



## Purification and Properties of Pullulanase from *Bacillus halodurans*

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### Abstract

A pullulanase-producing bacterium was isolated from soil and identified as *Bacillus halodurans* based on the microscopic examination and biochemical tests. Maximum pullulanase production occurred in the presence of soluble pullulan at 1.5% concentration and in the presence of 0.5% peptone at pH 10.0 and 37°C. The purified alkaline pullulanase had a molecular mass of  $37 \pm 1$  kDa, an optimum pH in the alkaline region (10.0) and optimum temperature of 50°C. The pullulanase activity was inhibited by  $Zn^{2+}$  and  $Cu^{2+}$  ions.  $Mg^{2+}$ ,  $Mn^{2+}$  and  $Fe^{2+}$  slightly inhibited the enzyme whereas  $Ca^{2+}$  had a stimulating effect on the enzyme activity. Ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMSF), tosylchloride methylketone (TLCK) and sodium azide (NaN<sub>3</sub>) did not obviously inhibit the enzyme whereas N-ethylmaleimide (NEM) and iodoacetic acid (IAA) had moderately inhibited the enzyme. N-bromosuccinimide (NBS) inhibited completely the enzyme activity suggesting that tryptophan is important for enzyme activity. The isolated enzyme was thermotolerant and alkalophilic which can thus be used in starch processing, detergent industry and other biotechnological applications.

**Keywords:** Pullulan, alkalophilic, thermotolerant, endodebranching enzyme, *Bacillus halodurans*

### Introduction

Pullulanases (E.C. 3.2.1.41,  $\alpha$ -dextrin 6-glucano-hydrolase) are endo-acting debranching enzyme capable of hydrolysing  $\alpha$ -(1,6)-D-glycosidic bonds in pullulan,  $\beta$ -limit dextran and amylopectin, forming maltotriose<sup>1</sup>. They are widely distributed in plants and bacteria<sup>2</sup>. Microbial pullulanase attracts more interest because of its specific action on  $\alpha$ -(1, 6) linkages in pullulan. Some of the *Bacillus* species that produce pullulanases are *Bacillus acidopullulyticus*<sup>3</sup>, *Bacillus deramificans*<sup>4</sup> and *Bacillus cereus* FDTA-13<sup>5</sup>.

Pullulanases are used in detergent industry as effective additives in dish washing and laundry detergents for the removal of starches under alkaline conditions<sup>6</sup>, in the starch processing industry for the production of maltose syrups and high purity glucose and fructose<sup>7</sup> and in the manufacturing of low caloric beer<sup>8</sup>. It is also possible to use pullulanase as a dental plaque control agent<sup>9</sup>. Because of the various important uses of pullulanase, the present study was conducted to purify and characterize a highly active alkaline pullulanase from *Bacillus halodurans*.

### Material and Methods

**Isolation, screening and identification of pullulanase-producing bacteria:** Soil samples were collected from various places of potato grown soil fields of Bangalore, mixed thoroughly in equal proportions and then screened for pullulanase-producing bacteria. 1 g of the soil sample was suspended in 10 ml of distilled water and serially diluted. After soil sedimentation, 0.1 ml of the supernatant was spread on the

surface of modified pullulan agar medium<sup>7</sup> containing (g/l) soluble pullulan (10), NaCl (2),  $MgSO_4 \cdot 7H_2O$  (0.1),  $K_2HPO_4$  (0.17),  $KH_2PO_4 \cdot 7H_2O$  (0.12) and agar (15), pH 10.0. The plates were then incubated at 37 °C till the colonies appeared. Colonies around which a clear hollow zone was observed were considered as pullulanase producers. The isolate was identified by microscopic examination and biochemical tests as described in the Bergey's manual of systematic bacteriology<sup>10</sup>.

**Crude pullulanase production and determination of crude pullulanase activity:** Submerged fermentation was performed by inoculating pure culture of the isolate into the fermentation medium which is same as the one used for screening except that it does not contain agar. The incubation was carried out at 37 °C for 3 days. The broth was centrifuged (REMI C-30 BL centrifuge, India) at 10,000 rpm for 10 min and the supernatant was used as crude enzyme. Pullulanase activity was assayed by measuring the reduced sugar released from pullulan<sup>11</sup>. The reaction mixture (1 ml), containing soluble pullulan (1%, w/v), 50 mM Tris-HCl (pH 7.0) and enzyme, was incubated at 50 °C for 20 min. The reducing sugar was measured by the dinitrosalicylic acid method<sup>12</sup>. One unit of pullulanase was defined as the amount of enzyme required to release reducing sugar equivalent to 1  $\mu$ mol of glucose per minute under standard assay conditions.

**Optimization of culture conditions for pullulanase production:** The effect of carbon source on the enzyme production was tested by using different concentrations of soluble pullulan in the production medium. The effect of different nitrogen sources was tested by adding individual nitrogen source viz. beef extract, tryptone, peptone and yeast

extract. The effect of pH and temperature was individually tested by taking the production media at different pHs and temperatures. The fermentation media were tested every day for pullulanase production till a decline was observed in the enzyme activity. For time course of pullulanase production, the isolate was grown in the optimized culture conditions and the activity was measured every day using a UV-spectrophotometer (SL 159, ELICO). The amount of protein present was assayed<sup>13</sup> using bovine serum albumin (BSA) as standard.

**Purification of pullulanase:** The cell free extract from fermentation broth was partially purified by acetone precipitation method<sup>14</sup> with slight modifications. Four times the volume of chilled acetone was added to the extract and it was allowed to precipitate overnight at -20 °C. A pellet was obtained by centrifugation at 10,000 rpm for 10 min. The pellet was dissolved in a minimum quantity of 0.2 M in Tris-HCl buffer of pH 8.5. Acetone precipitated sample was purified by anion exchange chromatography. The sample was redissolved in 0.2 M Tris-HCl buffer of pH 8.5, subjected to column chromatography with DEAE cellulose. The active fractions were eluted from the column and checked for enzyme activity. They were further lyophilized and used for characterization purpose.

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE):** The purified enzyme and known molecular weight markers were subjected to electrophoresis. SDS-PAGE was performed with 12% polyacrylamide gels<sup>15</sup>. After gel electrophoresis, the gel was stained with coomassie brilliant blue solution and destained using acetic acid/methanol (10% : 40%) solution. The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the relative mobility value. The following proteins were used as standards: phosphorylase (97.3 kDa), bovine serum albumin (66.6 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.1 kDa), soyabean trypsin inhibitor (20.1 kDa), and lysozyme (14.6 kDa).

**Hydrolysis of different types of substrates and chromatography of enzyme hydrolysed products:** The hydrolysis of starch substrates by the enzyme was studied by incubating the enzyme with 1% corn, amylopectin, potato starch and pullulan. The activity was then determined. Chromatography of pullulanase hydrolysed products was studied by thin-layer chromatography (TLC)<sup>16</sup>. Enzyme (0.5 ml) was incubated overnight with 0.5 ml of 1% pullulan dissolved in 0.2 M phosphate buffer (pH 7.0). Sample was spotted on the chromatographic plate. 1% glucose, starch, and maltose standards were also applied to the chromatographic plate. Chromatography was carried out using the solvent system: butanol: acetic acid: water (40 : 10 : 50, v/v/v). Carbohydrates were detected by staining with aniline-diphenylamine phosphoric acid reagent. The enzymatic products were visualized as blue spots after incubating the plate at 70°C for 5 min.

**Biochemical characterization of extracellular purified pullulanase:** The effect of pH was studied over a range of pH 3 to 11, using buffers of 0.2 M strength. 0.5 ml of enzyme was incubated with 1 ml of buffer containing 1% pullulan. Enzyme stability at optimum pH was studied and residual enzyme activity was measured at different time intervals. The effect of temperature was determined by performing the standard assay procedure at optimum pH with a temperature range of 0 °C to 100°C. Thermostability was determined by incubating the enzyme at optimum temperature at different time intervals. The effect of metal ions on the activity of enzyme was determined by adding metal ions (10 mM) to the enzyme and preincubating for 10 min. The selected ions were Cu<sup>2+</sup>, Fe<sup>2+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup> and Mn<sup>2+</sup>. The influence of different group specific reagents on the activity of pullulanase was studied using PMSF, DTT, IAA, NBS, NEM, NaI, TLCK and EDTA. They were individually added to the enzyme and preincubated for 10 min and the reaction was started by addition of the substrate. The activity was then measured as described earlier.

**Kinetic determination:** Michaelis-Menten constant ( $K_m$ ) and maximum initial velocity ( $V_{max}$ ) of the enzyme were estimated using soluble pullulan as substrate<sup>17</sup>.

## Results and Discussion

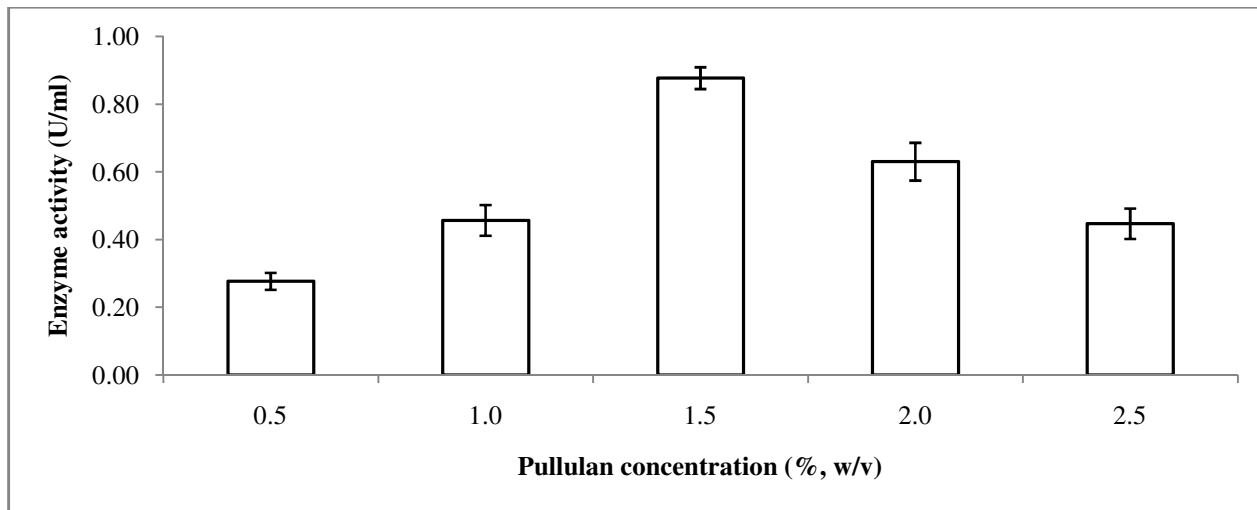
**Isolation, screening and identification of pullulanase-producing bacteria:** The bacteria were isolated from soil and the colony that showed a clear hollow zone around it after addition of KI solution on pullulan agar plate was regarded as pullulanase producer. Based on microscopic and biochemical tests, the isolate was identified as *Bacillus halodurans*. This finding is in agreement with the earlier reports that pullulanases are predominantly extracellular enzymes produced by a variety of bacteria, mainly by *Bacillus* sp.<sup>3,4,5</sup>

**Optimization of culture conditions for maximum pullulanase production:** Maximum pullulanase production occurred in the presence of soluble pullulan at 1.5% concentration (figure 1) and in the presence of 0.5% peptone (figure 2). An enhancement of pullulanase synthesis by pullulan in *B. cereus* FDTA-13 was also reported<sup>5</sup>. However, increasing pullulan concentration at higher level decreased enzyme production, suggesting substrate repression. Among the different nitrogen sources tested, peptone was more preferred for extracellular pullulanase production. Similar observation was noticed for *Clostridium thermosulfurogenes* SV9<sup>18</sup>. The pullulanase yields depend on strain, medium composition and culture conditions<sup>19</sup>.

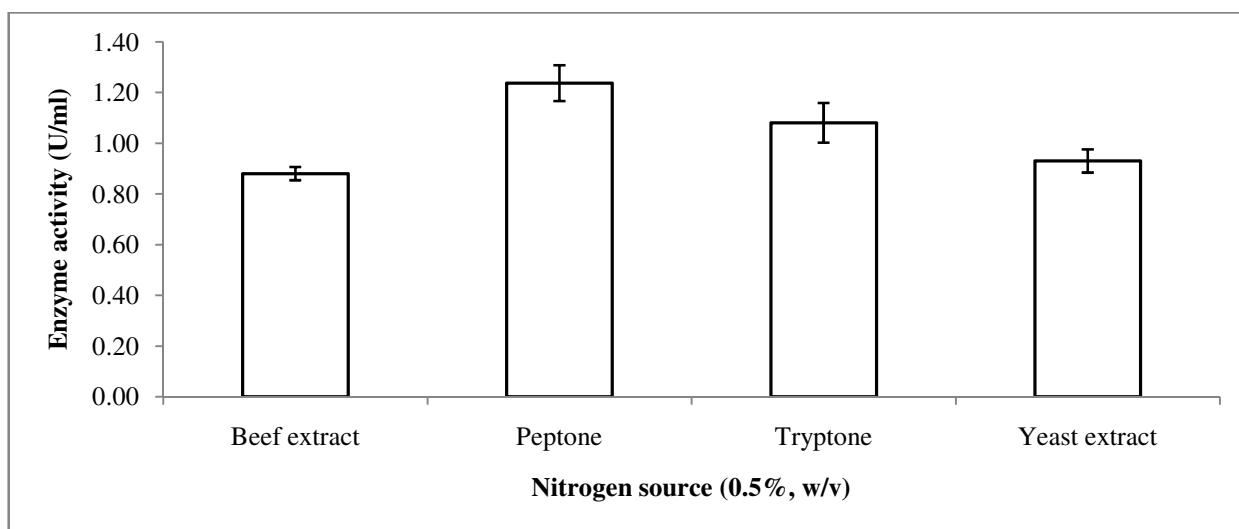
Maximum production was achieved at pH 10.0. The production of enzyme increased as pH of the medium increased but after pH 10.0 there was a decrease in enzyme production (figure 3). The isolate was capable of producing pullulanase in the range of 28 – 65°C with maximum production at 37°C (figure 4). However, increase in temperature beyond 37°C led to decline in

production of enzyme. The maximum production of pullulanase by *Bacillus amyloliquefaciens* was observed at 44 °C and pH 5.6<sup>20</sup>. The optimal range of initial pH and temperature for extracellular pullulanase production by *C. thermosulfurogenes* SV9 was between pH 7 to pH 9, and between 50 to 70 °C, respectively; at 55 °C, a good amount of enzyme (82%) was produced<sup>18</sup>. The isolate was studied for enzyme production under optimal conditions. Maximum production was observed on the 5<sup>th</sup> day (figure 5). This finding holds good with studies carried out on *Bacillus* sp. KSM-1876 wherein the pullulanase production increased sharply between 4 to 5 days<sup>21</sup>.

**Enzyme purification:** The specific activity and fold purification of the enzyme increased with each purification step (table 1) proving that acetone precipitation and anion exchange chromatography are appropriate methods of pullulanase purification. This finding holds well with results obtained for *Thermus caldophilus* GK-24 where thermostable pullulanase was purified to homogeneity with a specific activity of 86.2 U/mg protein and 13.2% recovery<sup>11</sup>. Similarly, a pullulanase from *Bacillus cereus* H1.5 with 8.987 U/mg specific activity, purification fold of 18.4 and yield of 10.9 % was reported<sup>22</sup>.



**Figure-1**  
Effect of different concentrations of soluble pullulan on pullulanase production. Y-error bar indicates the standard deviation from the mean of three independent replicates



**Figure-2**  
Effect of different nitrogen sources on pullulanase production. Y-error bar indicates the standard deviation from the mean of three independent replicates

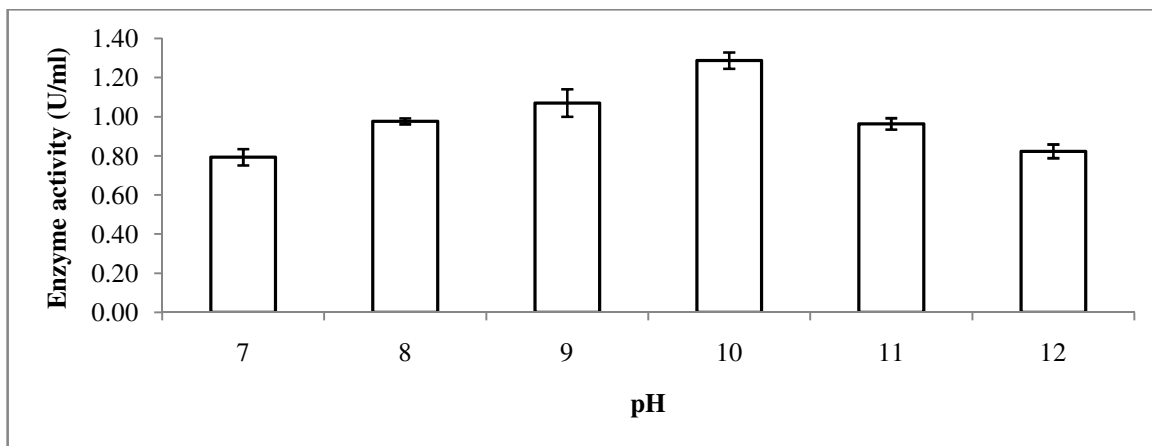


Figure-3

Effect of different initial pHs on pullulanase production by *Bacillus halodurans*. Y-error bar indicates the standard deviation from the mean of three independent replicates

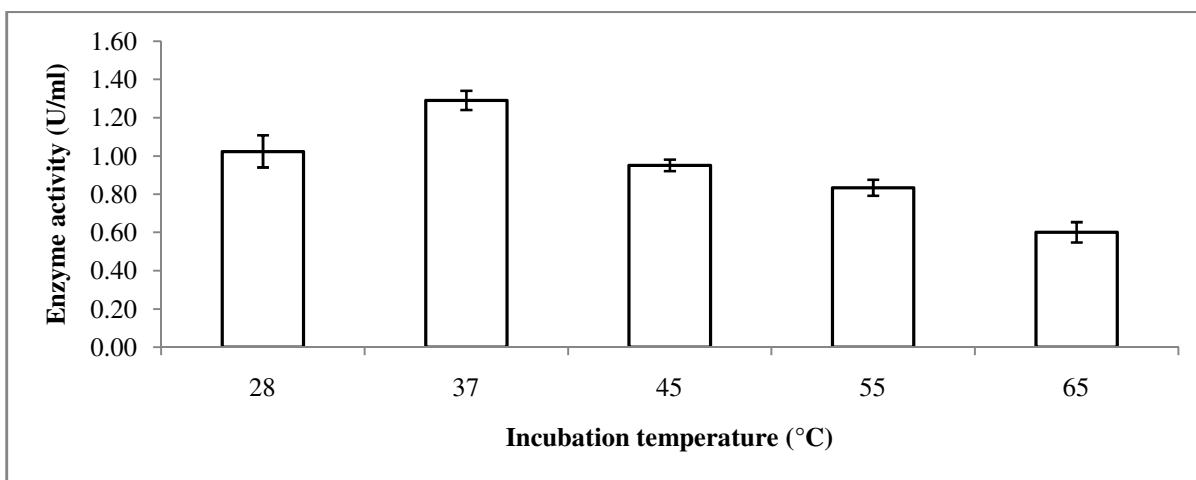


Figure-4

Effect of fermentation temperature on pullulanase activity.  $28 \pm 2$  °C is room temperature. Y-error bar indicates the standard deviation from the mean of three independent replicates

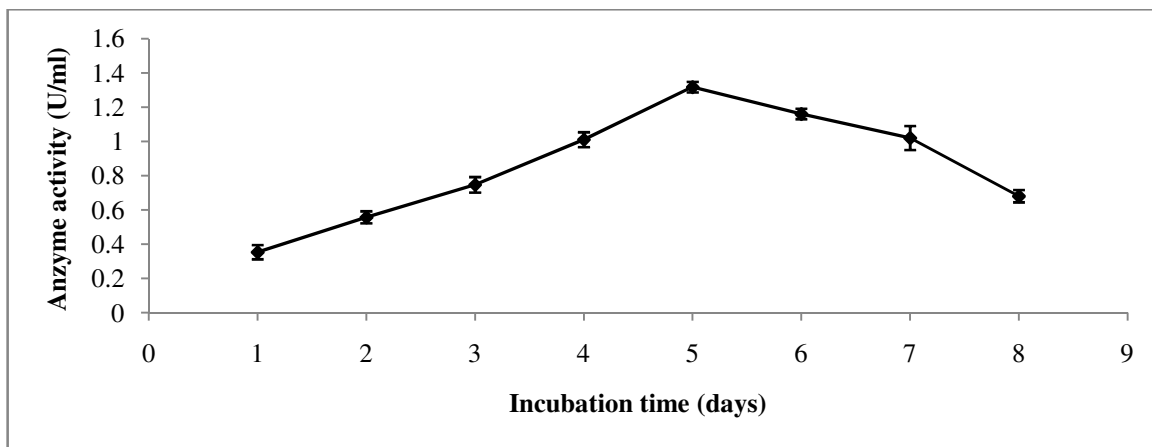


Figure-5

Time course of pullulanase production by *Bacillus halodurans* in submerged culture. Y-error bar indicates the standard deviation from the mean of three independent replicates

**Table-1**  
**Summary of purification of pullulanase from *Bacillus halodurans***

Fraction	Volume (ml)	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold	Recovery (%)
Cell free extract	250	509.3	32.46	15.69	1	100
Acetone precipitate	25	169.1	4.31	39.2	3.01	33.2
Purified by anion exchange chromatography	8	45.19	0.52	87.64	11.26	8.87

**SDS-PAGE:** The purified pullulanase showed a single band in between ovalbumin (44.3 kDa) and carbonic anhydrase (29.1 kDa) and had a relative molecular weight of  $37 \pm 1$  kDa (figure 6). A pullulanase from an alkalophilic *Bacillus* sp. S-1 showing a single band and a molecular mass of 140 kDa was purified<sup>23</sup>. Molecular mass of the purified thermostable pullulanase from *T. caldophilus* GK-24 was reported to be 65 kDa<sup>13</sup>. 210 kDa was recorded for pullulanase from *B. cereus* H1.5<sup>22</sup>. The majority of pullulanase enzymes so far studied were thus found to possess a high molecular mass. Very few reports suggest a low molecular mass of the alkaline pullulanase. The isolated pullulanase was a monomer which was further confirmed by activity determination of the band extracted from the gel (data not shown).

**Hydrolysis of different types of substrates and chromatography of enzyme hydrolysed products:** Among all the different starch substrates, pullulan gave high relative enzyme activity (90%) compared to others, but there is considerable good activity for corn (60%), amylopectin (75%) and potato starch (80%). This shows its broad substrate specificity. Similarly, corn, potato, sweet potato, topioca sago and wheat starches were saccharified to maltose using a mixture of  $\beta$ -amylase and pullulanase from *B. cereus* var mycoides<sup>24</sup>. The hydrolyzed products of enzyme substrate reaction were studied by performing TLC using standard sugar solutions. Hydrolysis products of soluble pullulan were maltose, unidentified oligosaccharides, and glucose. This showed that the purified enzyme is an endo-attacking enzyme. A purified amylopullulanase from *Geobacillus* sp. L14 was also reported as a specific debranching enzyme capable of producing glucose from pullulan<sup>25</sup>. Similarly, pullulanase was able to attack specifically the  $\alpha$ -1,6 glycosidic linkages in pullulan to generate maltotriose as the major end product<sup>3,26</sup>.

**Biochemical characterization of extracellular pullulanase:**  
**Effect of pH on the activity and stability of pullulanase:** The optimum pH of the purified pullulanase isolated from *Bacillus halodurans* was found to be 10 (figure 7). Similarly, studies on alkalophilic *Bacillus* sp. S-1<sup>23</sup> and also on *Bacillus* sp. KSM-1876<sup>26</sup> showed optimum pH 9.0-10.0. After incubation of the enzyme for 1 h at pH 10.0, 68% enzyme activity was recorded. After 2 h, nearly 50% of enzyme activity was lost; whereas 30% of activity was lost for overnight incubation (figure 8). Likewise, studies on *Bacillus* sp. KSM-1876 showed that after

incubation for 2 h at pH 10.0, enzyme activity was retained upto 65% and overnight incubation of enzyme lost 80% activity<sup>26</sup>. A stable pullulanase from pH 4.0 to pH 11.0 for 24 h was also reported<sup>23</sup>. Thus, the pullulanase of *Bacillus* sp. seems to be active at alkaline pH 10.0 for shorter period suggesting it to be a moderately alkalotolerant.

**Effect of temperature on the activity and stability of pullulanase:** The maximum activity was observed at 50 °C (figure 9). The isolated enzyme is therefore thermostable. Thermostability was studied at 50°C for an extended range of incubation period. Up to 45 min, incubation had less effect on enzyme activity. At 1<sup>st</sup> hour, 60% residual activity was observed, 2<sup>nd</sup> hour 46% residual activity and at overnight incubation, 17% residual activity was observed (figure 10). The data is in agreement with that of the pullulanase from *Aureobasidium pullulans* FB-1 where the optimum temperature was reported at ~ 50°C<sup>27</sup>. Similarly, a pullulanase from *Aerobacter aerogenes* that had an optimum temperature of 50 °C was purified and 90% of its activity was lost after 4 h of incubation at 50°C<sup>28</sup>. Likewise, a pullulanase was isolated from the supernatant of *Bacillus* species. After purification, the enzyme had optimum temperature and pH of 50 °C and 6.5, respectively<sup>29</sup>. An alkaline pullulanase from *Bacillus* species having excellent stability at 50 °C was also described<sup>26</sup>.

**Effect of divalent cations on the activity of pullulanase:** The pullulanase activity was inhibited by Zn<sup>2+</sup> and Cu<sup>2+</sup> ions. Mg<sup>2+</sup>, Mn<sup>2+</sup> and Fe<sup>2+</sup> slightly inhibited the enzyme whereas Ca<sup>2+</sup> had a stimulating effect on the enzyme activity (figure 11). Likewise, Zn<sup>2+</sup>, Cu<sup>2+</sup>, and Fe<sup>2+</sup> ions also inhibited the enzyme activity whereas Mn<sup>2+</sup> and Mg<sup>2+</sup> ions slightly inhibited the enzyme<sup>11</sup>. Similar results were observed wherein the pullulanase activity was inhibited strongly by Cu<sup>2+</sup>, and Zn<sup>2+</sup> ions, slightly inhibited by Mn<sup>2+</sup>, and Ca<sup>2+</sup> had a very strong stimulating effect on the enzyme<sup>22</sup>. A stimulation of pullulanase by Ca<sup>2+</sup> from thermophilic *Bacillus* strain 3183 was also noted<sup>30</sup>. Ca<sup>2+</sup> might thus be required for stabilization and maintenance of the enzyme conformation.

**Effect of group specific reagents on pullulanase activity:** Effect of group specific reagents on the enzyme was studied in the presence of soluble pullulan as substrate. EDTA, DTT, PMSF, TLCK and NaI did not obviously inhibit the enzyme; NBS inhibited completely the enzyme activity whereas NEM

and IAA had moderately inhibited the enzyme (figure 12). The inhibitory effect of some of the group specific reagents used in present work closely match with the studies<sup>21,22</sup> on *Bacillus* species wherein the NBS inhibited the activity of the alkaline pullulanase suggesting that tryptophan is important for enzyme activity. Studies on *Bacillus* strains<sup>11,23</sup> showed that pullulanase was not inhibited by EDTA. The lack of inhibition by EDTA which is strong metal chelating agent suggested that the isolated enzyme could not be a metalloenzyme. The enzyme was not inhibited by PMSF and TLCK suggesting that histidine and

serine residues were not present in the active site. The amylopullulanases are in general not serine enzymes<sup>22</sup>. As the enzyme had lost about 60% activity in presence of NEM and IAA, it can be concluded that a thiol group is involved in the catalytic action. This inference is based on the already available literature on the known group specific reagents and their target amino acid residues which get modified leading to enzyme inhibition.

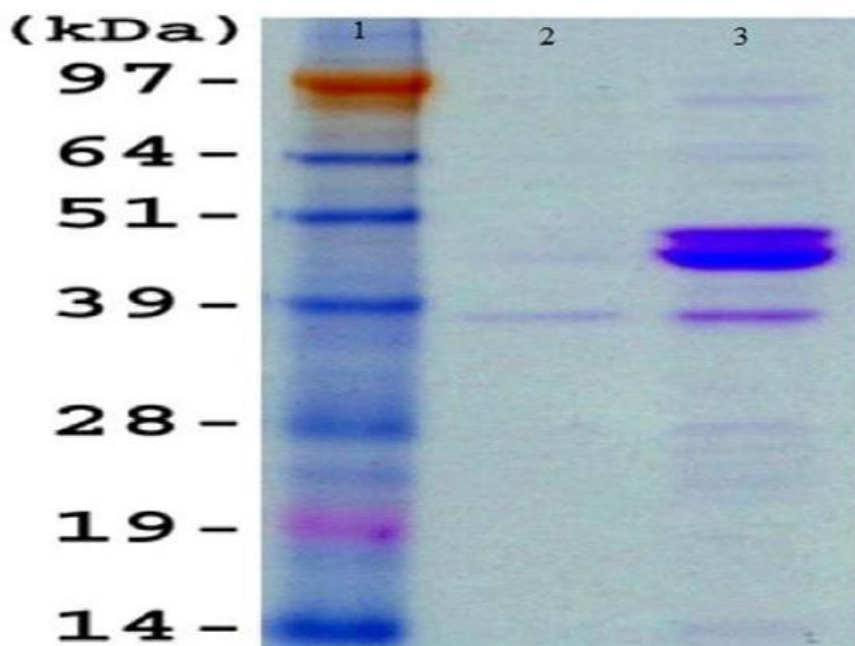


Figure-6

SDS-PAGE pattern of pullulanase isolated from *Bacillus halodurans*, Lane 1: molecular weight markers, Lane 2: purified pullulanase enzyme, Lane 3: partially purified enzyme

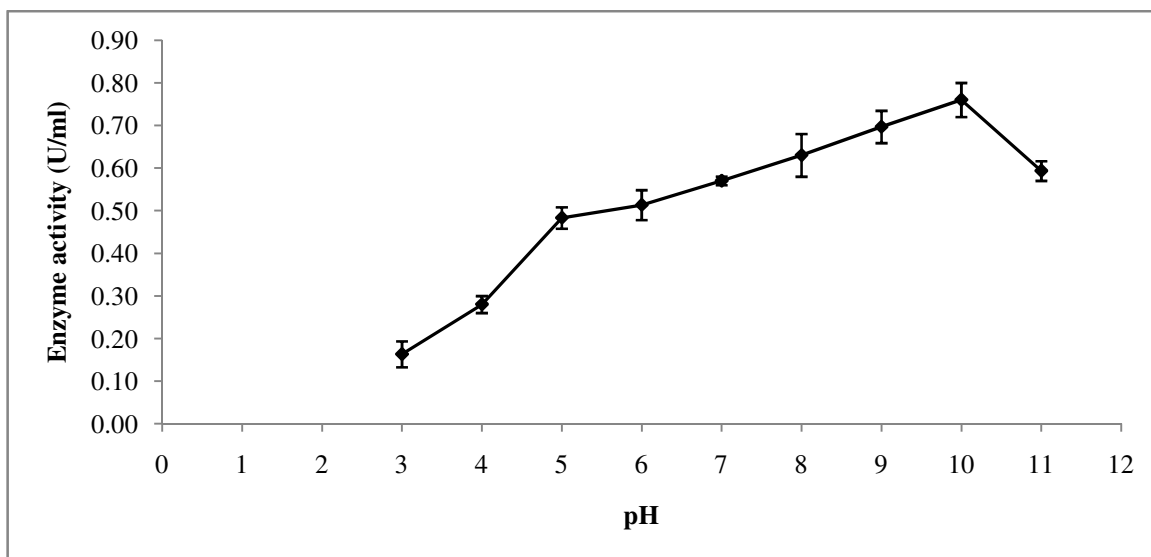


Figure-7

Effect of pH on the activity of alkaline pullulanase from *Bacillus halodurans*, Y-error bar indicates the standard deviation from the mean of three independent replicates

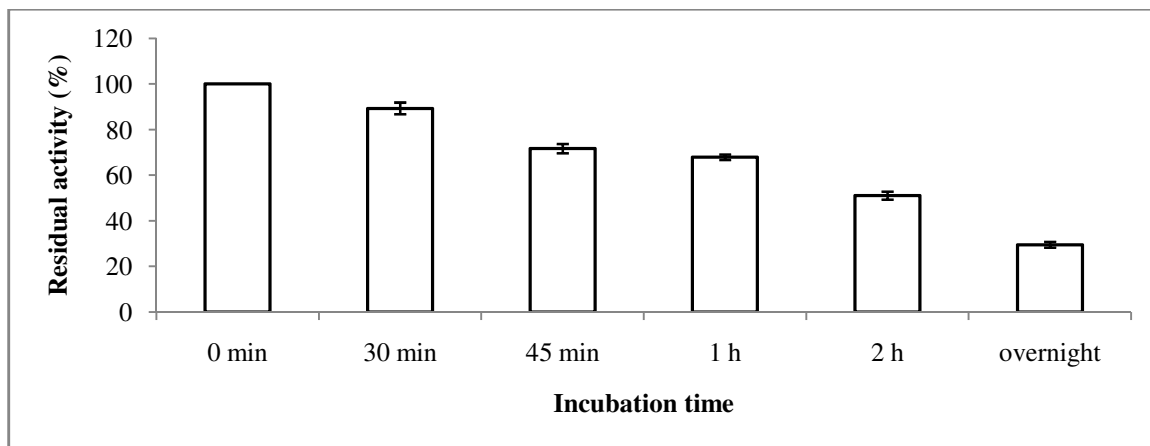


Figure-8

pH stability of pullulanase from *Bacillus halodurans* at pH 10.0. Y-error bar indicates the standard deviation from the mean of three independent replicates

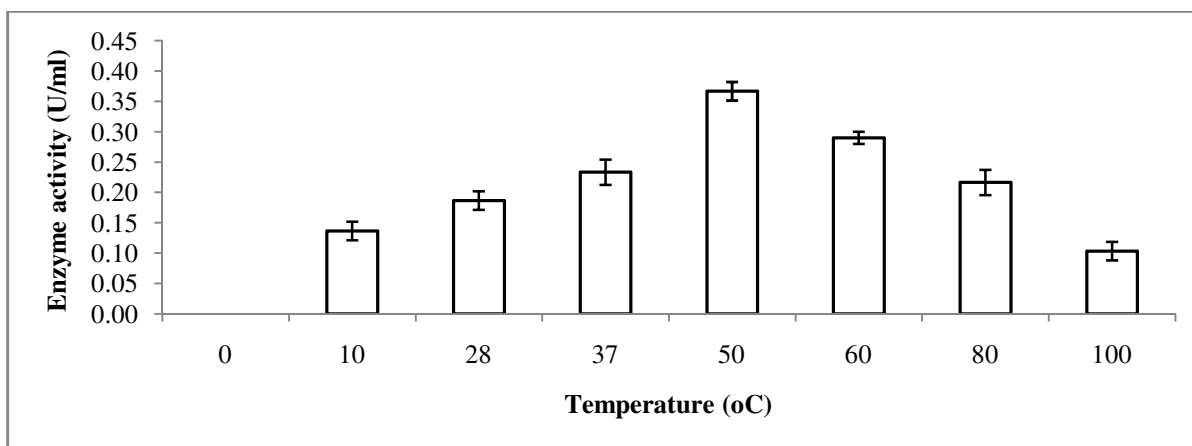


Figure-9

Effect of temperature on the activity of an alkaline pullulanase from *Bacillus halodurans*.  $28 \pm 2^\circ\text{C}$  is room temperature, Y-error bar indicates the standard deviation from the mean of three independent replicates

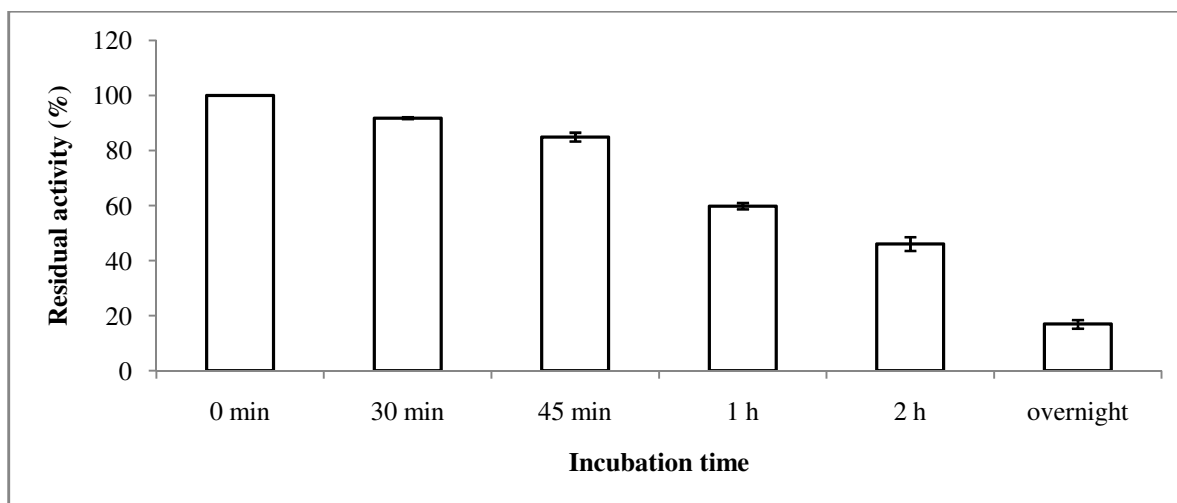
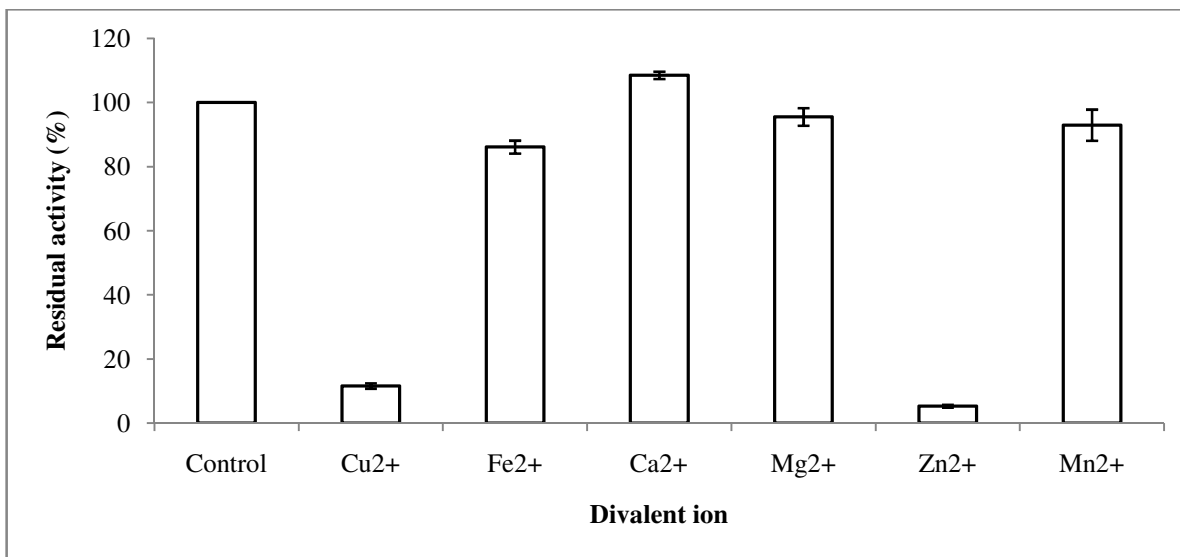
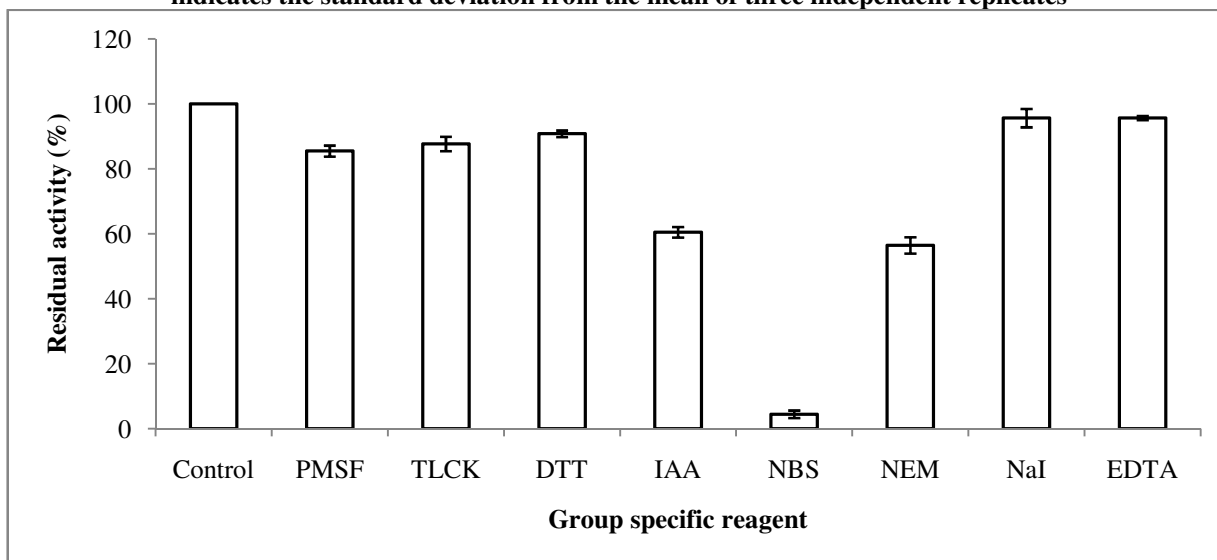


Figure-10

Temperature stability of pullulanase from *Bacillus halodurans* at  $50^\circ\text{C}$ . Y-error bar indicates the standard deviation from the mean of three independent replicates



**Figure-11**  
 Effect of various divalent cations on the activity of pullulanase from *Bacillus halodurans*, Y-error bar indicates the standard deviation from the mean of three independent replicates



**Figure-12**  
 The inhibition effect of various group specific reagents on the pullulanase activity from *Bacillus halodurans*, Y-error bar indicates the standard deviation from the mean of three independent replicates

**Kinetic Determination:** The  $K_m$  and  $V_{max}$  was calculated as 0.44 mg/ml and 36  $\mu\text{mol}/\text{min}$ . The lower  $K_m$  indicated that the isolated enzyme had a relatively high affinity for pullulan. This finding holds well with results obtained for an amylopullulanase where  $K_m$  and  $V_{max}$  values were 0.48 mg/ml and  $V_{max}$  44  $\mu\text{mol}/\text{min}$ , respectively<sup>25</sup>. Likewise, a  $K_m$  of 0.4 mg/ml was recorded with thermophilic *Bacillus* strain 3183<sup>30</sup>.

### Conclusion

A positive pullulanase-producing bacterium from soil was identified as *Bacillus halodurans* and was found to be a thermotolerant and alkalophilic which can therefore be utilized

in starch processing, detergent industry and other biotechnological applications. The alkaline pullulanase may therefore have wide scale application in pullulan processing industry on account of their thermostability and ability to degrade raw pullulan. Further isolating the gene and expressing it in some other organisms will be important for maximal production in order to get better yield of pullulanase for industrial applications.

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