeating units of α-maltotriose which are joined "head to tail" by α-1, 6 bonds. Pullulanase specifically act on α-1, 6glycosidic linkage and it can also attack on α-1, 4 glycosidic linkage with other residues and releasing valuable smaller molecular weight end products such as panose, isopanose, maltose and maltotriose.

In starch processing industry pullulanase could also be used to produce "Resistant starch" (Lu, e*t al.*, 2018). Resistant starch is that specific type of starch that escape from the digestive enzymes in small intestine and ends up in the large intestine, where the bacteria in the colon digest it and converts it into short chain fatty acids (SHFA) like butyrate, acetate and propionate (Topping and Clifton, 2001). Resistant Starch is classified into four groups i-e RS I, RS II, RS III, and RS IV : RS1; which is physically inaccessible starch for example whole grains: RS II; included uncooked or partially cooked starch food for example green banana, sweet potatoes: RS III; comprises of retrograded or recrystallized starch for example bread, corn flakes which can be synthesized by biocatalyst and chemical catalyst: RS IV; consist of chemically modified starch for example addition of ether or ester groups by cross linking, conversion or substitution in order to convert the

starch that prevent the digestion by amylase in intestine (DeMartino and Cockburn, 2020). Among four types of resistant starch RSI and RSII lose their resistivity during chewing and food processing before reaching to colon, while RS III have potential to retain its resistant ability in gastric environment due to its compact crystalline structure while RS IV has some security concerns (chemical nature) due to which RS IV use is limited in functional food products. Therefore, RS III is the only form of RS which is most commonly used in many food formulations and is mainly comprised of retrograded amylose because of its high affinity to reassociation. It can be synthesized by thermal, acidic, or enzymatic treatment (Shamai *et al.*, 2003). But among these, enzymatic synthesis is preferred due to its environmentally friendly or bio-based nature. Enzymatically synthesized resistant starch is free of any toxic residues as compared to chemically synthesized resistant starch because biocatalyst is highly selective and specific for their substrate and after reaction it can easily breakdown into simple nontoxic compounds and become inactive. Therefore, RS III is the only form of RS which is most commonly used in many food formulations and can safely be produced using debranching enzymes (Haralampu, 2000).

Wide applications of debranching enzymes in different industries have motivated its studies from various microorganisms isolated from different environments. But high production cost and very low yield of the enzyme limited its use in variety of applications. Culture conditions and medium composition has great effect on production cost of pullulanase. Therefore, for increase production of pullulanase proper medium development is an important step (Akassou and Groleau, 2019). For the bulk production of the enzyme at low cost, one of the strategies is to use raw cheap substrates rich in starch. This strategy was also applied in the current study and cheap substrates of different agricultural by-products (Naik *et al*., 2019) such as corn bran, wheat bran, rice bran, two pest plants i.e. *Eichhornia crassipes* (locally known as water hyacinth) and *Pistia stratiotes* (locally known as water cabbage) and commonly grown plant i.e. *Phaseolus vulgaris* (Local red kidney beans) were used.

To the best of our knowledge this study reports for the first time selective combination of raw substrates for production of pullulanase. The present study was designed to evaluate the effect of raw carbon source on production of debranching enzyme pullulanase from indigenously isolated *B. licheniformis* strain. The enzyme was purified and biochemically characterized with unique calcium independent nature compared to previously reported pullulanase and finally, it was utilized for synthesis of resistant starch.

MATERIALS AND METHODS

***Isolation of B. licheniformis Strain*:** *B.licheniformis* the producer of pullulanase was isolated from hot spring water (choothurn: Skardu, Pakistan). The strain was preserved from the seed culture when the growth was in log phase (0.06-0.08 OD) and was maintained in 15% glycerol broth at −20°C.

***Qualitative Assays for Pullulanase Activity*:** *B. licheniformis* was sub-cultured on LB agar plates and for qualitative tests it was point inoculated on modified pullulan agar medium for pullulanase activity. The pullulan agar medium composition used for plate assay was in (g/l) as; $MgSO_4.7H_2O$ (0.01), soluble pullulan (10), K_2HPO_4 (0.17), NaCl (2), $KH_2PO_4.7H_2O$ (0.12) and agar (15) and made the final volume to 1000 ml with distilled water. These plates were incubated at 50°C for 24 hr to check the hydrolysis zones. For confirmation of pullulan hydrolyzing ability plates were flooded with iodine solution after incubation.

***Quantitative Assay for Pullulanase Activity*:** For quantitative determination of pullulanase activity, release of reducing sugar was measured from pullulan used as a substrate. Enzyme assay was performed by incubating pullulanase (100µl/sample) and pullulan (900µl, 0.5% w/v) for 30 min at 60°C. After that reaction was stopped by addition of 1.5 ml of DNS reagent and heated at 99.9°C for 10 min. Then cooled at room temperature and optical density was measured by taking the absorbance at 540 nm as compare to control using UV-visible spectrophotometer.

The enzyme activity was assayed as the rate of release of reducing sugar (1mg/ml) in 30 min at 50°C and total protein concentration was calculated by Lowery method. Specific activity (U/mg) was determined by the following formula

Specific activity(U/mg) = Enzyme activity (U/ml)/ protein concentration (mg/ml)

***Biomass/ Cell mass*:** Growth O.D was taken using spectrophotometer at wavelength 600 nm was used to observe the biomass of the bacterial culture.

***Inoculum Preparation*:**

Inoculum was prepared by inoculating 2-3 full loops of *B. licheniformis* in 100 ml nutrient broth and incubated at 50°C for 16 hr at 150 rpm.

***Optimization of Media Components and Culture Conditions for Pullulanase Production*:** Indigenous carbon sources including wheat bran, corn bran, rice bran, water hyacinth, water cabbage and local red kidney beans were screened for production of pullulanase enzyme. *P. stratiotes* (Water cabbage) and *E. crassipes* (Water hyacinth) were chopped and dried in oven at 40°C overnight and utilized as substrates for pullulanase production. Local red kidney beans *(P. vulgaris)* were crushed and finally used as an organic substrate for pullulanase production. Corn bran, wheat bran and rice bran were taken from local market and were used as substrates. The raw substrates prepared were stored in air tight bottles separately for further use.

Various media components and culture parameters were optimized, for enhance production of pullulanase. Different raw carbon sources 5 g/l (wheat bran, wheat husk, rice husk,

corn bran, grounded red kidney beans, pest plants like water hyacinth and water cabbage) were exploited as nutritive substrates for submerged fermentation.

Effect of different temperature ranges (40°C, 45°C, 50°C, 55°C, 60°C) on pullulanase production were checked. The shaker incubator was set at 150 rpm and incubated the culture broth for 96 hr. After every 24 hr sample was taken for measuring pullulanase activity and total protein quantity.

For pullulanase production the effect of varying pH (5, 6, 7, 8, 9 and 10) was also studied. The fermentation medium with different pH was incubated at optimum temperature 50°C for 5 days. After every 24 hr enzyme activity and protein estimation were determined.

The effect of varying concentrations of starch (1 g/l, 2.5 g/l, and 5 g/l) were considered for pullulanase production and different concentrations of starch in media were used with optimum pH 6 and temperature 50°C for 5 days. After every 24 hr enzyme activity and protein estimation were carried out.

The effect of different organic nitrogen sources (peptone and yeast extract) were determined for pullulanase production. That were carried out by adding 5g/l of nitrogen sources in media under optimized conditions. After every 24 hr enzyme activity and protein estimation were analysed.

To estimate the time required for maximum pullulanase production bacterial culture was grown and analysed at 50°C for 24-96 hr. After every 24 hr enzyme activity and protein estimation were measured.

***Experimental Design using Statistical Tool Preliminary Screening by Plackett-Burman Design*:** To determine which factors significantly affect pullulanase production by *B. licheniformis*Plackett-Burman design of 15 runs were established with various concentrations of eleven factors including carbon sources (Corn Bran, Orange Peel, Wheat Bran, Starch) nitrogen sources (Beef Extract, Soya Bean, potassium nitrate), and different salts (K_2HPO_4, KH_2PO_4, NaCl and $MgSO_4.7H_2O$).

The design expert 9 Stat-Ease software, USA was used to generate design with 15 experimental runs for 11 factors with three central points. All the factors were kept either at low or high level.

***Purification of Pullulanase*:** The crude enzyme was partially purified by acetone precipitation. Cell free supernatant from fermentation medium after centrifugation was mixed dropwise with chilled acetone (kept at -20°C for 24 hr) by constant stirring. The supernatant was kept on ice while adding acetone until precipitate formation. The solution was kept for 30 min and centrifuge at 12,000 rpm for 20 min at 4°C. The formula used for calculating the amount of organic solvent to be added as;

$$\text{Volume to be added to 1 litter to make \% from x to y} = \frac{1000\ (y - x)\ \text{mL}}{100 - y}$$

where X= initial % of acetone in supernatant; Y= required % of acetone in supernatant

The pellet was then air dried to remove any trace amount of acetone and dissolved in 2 ml of 0.01 M sodium phosphate buffer and stored at -4°C.

Different types of protein present in the sample were separated based on their molecular weight on the gel permeation chromatography using sephadex G-100. After calibration of the column with buffer, 2 ml of filtered sample (precipitate dissolved in 0.01 M sodium phosphate buffer) was applied to the glass column containing Sephadex G-100. The sample was then allowed to pass through the gel and fractions (3 ml/15 min) were collected separately. For every fraction enzyme assay and protein estimation (lowery method at 650 nm) were carried out. Those fractions having high activity were pooled together, lyophilized and processed for further analysis.

***Biochemical and Biophysical Characterization of Pullulanase*:** Purified pullulanase was characterized at different pH (3, 4, 5, 6, 7, 8, 9), and temperature (40, 45, 50, 55, 60°C). Molecular weight of the purified enzyme was estimated from SDS-PAGE. The effect of various substances such as enzyme inhibitors, metal ions, detergent, and modifying agents were investigated on pullulanase activity.

***Substrate Specificity and Hydrolytic Property of Pullulanase*:** The substrate specificity of pullulanase was determined by measuring its activity using different substrates such as pullulan, soluble starch, glycogen, and raw maize. All the substrates were dissolved in 0.1 M citrate buffer (pH 5) and the stock solution 10 mg/ml was prepared. Finally, activity of the enzyme was determined at 50°C for 30 min. The amount of released reducing sugars was determined by the DNS method. One unit (U) of pullulanase activity was defined as the amount of the enzyme that required releasing 1 mg of glucose equivalent reducing sugar under the assay conditions. The hydrolysis product of pullulanase was analyzed using Thin Layer Chromatography. Pullulanase reaction was conducted using standard pullulan (1%) under standard conditions and reaction was stopped at boiling temperature for 5 min. Three microliters of pullulanase product and maltose standard (5mg/ml) were spotted on TLC plate. The plates were developed with solvent system 1-butanol, acetic acid, and water (3:1:1). The plate was then sprayed with sulfuric acid: water (95:5) and visualized by heating in oven for 5 min at 120°C.

***Synthesis of Resistant Starch by Pullulanase*:** The synthesis of resistant starch from maize starch was carried out using 5 g starch dispersed in 0.5 M citrate buffer (pH 5), and the mixture was then pregelatinized by autoclaving at 121°C for 20 min. The gelatinized slurry of starch was cooled to 60°C and purified pullulanase was added. After incubation, the starch paste was heated at 100°C in order to inactivate the enzyme. Then second cycle of autoclave (121°C, 15 psi) for 1 hr was carried out and samples were retrograded at -4°C for 24 hr. Finally, the treated starch samples were grinded and

stored for further use. All the experiments were carried out in triplicate.

The resistant content of samples, (Native maize flour (A), enzymatically/pullulanase treated starch (B), and heat-treated starch was further determined by Megazyme Resistant Starch Assay Kit.

***Light Microscopy*:** Light microscopy was performed for the investigation of morphological and structural characteristics of the untreated (A) and enzymatically treated starch (B). Samples were prepared for microscopic study by suspending the starch samples (A and B) in a drop of 50% glycerol (1:1 glycerol/water (v/v) over on a microscopic slide properly and covered the sample with a glass coverslip. The samples were observed within 30 min after the slide preparation. Microscopic examination was carried out on 40 X resolution and images were captured.

RESULT AND DISCUSSION

***Isolation and Characterization of Bacterial Strain*:** *B. licheniformis* has been previously isolated from hot spring located at choothurn Skardu (Pakistan). Isolated strain was further sub-culture on nutrient agar plate and purified. Strain identification was carried out morphologically, biochemically, and molecularly. Through 16S rRNA phylogenetic analysis it proved that strain belongs to genus *Bacillus*. The GenBank accession number for the *B. licheniformis* is (KP342533). *B. licheniformis* was streak on pullulan agar medium. The colony which showed clear hollow zone (3 cm) around it resulted in the hydrolysis of pullulan by pullulanase (Fig.1).

Figure 1. Zone of hydrolysis of pullulan as a substrate by *B. licheniformis* (3 cm) around the colony by using potassium iodide solution.

***Optimization of Culture Conditions for Pullulanase Production*:** Selection of appropriate organic substrate was an important factor to produce pullulanase. So, screening of suitable organic substrate was carried out for maximum pullulanase production. Carbon containing components such as oligosaccharides starch and sugars are major and most important sources of cellular carbon and a good source of energy (Wang *et al.*, 2019). In current study six organic substrates were used for pullulanase production in submerged fermentation. The selection of organic substrates depends on their availability, starch content and their previous use for pullulanase production.

It was determined that the bacterium grew well in the presence of all selected organic substrates except for rice husk-containing medium where the biomass was considerably lower than all other selected organic substrates used in fermentation medium. The growth trend was in the following sequence CB> WC>WH>RB>WB>RH Fig. 2A. Initially for corn bran, red beans and wheat bran growth rate and pullulanase production was increased at initial stages of fermentation (24 hr of cultivation) and reached a stationary phase after 48 hr of incubation while in case of water hyacinth, water cabbage and rice husk maximum growth were obtained after 72 hr of incubation. Therefore, it can be concluded that extended incubation time for pullulanase production may be due to substrate structure and its starch composition. These substrates were not easily hydrolyzed by the enzyme and thus required longer incubation time by the bacterium to build-up their biomass and release enzymes. The maximum extracellular pullulanase production was observed in the fermentation medium using corn bran as sole carbon source, with maximum pullulanase activity of approximately 2.37 U/mg Fig. 2B. The result obtained suggested that corn bran based fermentation medium could be selected as an appropriate fermentation medium for the growth as well as for enzyme production by *B. licheniformis*. This result also depicted the enzyme specificity towards corn starch and therefore acts as leading source for production of resistant starch type III (RSIII) from such substrate during the current study. From the analysis of the results it is concluded that, variation in pullulanase production occurred when different type of organic substrates was used in fermentation medium by *B. licheniformis*, it may be due to the different types of organic substrate.

Therefore, it was concluded that *B. licheniformis* was an appropriate microorganism to produce pullulanase by using corn bran as a carbon source. These findings are helpful in reducing the overall production cost for the pullulanase production because corn bran is regarded as cheap substrate as compared to pullulan. Furthermore, corn bran is an agricultural by-product abundantly produced in Pakistan during wet milling and can be used as an effective substrate for fermentation of pullulanase in starch industries.

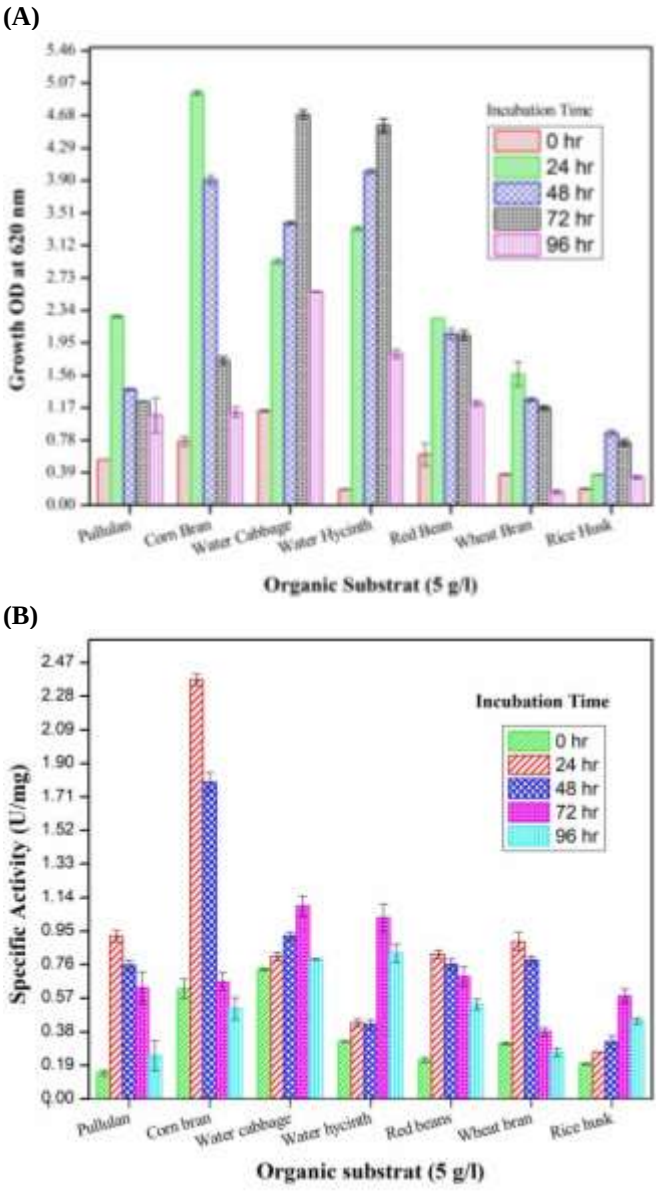


Figure 2. (A) Qualitative test for pullulanase production (U/mg) by *B. licheniformis* using different organic substrate (Pullulan, Corn Bran, Water Cabbage, Water Hyacinth, Red Beans, Wheat Bran and Rice Husk). (B) Growth of *B. licheniformis* using different organic substrates (Pullulan, Corn Bran, Water Cabbage, Water Hyacinth, Red Beans, Wheat Bran, Rice Husk).

The incubation time is another important factor for enzyme production. Maximum pullulanase production was observed at 24-48 hr of incubation when corn bran was used as a substrate Fig. 3. By further increasing the incubation time decline in growth as well as in enzyme production was observed. The possible reason for this decline is depletion of nutrients, production of some toxic metabolite or by-products formation like proteases in fermentation media or it may be due to denaturation/inhibition of pullulanase by production of other compounds in medium. In some studies, it was also reported that end product repression like glucose may also be the reason for decline in pullulanase production during batch culture (Gurung *et al.*, 2013).

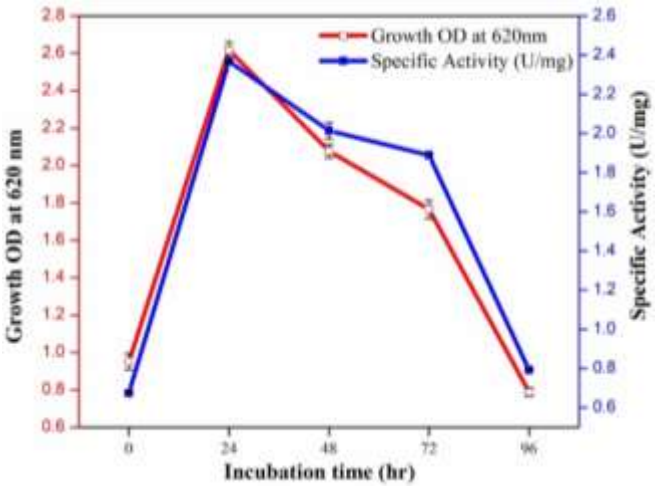


Figure 3. Qualitative test for pullulanase production by *B. licheniformis* using Corn bran as carbon source determining the incubation time for maximum growth and specific activity (U/mg) for 5 days (96 hr). Y-error bar determined the standard deviation.

Optimum temperature and pH were required for maximum production of enzymes. Raising temperature generally speeds up metabolic activities, and lowering temperature slows down. Similarly, each enzyme has an optimum pH range. Changing the pH outside of this range will slow enzyme activity (Robinson, 2015). However, at extreme temperature and pH conditions enzyme loses its shape (denature) and stop working. Therefore, pH and temperature of fermentation medium were optimized for maximum production of pullulanase. Generally, pH directly affects the production of enzyme. Variation in pH in fermentation medium not only affects the structure of enzyme but also cause variation in catalytic properties of enzymes, which ultimately resulted in reduction of enzyme activity. Every enzyme has optimum pH for maximum activity, while, in case of pullulanase enzyme production optimum pH ranges varied between pH 4.0-7.0 and maximum pullulanase activity of 2.36 U/mg was obtained using *B. licheniformis* at pH 6.0 Fig. 4A. Furthermore, higher or lower pH might have affected the pullulanase production by inducing the activation of certain enzymes (protease) that might have caused deformation of the molecular shape and distortion of the structure of pullulanase (Yue Wang *et al.*, 2019).

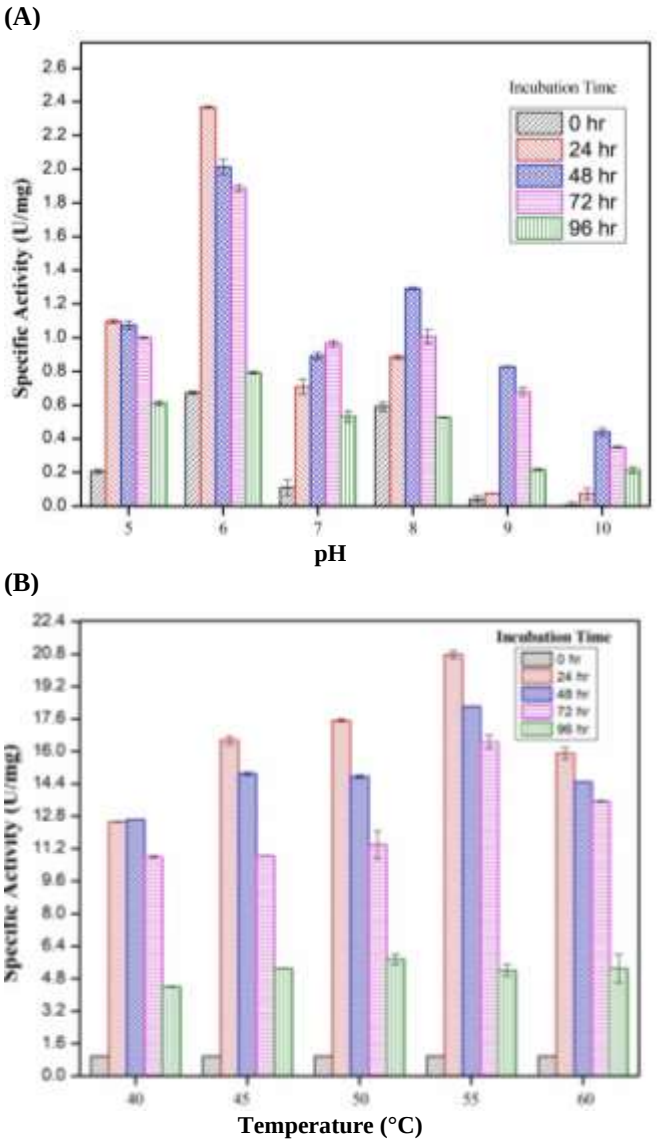


Figure 4. (A) Effect of different pH (pH 5.0-10.0) on the production of pullulanase from *B. licheniformis*-C1. (B) Effect of temperature (45°C to 60°C) on the production of pullulanase from *B. licheniformis*.

Change in temperature significantly affects growth and enzyme production. Generally, temperature has a direct effect on metabolism of microorganism required for the production of enzymes. During the study, *B. licheniformis* was incubated at 40°C to 60°C and maximum enzyme production was obtained at 55°C and further increase in temperature resulted in decrease of growth and production of enzyme Fig. 4B. The possible reasons for decrease in pullulanase activity at high temperatures could be reduction in transport of soluble compounds between interior and exterior of the cells, which eventually increased the accumulation of toxic metabolites inside the cells (Singh *et al*., 2010). The lower biomass at higher temperatures could arise from shortening of exponential growth phase at unfavorable temperatures (Kirchman *et al*., 1995). Enzyme stability at higher temperature is depending on their structural flexibility. A research conducted by Fitter described conformational entropy of enzyme from both mesophilic and thermophilic sources elucidate that in folded stage, thermophilic proteins have more flexibility as compared to mesophilic proteins (Fitter, 2003).

The effect of starch supplementation on enzyme production was also studied. According to the results, it was perceived that when production media was supplemented with starch the production and growth of enzyme increased. The maximum specific activity of 8.99 U/mg was obtained when production medium was supplemented with 5 g/l of starch after 48 hr of incubation (Table 1) and by decreasing concentration of starch specific activity gradually decreased. Starch is an important biopolymer and consists of amylopectin and amylose. Amylopectin is a large macromolecule and have highly branched ends having both α-1, 6 linked branches and -α-1, 4 linked glucose while in case of amylose linear α-1, 4 linked glucan. Therefore, high content of amylopectin in starch is responsible for the induction of debranching enzymes which results in the hydrolysis of starch. Antranikian *et al.* (1987) has also found increased production of pullulanase by starch induction. It was observed that starch had the maximum capabilities for inducing the production of pullulanase by *Clostridium sp*. strain EM1. Similarly, Reddy *et al*. (2000) also reported that starch has inducing effect on the production of pullulanase from *Clostridium thermosulfurogenes* SV2.

Nitrogen sources like peptone and yeast extract effects both growth and production of pullulanase. As different nitrogen sources have different types and amount of amino acid present which might be one of the reasons for the increased or decreased production of some enzymes by different microorganisms. As reported by Antranikian *et al*. (1987) that *Clostridium thermohydrosulfuricum* DSM 567 released 90% of enzymes into the cultivation media when it contains organic nitrogen source.

Table 1. Effect of starch supplementation on pullulanase activity

	Specific activity (U/mg)			
	24 hr	**48 hr**	**72 hr**	**96 hr**
Control	0.40±0.012	0.96±0.040	0.86±0.03	0.79±0.020
Starch (1.0g/l)	2.62±1.6E-4	2.81±0.001	2.72±0.06	2.48±0.003
Starch (2.5g/l)	2.90±0.02	7.19±0.040	6.68±0.01	3.42±0.007
Starch (5.0g/l)	7.70±1.2E-4	8.90±0.040	4.74±0.04	4.11±0.001

During current studies among different nitrogen sources tested, peptone was more preferred for extracellular pullulanase production. The peptone containing production

medium results in production of enzyme with maximum sp. activity of 4.40 U/mg after incubation period of 48 hr as compared with yeast extract which have sp. activity of 2.4 U/mg after incubation of 48 hr (Table 2). The reason for the utilization of the peptone is its molecular weight, which can be easily degraded by the enzyme produced by microorganisms and therefore can easily be absorbed and utilized by microorganisms. Yeast extract is known as a complex medium, this not only comprises of nitrogen source, but also comprises of salts and other important nutrients.

Table 2. Effect of nitrogen source on pullulanase activity

	Specific activity (U/mg)			
	24 hr	**48 hr**	**72 hr**	**96 hr**
CB*	0.24±0.012	2.28±0.040	1.57±0.03	1.20±0.020
P+CB *	0.07 ±1.6E-4	0.08±0.001	0.31±0.06	0.15±0.003
S+P+CB*	2.01 ±0.02	4.40±0.040	3.87±0.01	2.48±0.007
YE+CB*	0.52 ±1.2E-4	0.55±0.040	0.68±0.04	0.63±0.001
S+YE+CB*	2.31 ±4.3E-4	2.40±0.020	1.75±0.02	1.27±0.004

CB*=Corn bran, P*=Peptone, S*=Starch, YE*=Yeast extract

Therefore, during current study, it was observed that when both nitrogen source and starch were added to fermentation medium it results in significant increase in growth and enzyme production. The same combined effect of nitrogen source and starch in the fermentation medium were observed by Hii *et al*. (2009). The possible reason for this effect is that during cell growth and pullulanase production processed organic nitrogen source in culture medium influenced the hydrolysis rate of starch.

Statistical analysis is important in analyzing interaction between different components of production media for optimum production of pullulanase. Eleven factors were tested in Plackett-Burman design. These factors were carbon sources (Corn Bran, Orange Peel, Wheat Bran, Starch) nitrogen sources (Beef Extract, Soya Bean, potassium nitrate), and different salts (K_2HPO_4, KH_2PO_4, NaCl and $MgSO_4.7H_2O$) which are pre-screened through one factor at a time analysis. Analysis of responses of design showed that three factors were the most significant for production of pullulanase and included orange peel, wheat bran and corn bran (Carbon source). Significance level of this model is concluded from its p-value that was 0.0003. The Model F-value of 6.55 implies the model is significant. The "Lack of Fit F-value" of 0.55 implies that it is not significant relative to the pure error. After statistical model optimization of fermentation media, specific activity of pullulanase was increased from 16 U/mg to 25 U/mg. Significant factors were analyzed form ANOVA results and were found to be orange peel, calcium chloride and potassium nitrate. Therefore, it was concluded that pullulanase yields was highly dependent on medium composition (Izmirlioglu and Demirci, 2016).

***Purification of Pullulanase from Thermophilic B. licheniformis*:** Saturation of culture filtrate of pullulanase was done by using solvent precipitation and then subjected to size-exclusion chromatography on Sephadex G-100. The pullulanase from *B. licheniformis* from culture filtrate was concentrated using 80% of chilled acetone precipitation. The finding showed similarity with the results obtained by Odibo and Obi (1988) which concentrated pullulanase from *Thermoactinomycesthalpophilus* No.15 culture filtrate by 80% acetone. Precipitated enzyme (80% precipitated with acetone) was then applied to size-exclusion chromatography using Sephadex G-100 and as a result single peak obtained demonstrating pullulytic activity with an overall increase in specific activity of about 80 folds. In the present study pullulanase was purified with specific activity of 190.9 U/mg and 9.42% recovery yield with 6.35 purification fold (Table 3). The specific activity and purification fold of enzyme were increased with each purification step verifying that acetone precipitation and gel filtration chromatography were suitable methods for pullulanase purification.

***Biophysical and Biochemical Characterization of Pullulanase*:** Pullulanases from thermophiles were interesting both from an enzyme stability perspective and also these enzymes can potentially be applied in the industrial starch hydrolysis process. From the findings of the present study it was revealed that crude, partially purified and finally purified pullulanase from *B. licheniformis* showed activity and stability to varied temperature in the range of 30°C to 80°C and pH 3 to pH 10.

Generally, pH directly affects the activity of enzyme. Variation in pH affects the structure of enzymes which were responsible for their maximum activities. Optimum pH for purified pullulanase was 5 with maximum specific activity of 117.4 U/mg Fig. 5A. Therefore, it was concluded that for maximum enzyme activity optimum pH was most significant factor. The reason is that due to change in pH from optimum resulted in conformational changes in the protein which is

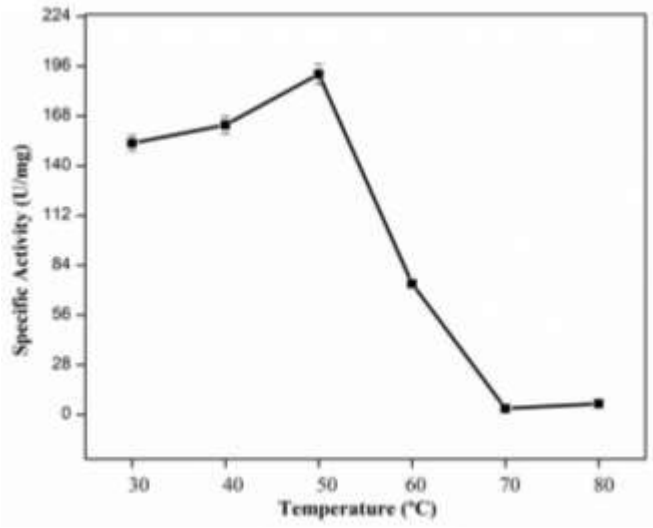
Table 3. Summary of purification step

Pullulanase obtained from *B. licheniformis* C1	**Total activity (AU)[a]**	**Total protein (mg)[b]**	**Specific activity (AU/mg)**	**Recovery (%)**	**Purification (fold)**
Cell free supernatant	427.20	26.70	16.0	100.00	1.00
Acetone precipitation	235.08	7.83	30.0	55.02	1.87
Purified by gel filtration chromatography (G-100)	22.15	0.12	190.9	9.42	6.33

a. Enzyme activity was measured in 0.1 M citrate buffer pH 5 at 50°C using 1% (w/v) pullulan as the substrate; b. Protein concentration was measured by the Lowry method, using BSA as the standard.

directly related to enzyme activity. By changing pH ionization state of amino acid side chain in enzyme structure is affected which are responsible for holding overall active protein confirmation (Mazzei *et al.*, 2019).

(A)



(B)

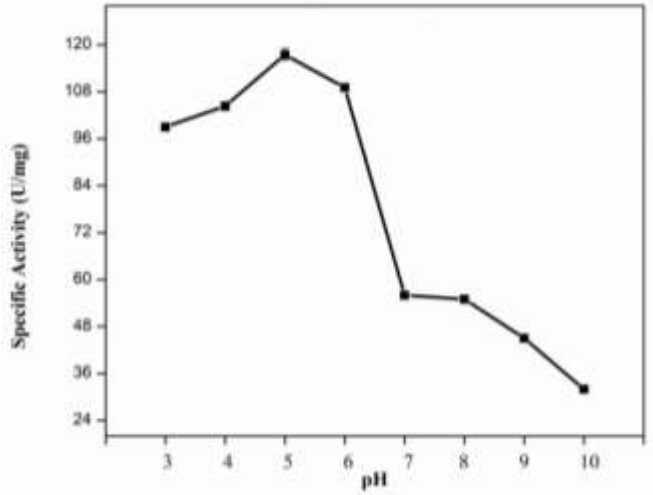


Figure 5. (A) Effect of different temperature on pullulanase activity.(B) Effect of different pH on pullulanase activity.

Temperature is another most significant factor for maximum enzyme activity. Optimum temperature signifies enzymatic thermodynamic properties which resulted in increase of reaction rate. In our findings the optimum temperature for purified pullulanase was 50°C with maximum specific activity of 191.4 U/mg Fig. 5B. Therefore, it can be concluded that thermophilic enzymes have the potential to be used in starch processing industries significantly in saccharification process as the scarification process of starch was carried out at around 60°C.

In present study, effect of various substances like inhibitors, metal ions, detergent and modifying agent were analyzed on the pullulanase activity. According to our findings Fe^{+2}, Hg^{+} and anionic detergent like SDS completely inhibit enzyme activity, while in case of Zn^{+2}, Mg^{+2} and NaI slight inhibition was observed on pullulanase activity. However, Hg^{+2} having ability to act on SH bonds, specifically act on thiol group. Therefore, Hg^{+2} affects activity of pullulanase which indicates that these thiol groups are responsible for maintaining structure of enzyme. The inhibitory or negative affect of anionic detergent like SDS suggested that this enzyme is not a good candidate to be used in detergent industries. In addition to this Ca^{+2} and EDTA has no noticeable effect on enzyme activity (Table 4). These results suggesting that Ca^{+2} is not required for activity of pullulanase and no effect of chelating agent suggested that it is not a mettalo enzyme. These results were in accordance with Saha *et al*. (1988) reported that, pullulanase activity was independent of Ca^{+} ions in the reaction mixture.

Table 4. Effect of various metal ions and other reagents on pullulanase activity

Metal Ions	Specific activity (U/mg) *	Relative activity (%)
Control	191.0±0.98	100.0
Mg^{+4}	67.60±0.03	35.4
Zn^{+4}	52.20±0.04	27.3
Fe^{+4}	0.50±0.05	0.5
Ca^{+2}	189.8±0.06	99.3
EDTA	183.2±0.07	95.9
NaI	64.10±0.04	33.6
SDS	0.80±0.03	0.8
Hg^{+}	16.50±0.03	8.6

*The effects of various metal ions and other reagents on PUL activity were determined by incubating the purified enzyme in 0.1 M Sodium citrate buffer (pH5) at 50°C for 30 min in the presence of 10 mM of different metal ions as well as other reagents.

Therefore, it was concluded that pullulanase enzyme from *B. licheniformis* is a best attractive enzyme to be used in starch industry. The most highlighted reason was the use of enzyme in starch industries because its Ca^{+2} independent nature. If Ca^{+2} ions were added in starch scarification process, it inhibits other enzymes used in process like glucose isomerase especially in case of high fructose syrup formation. Another noticeable disadvantage of using Ca^{+2} ions in starch processing industries is calcium oxalate production as a byproduct during reaction process and become deposited in pipes of fermenter and heat exchangers. Due to this deposition blocking of pipes occurs, which resulted in overall increase of production cost of the process. So, this enzyme with such novel attracted properties is the best alternative and most preferred candidate to overcome all these problems in starch industries. The purified pullulanase migrated as a single band

on SDS-PAGE with a molecular mass of approximately 75 kDa Fig. 6, suggesting that the enzyme is a monomer.

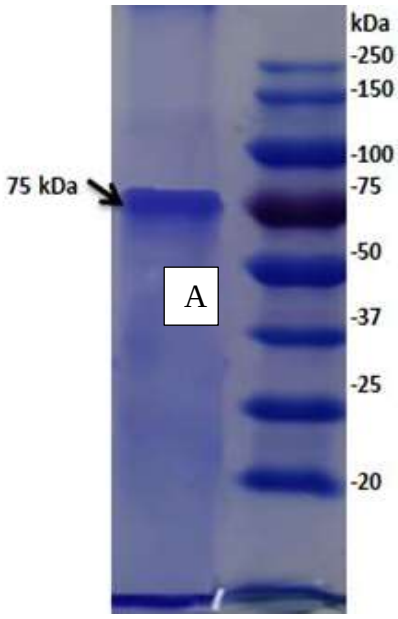


Figure 6. SDS-PAGE analysis of pullulanase from *B. licheniformis*. Lane M broad molecular weight protein standards (bio-rad # 61-03377); lane 1 purified pullulanase

***Substrate Specificity and Analysis of Hydrolysis Products by Thin Layer Chromatography*:** Generally, pullulanases showed broad substrate specificity towards polysaccharides such as pullulan (190 U/mg), glycogen (68 U/mg), starch (42 U/mg) and raw maize (23 U/mg) that have both α-1, 4-glycosidic bonds and α-1, 6-glycosidic bonds in their structures.

Because of broad substrate specificity, the enzyme pullulanase action tailored complete hydrolysis of polysaccharide into small molecular weight sugars without any aid of other starch hydrolyzing enzymes including α and β amylases. This type of pullulanase having such unique characteristics was designated as pullulanase type I (Liu *et al.*, 2017). The hydrolysis product of pullulanase utilizing pullulan as substrate was analyzed by TLC Fig. 7. The reaction product of pullulanase on pullulan resulted in the production of maltotriose as analyzed by TLC Fig. 7 and therefore, it was classified with pullulanase type I.

***Application of Pullulanase for Synthesis of Resistant Starch III*:** RSIII has attracted great interests amongst the nutritionists and food industry, due to its reduced levels of plasma glucose and insulin, increased faecal bulk, and short-chain fatty acid (SCFA) production through fermentation in the large intestine (Raigond *et al.*, 2015). Application of purified pullulanase on resistant starch type III synthesis from maize starch (Obtained from local market) was estimated and production condition was optimized Fig. 8. In optimized conditions RSIII yield increased significantly from 1.34% to 14.32% while heat treated starch have 4.25% RS. Resistant starch III can be synthesized from different starch sources by physical and enzymatic treatments, in which pullulanase has attracted considerable attention in increasing resistant starch III yield (Lovegrove *et al.*, 2017). Infact, pullulanase can hydrolyze alpha-1,6 glycosidic bonds present at branching points of amylopectin. An increase degree of debranching with proper amylose chain length enables to align and form double helix crystalline structures, therefore leading to increase yield of resistant starch III.

Figure 7. Thin-layer chromatography (TLC) of hydrolysis products of pullulanase from pullulan (lane 2) and standard maltotriose (lane 1)

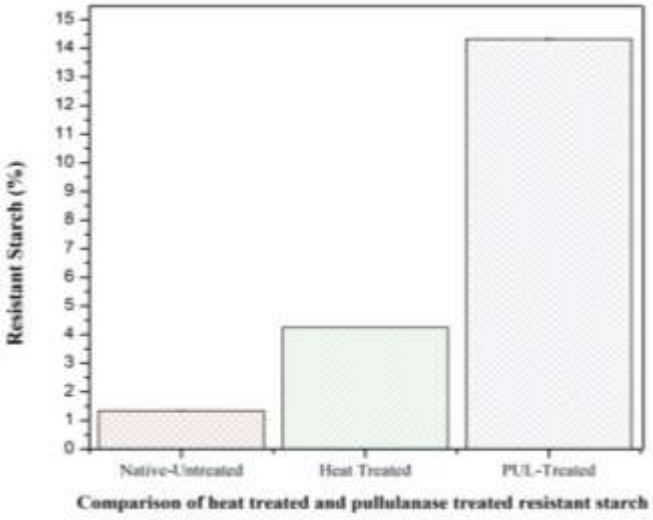


Figure 8. Comparison of resistant starch (RSIII) yield in native, heat treated and pullulanase treated maize starch

***Structural Characterization of Pullulanase treated Resistant Starch type III*:** The structural characteristics of native maize starch (A) and pullulanase treated starch (B) were analyzed by light microscope Fig. 9. The results revealed that native maize starch granules have smooth surface with clear polygonal shape and have no sign of surface destruction (Román *et al.*, 2017). However, pullulanase treated starch granules have clear changes in morphology with damaged irregular surface with dense matrix as compared to native maize starch. The morphological differences between treated and untreated starch was possibly due to reassociation and double helical formation of amylose chain during pullulanase treatment and retrogradation. These characteristics depicted increase resistivity of pullulanase treated maize starch from gastric conditions and were also responsible for dense crystalline structure.

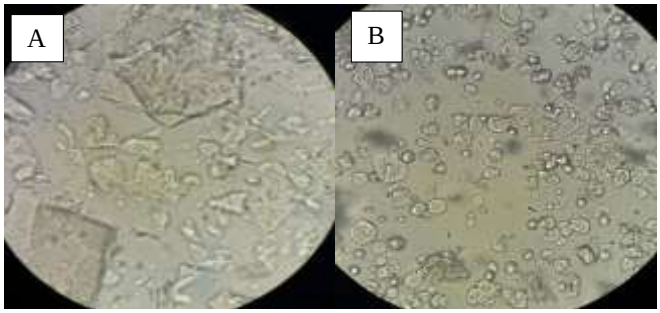


Figure 9. Light microscopy of (A) Native maize Starch (B) Pullulanase treated maize Starch

From the current study it was concluded that *B. licheniformis* is an efficient producer of pullulanase. Among all selected organic substrate corn bran was explored to be an efficient and inexpensive source to produce pullulanase. Pullulanase from *B.licheniformis* was considered as thermophilic and acidophilic enzyme. Pullulanase was growth associated enzyme. Pullulanase enzyme was partially purified by 80% acetone and further purified by gel filtration chromatography. Biochemical characterization of purified enzyme was optimized at 50°C and pH 5.0. Pullulanase belongs to amylopullulanase is an acidic enzyme and a best candidate to be used considerably for the synthesis of resistant starch type III. The unique properties of the pullulanase make it attractive and preferred candidate in food and starch industries.

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