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Original article

Isolation, characterization, and cloning of thermostable pullulanase from *Geobacillus stearothermophilus* ADM-11

Dilara Abbas Bukhari [a,*], Zuhra Bibi [a], Arif Ullah [a], Abdul Rehman [b]

[a] Department of Zoology, Government College University, Lahore, Pakistan
[b] Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan

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ABSTRACT

This study aimed to identify thermo-stable pullulanase-producing bacteria in soil samples of potato fields and food-producing companies. Pullulan agar medium was used to screen 17 bacterial strains, which were incubated at 65 °C. The isolate with the maximum activity (375U/ml) was selected and recognized as *Geobacillus stearothermophilus* ADM-11 by morphological, biochemical characterization, and 16S rRNA gene sequencing. The pullulanase production required optimum pH of 7 and temperature of 75 °C, respectively. The electrophoresis of purified pullulanase on SDS-polyacrylamide gel revealed 83 kDa of a molecular weight that is active at 70 °C and pH 7.0. It was also stable at 90 °C but its activity was decreased by 10 % at 100 °C. The action of pullulanase was increased and stabilized by Ca^{+2} among the metal ions. Beta and gamma-cyclodextrins inhibited enzyme activity while ethylenediaminetetraacetate (EDTA) and phenylmethylsulfonyl fluoride (PMSF) have no significant effect on pullulanase activity. A full-length pullulanase gene was amplified from *G. stearothermophilus* ADM-11 using genomic DNA 2.1 kb of PCR product which was then purified and ligated in the cloning vector pTZ57R using the TA cloning technique. Colony PCR confirmed cloning on the positive clones after the pullulanase gene had been ligated and subjected to restriction digestion. It revealed 74 % similarity with the reported pullulanase gene from *Geobacillus* sp. 44C. The thermostability of pullulanase and its ability to degrade raw pullulan may therefore have wide-scale applications in starch processing, the detergent business, and new biotechnological applications.

1. Introduction

The direct starch-debranching enzyme pullulan-6-glucanohydrolase, also known as pullulanase, is found in a varied diversity of microbes, plants, and animals. This enzyme belongs to the family of amylases (Kahar et al., 2022). Pullulanases are frequently used to synthesize oligosaccharides, resistant starch for baked goods, and starch saccharification (Hii et al., 2012; Yang et al., 2022). It can create oligosaccharides because of its hydrolytic abilities. For instance, type 1 pullulanase produces maltotriose (Messaoud et al., 2002; Mishra et al., 2016), whereas neopullulanase and isopullulanase, respectively, cause panose and isopanose production (Nisha and Satyanarayana, 2016). However, it is also possible to produce oligosaccharides utilizing the enzyme's transglycosylation characteristics, which results in oligosaccharides with novel structures and relatively few byproducts, particularly when generating oligosaccharides with an elevated level of polymerization (Li et al., 2017). Numerous pullulanase can transglycosylate oligosaccharides, for instance, type 1 pullulanase from *Thermotoga neapolitana* produces α-maltotriose-(1,6)-aesculin by forming 1–6 glucosidic linkage (Kudanga et al., 2011; Park et al., 2018).

Neopullulanase from *B. stearothermophilus* forms α-1,4/α-1,6-glucosidic linkages to create malt-oligosaccharides and isomalt-oligosaccharides (Kamasaka et al., 2002). Pullulanases are widely utilized in starch fermentation, decreasing glucoamylase consumption by less than 50 % and the overall reaction period of the starch transformation procedure (Reddy et al., 1999a,b; Nisha and Satyanarayana, 2016). Glucose typically involves the saccharification phase, and the optimal characteristics for the melting stage are about pH 6.0 and 105 °C, whereas the subsequent process of saccharification happens at approximately pH 4.5 and 60 °C (Jiao et al., 2011).

The pullulanase gene was reported in mesophilic bacteria such as *Bacillus pseudofirmus* 703 (Lu et al., 2018) and *Bacillus* strain KSM-1876 (Hatada et al., 2001), thermophilic bacteria such as *Thermotoga maritime* MSB8 (Zhao et al., 2023), hyperthermophiles (such as *Rhodothermus*

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* Corresponding author at: Department of Zoology, Government College University, Lahore, Pakistan.
E-mail address: dr.dilaraabbas@gcu.edu.pk (D. Abbas Bukhari).

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marinus (Gomes et al., 2003), and *Thermococcus siculi* HJ21 (Wu et al., 2023). Extremophilic microorganisms, which thrive under severe conditions, can be classified into 7 families; acidophiles, alkaliphilies, halophiles, metalopiles, psychrophlies, and thermophiles (Taha et al., 2020). These thermophiles prefer a temperature of 50 °C or higher for the production of enzymes such as pectinase, cellulose, chitinase, amylase, lipase, DNA polymerases, etc. which possess unique properties at high temperatures (Reddy et al., 1999a,b; Mohammad et al., 2017). The discovery and application of pullulanase have a transformative impact on multiple industries, addressing existing challenges and providing solutions that lead to more efficient, sustainable, and high-quality products and processes. It will prove a better enzyme in different food processing industries and will surely reduce the cost of products (Naik et al., 2023).

Halophiles, specifically, can survive extreme salinity and thrive under such conditions (Ferreira et al., 2018). Studying thermophilic organisms in geothermal conditions provides information on the origin of life and has also opened doors to utilizing primary resources for future applications in the field of biotechnology (Yadav et al., 2018). However, thermophilic bacteria can only thrive in specific environments. Many mesophiles and thermophiles have had their pullulanase genes successfully cloned and characterized (Park et al., 2023).

Very few reports are available on pullulanase production from native strains because of low-yield issues, and increasing demands for pullulanase, it has become important to search for novel pullulanase-producing microorganisms with high yields. Moreover, high production costs and low yield are major limitations in the industrial production of pullulanase enzymes (Oğuzhan and Yangılar, 2013).

In this study, the thermostable pullulanase from a locally identified strain, *G. stearothermophilus* ADM-11, was identified and purified. The purified pullulanase exhibited a tenfold increase in activity during enzyme assays. Furthermore, the pullulanase gene was sequenced, cloned, and characterized, leading to the identification of purified thermostable pullulanase. These findings contribute to a better understanding of pullulanase's action and response processes through molecular analysis of the pullulanase gene.

2. Materials and methods

2.1. Isolation of pullulanase-producing Bacillus sp

Twenty-five soil samples were collected from potato fields and processing plants in Lahore. Physiological parameters such as pH and temperature were recorded during sample collection. Approximately, 1 g of each soil sample was mixed in 10 ml autoclaved water to isolate thermophiles and incubated at 90 °C for 24 h. After that, 100 ml of the diluted sample was plated on a red pullulan agar medium and set at 65 °C. The disappearance of the red color surrounding bacterial colonies indicated pullulanase hydrolysis. Isolates showing a significant clear region were selected for quantitative pullulanase evaluation (Krishnan et al., 2023).

2.2. Screening of pullulanase-producing bacterial isolates

The isolated bacterial community was put into a flask having PYE broth to screen bacterial isolates. The medium was boiled and autoclaved before use. The culture was placed in a shaking incubator at 37 °C and 200 rpm for 18 h. Centrifugation was performed at 4 °C and 5000 rpm for 5 min to separate the culture, and the pellet was washed with saline water. Bacterial cells with an OD of 0.5 at 550 nm were suspended in a saline solution. The supernatant containing crude enzyme was used for pullulanase investigation following the protocol of Reddy et al., (1999a,b).

2.3. Pullulanase assay

Quantitative pullulanase assay was performed using the method described by Roy et al. (2003). The amount of reducing saccharides produced was determined using 3, 5-dinitro salicylic acid (DNS) and calculated based on Miller's (1959) method. The pullulanase activity is defined as the amount of pullulanase required to produce 1 M of reducing sugars per minute under controlled conditions.

2.4. Biochemical and molecular characterization

The bacterial isolate ADM-11 was subjected to morphological and biochemical characterization following the protocol described by Al-Mamoori et al. (2023). The 16S ribosomal rDNA full-length gene (1.5 kb) of the selected bacterium ADM-11 was amplified using universal forward (5′-AGA GTT TGA TCC TGG CTCAG-3′) and reverse primers (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg et al., 1990). The PCR reaction was performed using a Thermocycler thermal cycler (Applied Biosystem 2720) PCR machine. The gel bands were cleaned using the Fermentas gene clean kit (# K0513), following the procedure outlined by Sambrook et al. (1989) and Mohammad et al. (2017).

2.4.1. Analysis of 16S rRNA gene sequence

The full-length sequence of the 16S rRNA gene from bacteria ADM-11 was compared with other microbial rRNA sequences deposited in the International Nucleotide Sequence Database (GenBank/EMBL/DDBJ). The CLUSTAL W program, available on the web, matched and aligned the sequence, finding the closest 16S rRNA sequence to ADM-11. Additionally, MEGA (11.0.13) was used to construct the phylogenetic tree.

2.5. Optimization of growth parameters for pullulanase production

The synthesis of pullulanase from *G. stearothermophilus* ADM-11 was optimized by investigating the effects of carbon and nitrogen supplies, pH, temperature, fermentation period, and inoculum size, following the protocols of Chaubey et al. (2019) and Yang et al. (2010).

2.6. Purification and physicochemical properties of pullulanase

The molecular weight of pullulanase was determined by using SDS-PAGE, following the methods described by Zhang et al. (2022). The effect of metal ions, inhibitors, pH, and temperature on the purified pullulanase activity was determined according to the procedure described by Masood et al. (2015).

2.7. Pullulanase gene cloning

For molecular characterization, the pullulanase gene was amplified, cloned, and sequenced. The bacterial strains and plasmid used for cloning the pullulanase gene were *G. stearothermophilus* ADM-11, maintained on a pullulan agar medium. The competent cells of *E. coli* DH5α and pTZ57R/T were used to clone and sequence the amplified pullulanase gene. Positive transformants of *E. coli* were selected based on the presence of the pullulanase gene using X-gal, IPTG, and ampicillin. The following steps were performed to amplify the pullulanase gene from *G. stearothermophilus* ADM-11.

2.7.1. PCR-based amplification of pullulanase gene

The full-length (2.1 kb) pullulanase gene was amplified using a polymerase chain reaction, employing the forward (5′-ATGAG-GACGGTTTTAGTGCGAGAGTCG-3′) and the reverse primer (5′-TTATGATTGGGGCACAAGCACGCAG-3′) which were designed on the base of previously reported pullulanase gene sequence (accession no E03513) from the GenBank DNA database. The pullulanase gene was amplified in a thermal cycler (Applied Biosystem-2720) using a protocol

followed by Zhang et al. (2022).

2.7.2. Assembly of the cloned vector comprising pullulanase gene

The pullulanase gene was cloned in a T/A cloning vector (pTZ57R) digested with *EcoRI* and *HindIII* and then used to ligate PCR products with DNA ligation Fermentas kit (# K1214). The competent cells of *E. coli* DH5α were prepared by the protocol followed by Sambrook et al. (1989). Cloning was verified by restriction digestion and colony PCR (Yang et al., 2017).

2.7.3. Analysis of pullulanase gene sequence

Cloned pullulanase genes (2.1 kb) from *G. stearothermophilus* ADM-11 were sequenced in a DNA sequencer (CEQ System, Ver.9.0.25) as described by Lee et al. (1997). After sequencing processing, the pullulanase gene sequence was added to the NCBI DNA database.

2.8. 3D structure of pullulanase

A 3D structure of pullulanase from assumed amino acids was displayed by the Swiss Model (https://swissmodel.expasy.org) and the feature of the model was evaluated with QMEAN cluster as determined by Benkert et al. (2009) and Bartuzi et al. (2023).

2.9. Statistical analysis

Statistical analysis was conducted on the activity of pullulanase activity. The mean value graph was constructed by Microsoft Excel along with an error bar.

3. Results

3.1. Isolation and screening of a heat-resistant Bacillus species producing pullulan

In this investigation, 50 microbial isolates were isolated from soil samples obtained from various food industries, potato processing industries, and potato fields in Lahore, Pakistan. These strains were screened using a pullulan agar medium to observe the formation of clear zones with different widths. Among the isolated strains, 17 distinct microbial strains were identified. The bacterium isolated from the potato field exhibited the highest pullulanase activity, reaching 375 U/ml, and was subsequently chosen for further analysis.

3.2. Identification of a pullulanase-producing bacteria

The bacterial strain displaying the highest pullulanase activity exhibited certain morphological and biochemical features. After 24 h of growth on pullulan agar media, this strain formed large, flat, regular colonies with an off-white color (Fig. 1). Gram staining revealed that the bacterial isolates were gram-positive. Further biochemical characterization was conducted, indicating catalase-positive activity and positive for the Voges-Proskauer test. Additionally, the strain showed the ability to utilize citrate and reduce nitrate. It demonstrated the hydrolysis of casein and starch and the breakdown of tyrosine.

PCR-based amplification of the 16S rRNA gene using universal primers was conducted for molecular characterization. A BLAST search identified the unknown microbe as *G. stearothermophilus* ADM-11 by aligning its sequence with the closest match. The 16S rRNA gene sequence of the local *G. stearothermophilus* ADM-11 was deposited in the GenBank sequence database with accession number OR239416. The family tree of *G. stearothermophilus* ADM-11 was constructed using MEGA (11.0.13) (Fig. S1).

3.3. Optimum growth conditions for pullulanase production

The optimal growth conditions for *G. stearothermophilus* ADM-11 were determined, including the influence of carbon and nitrogen sources, pH, temperature, fermentation period, and inoculum size. Potato starch and potato dextrin showed the highest enzyme units among various carbon sources. Different organic and inorganic nitrogen sources were tested; soy flour and ammonium carbonate were most effective in the production of pullulanase. The optimal pH was 7 for the enzyme activity while its activity decreased at alkaline pH. The optimum temperature was 75 °C, which supported the highest growth and enzyme activity of *G. stearothermophilus* ADM-11. The inoculum of 1 % to 10 % was evaluated, and the best enzyme activity was observed with a 5 % inoculum size from an 18 h old inoculum. Fermentation periods ranging from 6 to 72 h were tested, and the highest enzyme production occurred at the 48 h mark.

3.4. Purification of native pullulanase from G. stearothermophilus ADM-11

After 48 h of fermentation, the supernatant was used to purify pullulanase from *G. stearothermophilus* ADM-11. The enzyme was precipitated overnight at 4 °C with ammonium sulfate and then dialyzed against 50 mM Tris-HCl and loaded into Q-Sepharose to purify pullulanase. The protein elution was conducted using a linear gradient of NaCl (0.1–0.6 M). The purified enzymes were concentrated and their activity was determined using a pullulanase assay. The molecular weight of pullulanase was 83 kDa, confirmed through SDS-PAGE analysis (Fig. 2). The most effective purification and activity were achieved with the active fraction.

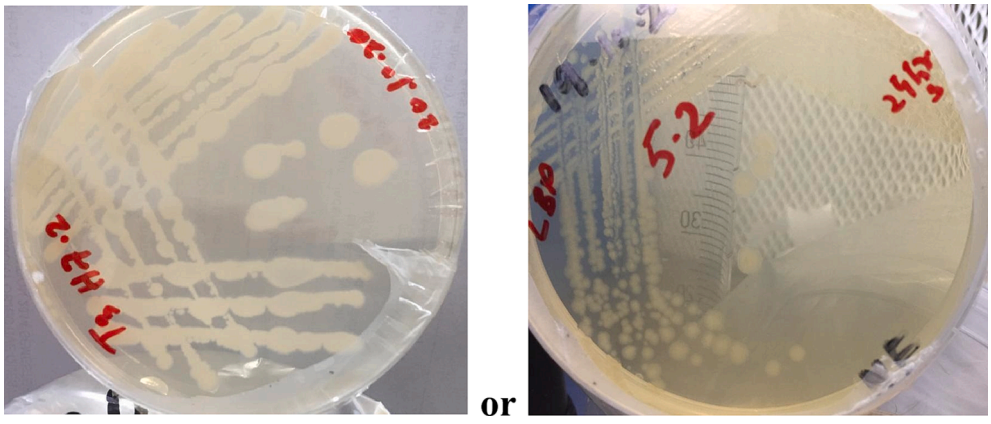

Fig. 1. *Geobacillus stearothermophilus* ADM- 11 colony morphology after 24 h of growth on pullulan agar medium.

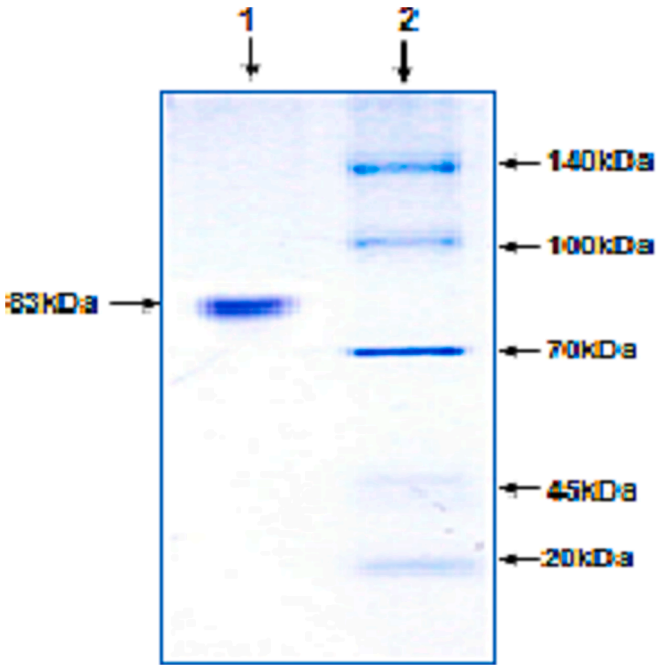

Fig. 2. SDS-PAGE (12%) of purified pullulanase from *G. stearothermophilus* ADM- 11, lane 1, purified pullulanase presenting the single band and lane 2, protein marker.

3.5. Characterization of purified pullulanase from G. stearothermophilus ADM-11

The stability and activity of the purified pullulanase from *G. stearothermophilus* ADM-11 were investigated using pullulan as the substrate. Optimal temperature, pH ranges, and the enzyme's strength under different conditions were determined. The purified pullulanase showed maximum performance at 70 °C and a pH of 7. It showed stability even at 90 °C, but a 10 % loss in activity was observed at 100 °C (Fig. 3a,b).

Various ions were examined to assess the influence of metal ions on the purified pullulanase. The enzyme's function was enhanced and stabilized in the presence of Ca^{+2}. However, the addition of Sr^{+2} and Ni^{+2} completely deactivated the enzyme. Mg^{+2} and Mn^{+2} had no significant effect on pullulanase activity (Fig. 3c). Furthermore, the purified pullulanase was subjected to inhibition tests using N-bromosuccinimide, iodoacetamide (N-BSIA), α, β, and γ-cyclodextrins, indole acetic acid (IAA), ethylenediaminetetraacetate (EDTA) and p-chloromercuribenzoate (p-CMB). It was slightly inhibited by cyclodextrins and not affected by EDTA and PMSF (Fig. 3d).

3.6. Cloning of pullulanase gene from G. stearothermophilus ADM-11

The full-length pullulanase gene (2.1 kb) was amplified using DNA extracted from *G. stearothermophilus* ADM-11. Electrophoresis of the pullulanase gene PCR product revealed a band of approximately 2100 bp, as depicted in Fig. 4a. A 0.8 % agarose gel was employed to separate the PCR product. The pullulanase gene PCR product was ligated into the pTZ57R (T/A cloning vector) to generate recombinant DNA. The resulting construct was introduced into DH5α *E. coli* cells. The combination of cells and X-gal, IPTG, and ampicillin was plated. Positive transformants were identified by selecting white colonies containing the recombinant plasmid. Ten colonies were observed on the plate.

Subsequently, plasmid synthesis was carried out for multiple colonies. The recombinant plasmid preparations were subjected to double digestion using *EcoRI* and *HindIII* enzymes to screen for recombinant plasmids containing the pullulanase gene (2.1 kb). Fig. 4b shows two bands of approximately 2.3Kbp (pTZ57R) and a 2.1Kbp pullulanase gene was detected to further confirm the pullulanase gene in DH5α, colony PCR was performed. The PCR product, visualized on a 0.8 % agarose gel, confirmed the existence of the pullulanase gene.

3.6.1. Sequence and analysis of pullulanase gene

The full-length pullulanase gene from the local *G. stearothermophilus* ADM-11 isolate was sequenced. The gene had a total length of 2.1 Kb that encode a protein of 83 kDa protein having 727 amino acids. Sequence analysis of the full-length pullulanase gene (2100 bp) was performed. The pullulanase obtained from Geobacillus stearothermophilus ADM-11 belongs to type I pullulanase with a similarity of 61–63 % and type II pullulanase with a similarity of 25–31 % (Fig. S2).

3.6.2. Phylogenetic relationships of pullulanase gene from G. stearothermophilus ADM-11

The sequence homology of the pullulanase gene from *G. stearothermophilus* ADM-11 with other pullulanase genes was examined. The phylogenetic neighbor-joining tree was constructed using MEGA (11.0.13), based on gene sequences of diverse bacterial pullulanase genes (Fig. 5a), revealing a close relationship (74 % homology) with the pullulanase gene of *Geobacillus* sp. 44C (accession no. CP061475).

3.7. 3D structure of pullulanase on the base of homology

The 3D structure of pullulanase from G. *stearothermophilus* ADM-11 on the base of homology base was predicted using the presumed amino acid sequence (Fig. 5b). The system consisted of four domains: Domain A, B, C, and N. Domain N contained an antiparallel β-sheet, Domain A had both β and α elements, Domain B consisted of α-helices and Domain C contained a β-sheet with eight antiparallel strands. The active region of pullulanase was located on the C-terminal aspect of the core β-sheet, forming a cleft composed of domains A and B. Notably, the active site of pullulanase, which is broader than that of other 1,4-specific enzymes like amylase, accommodates the substrate pullulan with its 1,6-glucosidic linkages. Critical amino acids involved in the active site included Asp328, Glu357, and Asp424, which are conserved in other starch-degrading enzymes. These amino acids were situated within four conserved regions, exhibiting a conserved spatial arrangement observed in other α-amylase families.

4. Discussion

Pullulanase is employed to produce high glucose and high-maltose syrups (Costa et al., 2020). Pullulanase is used in the detergents industry for the removal of starch-based stains on clothing, making it easier to remove them during washing (Al-Ghanayem et al., 2022). In biotechnological industries, pullulanase is utilized to produce high-value carbohydrates and bio-based products (Bilal and Iqbal, 2019). In the current study, a thermostable pullulanase from a locally identified *G. stearothermophilus* is attempted to be isolated and characterized. The isolate that displayed the highest activity (375U/ml) out of a total of 17 tested isolates was chosen for additional research. It has been shown that bacteria can be identified using morphological, biochemical characterization, and assimilation assays (Cai et al., 2020; Masi et al., 2021). The most accurate way to determine the bacteria is through ribotyping. The *Bacillus* spp. ADM-11 was recognized as *G. stearothermophilus* ADM-11 after molecular analysis. These results agree with those of (Nair et al., 2007; Uhlig, 1998).

According to Ara et al. (1992), pullulanase production varies on the strain, medium composition, and culture conditions. Optimum growth conditions like the effect of carbon and nitrogen sources, pH, temperature, fermentation period, and inoculum size for *G. stearothermophilus* ADM-11 were determined as reported by Gantelet et al. (1998), and Devi and Yogeeswaran (1999). The maximum pullulanase production was

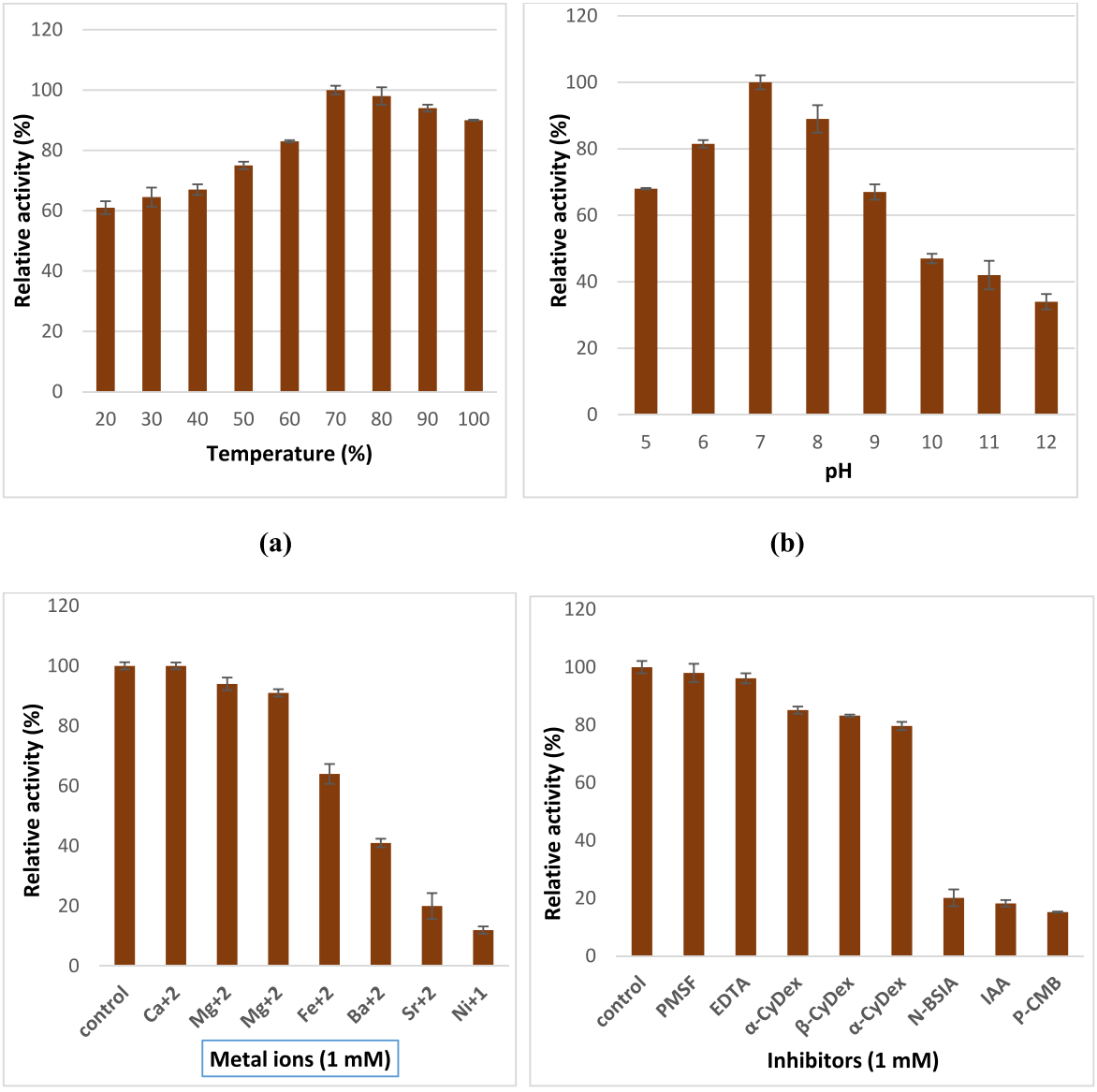

Fig. 3. **(a)** Pullulanase relative activity at temperature, **(b)** Pullulanase relative activity at pH, **(c)** Pullulanase function was enhanced and stabilized when Ca^{+2}. Mg^{+2} were added and Mn^{+2} had no significant effect on pullulanase activity. **(d)** The purified pullulanase was repressed with N-BSIA, IAA, and P-CMB. PMSF and EDTA have no significant effect on pullulanase activity.

achieved at pH 7.0. The production of enzymes decreased as the pH of the medium increased and at pH 10.0 there was a decrease in enzyme production this finding is in agreement with Asha et al. (2013), who reported the alkaline pullulanase by *B. halodurans*.

Purification of native pullulanase from *G. stearothermophilus* ADM-11 showed 18 fold increases in activity as compared to the crude pullulanase enzyme. Kim et al. (1996) reported the purification of thermostable pullulanase from bacterial strain *Thermus caldophilus* GK-24, having a specific activity of 86.2U/mg, and pullulanase from *Bacillus cereus* H1.5 with a specific activity of 8.987 U/mg with 18.4fold increases in activity was reported by Ling et al. (2009).

The purity of pullulanase was compared to 12 % by SDS-PAGE showed the presence of a single band 83 kDa. The purified pullulanase from different organisms showed great variation in molecular weight, such as purified pullulanase of 65 kDa from *T. caldophilus* GK-24 (Ling et al., 2009), 68.25 kDa from B. *cereus* (Park et al., 2023), and 76 kDa for *Bacillus stearothermophilus* (Kuriki et al., 1990).

The pullulanase activity is limited to pullulan and starch residue only. It is affected by any change in temperature, pH, and cofactors that limit its activity. Pullulanase production and purification is costly (Linares-Pasten et al., 2014). The purified pullulanase from *G. stearothermophilus* ADM-11 was characterized, and the optimum pH of the purified pullulanase was found 7.0. Some alkaline pullulanase is reported to be stable at alkaline pH like *Bacillus* sp. S1 and *Bacillus* sp. KSM-1876 have optimum pH of 9, and 10, respectively (Ara et al., 1992). Kim et al. (1993) have reported some pullulanase stable at a range of pH (4–11) for 24 h.

The optimum temperature of purified pullulanase was 70 °C, but it was stable at 90 °C and lost its activity by 10 % at 100 °C. A purified pullulanase from *Bacillus vallismortis* RG07 (Gaur and Tiwari, 2015), *A. gottschalkii* (Bertoldo et al., 2004), *Bacillus* sp. strain 1 (Lee et al., 1997), and *F. pennivorans* (Bertoldo et al., 1999) were active at the temperature range of 60–70 °C.

The effects of metal ions on purified *Geobacillus stearothermophilus* ADM-11 pullulanase showed that Ca^{+2} ions increased enzyme activity and Ba^{+2} and Fe^{+2} reduced activity by 40 and 55 %, respectively while Mn^{+2} and Mg^{+2} had no significant effect on activity while Sr^{+2} and Ni^{+2} completely inactivated the enzyme. The mechanism of $Ca^{+}2$ on

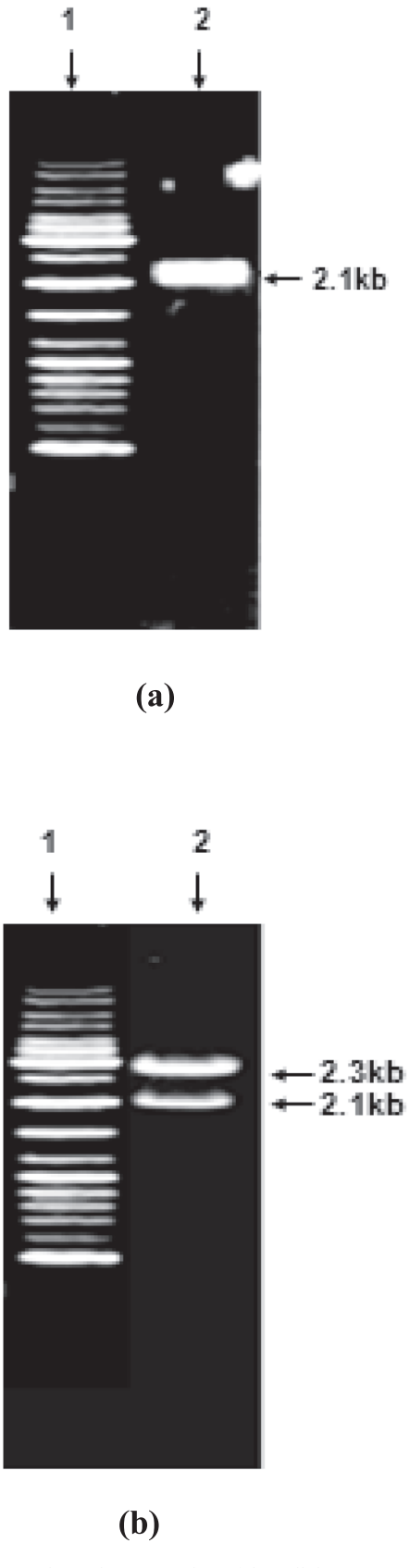

Fig. 4. **(a)** Lane 2 shows the PCR product of the pullulanase gene (2.1 kb) from the *G. stearothermophilus* ADM-11 isolate and **(b)** Lane 2 represents the restriction digestion of the recombinant plasmid containing the 2.1 kb pullulanase gene from *G. stearothermophilus* ADM-11 using *EcoRI* and *HindIII* enzymes. Lane 1 in both figures represents the DNA ladder (Fermentas).

pullulanase activity involves its interaction with the enzyme's active site and structural stabilization. Calcium ions bind to specific sites on the pullulanase enzyme, known as calcium-binding sites composed of negatively charged amino acid residues, like glutamate and aspartate which coordinate with $Ca2^{+}$. This binding of $Ca2^{+}$ to the enzyme's active site activates the enzyme, making it more effective in breaking down pullulan and other starches (Mikami et al., 2006).

Ling et al. (2009), Arikan (2008), and Özdemir et al. (2012) reported that the pullulanase activity was slightly inhibited by Mn^{+2}, completely inhibited by Cu^{+2} and Zn^{+2} while strongly stimulated by Ca^{+2}. Acer et al. (2016) also reported that pullulanase from *Anoxybacillus*-AH1 showed an inhibitory effect with exposure to Cu^{+2} whereas activated by Mg^{+2} and Ca^{+2}. EDTA and PMSF have no significant effect on the inhibition of purified pullulanase which showed that the enzyme was not a metalloenzyme or serine protease enzyme. The lack of inhibition of purified pullulanase showed that this enzyme was not a metalloenzyme and is not affected by a strong chelating agent resembles Lu et al. (2018). According to the literature the amylopullulanases are generally not serine enzymes (Kahar et al., 2016; Ling et al., 2009).

A full-length pullulanase gene (2.1 kb) was amplified from *Geobacillus stearothermophilus* ADM-11 resembles Su et al. (2010) who cloned and expressed the 2.532Kbp pullulanase gene (pulA) from T. *maritima* MSB8. The overall homology-based three-dimensional structure of pullulanase from *G. stearothermophilus* ADM-11 was made from deduced amino acid sequence and it was found that the single polypeptide folds consisted of 4 domains (A, B, C, and N). The active site of pullulanase is situated between domains A and B. The shape of the furrow which is the active site of the pullulanase is much wider than α-amylase other-1, 4 specific enzymes which are in agreement with Imanaka and Kuriki (1989). The catalytic site has some important amino acids Asp328, Glu357, and Asp424 like other starch-degrading enzymes like other α -amylase families which showed conserved spatial arrangements (Katsuya et al., 1998; Habib-ur-Rehman et al., 2018).

5. Conclusion

In conclusion, the bacterium showed maximum activity of 375U/ml and was identified as *Geobacillus stearothermophilus* ADM-11 on the basis of morphological, biochemical characterization, and 16S rRNA gene sequencing. The optimum conditions required for enzyme production were pH of 7 and temperature of 75 °C. The electrophoresis of purified pullulanase on SDS-polyacrylamide gel revealed a band of 83 kDa that was active at 70 °C and pH 7.0. It was also stable at 90 °C but its activity was decreased by 10 % at 100 °C. The enzyme activity was enhanced and stabilized by Ca^{+2} among the metal ions used. Beta- and gamma-cyclodextrins inhibited pullulanase activity while EDTA and PMSF have no significant effect on pullulanase activity. A full-length pullulanase gene was amplified from *G. stearothermophilus* ADM-11 which was then purified and ligated in the cloning vector pTZ57R using the TA cloning technique. It revealed 74 % similarity with the reported pullulanase gene from *Geobacillus* sp. 44C. This enzyme was purified and characterized from the organism and molecular characterization of the pullulanase gene producing thermostable pullulanase was done. The enzyme thermostability and its potential to degrade raw pullulan may therefore have wide-scale applications in starch processing and biotechnological applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.

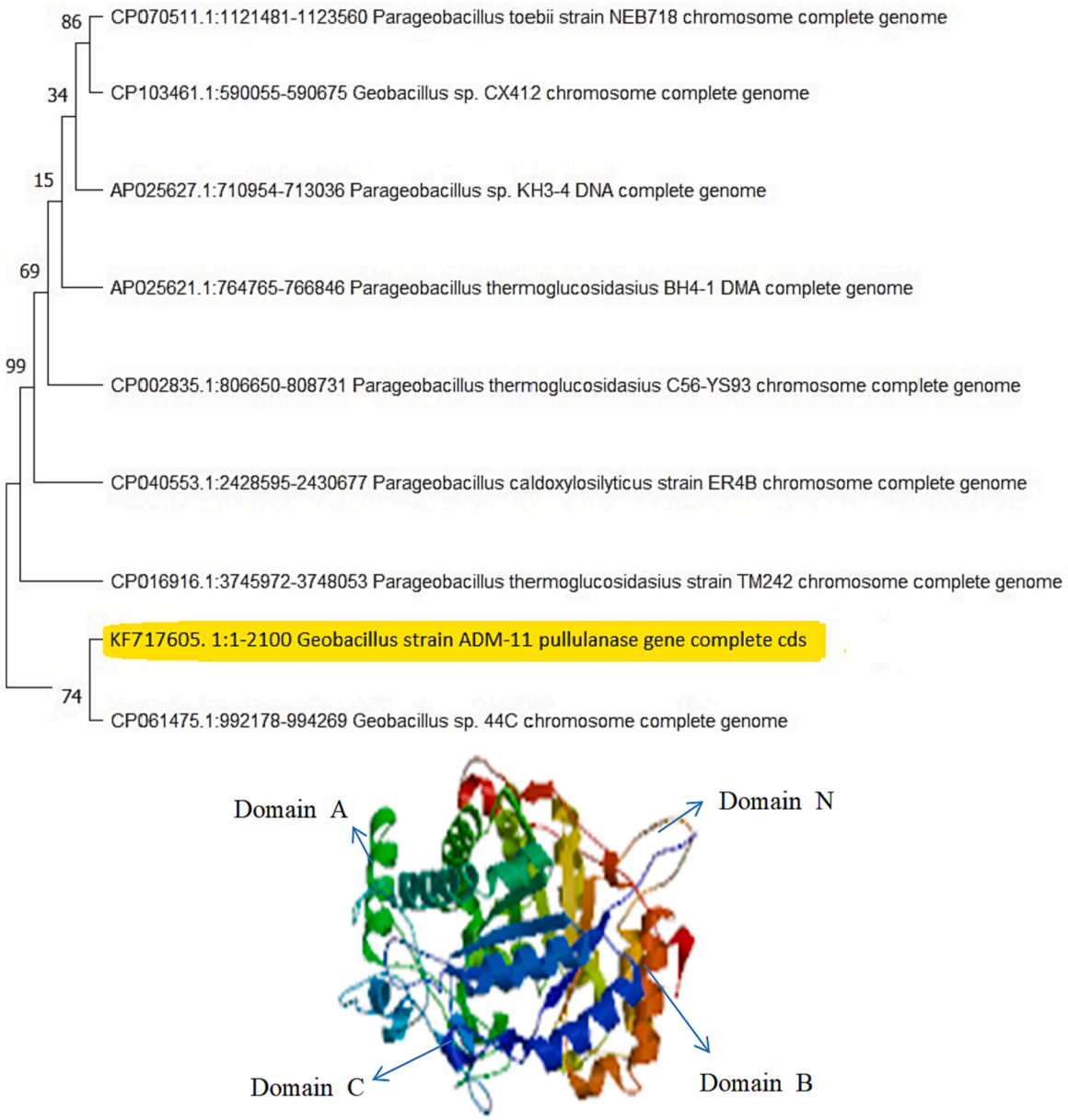

Fig. 5. **(a)** The phylogenetic neighbor-joining tree, constructed using MEGA (11.0.13), based on gene sequences of diverse bacterial pullulanase genes, revealing a close relationship (74% homology) with the pullulanase gene of *Geobacillus* sp. 44C (accession no. CP061475) **(b)** Illustrates the homology-based three-dimensional shape of pullulanase simulated using the Swiss Model (http://swissmodel.org).

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