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Research article

Isolation, identification, and characterization of neopullulanase from *Thermomonas hydrothermalis* GKE 08

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Abstract

The production of neopullulanase from thermophiles, such as *Thermomonas hydrothermalis* GKE 08, has great importance due to the enzyme's unique thermophilic nature. This characteristic results in enhanced stability and functionality at elevated temperatures. It is known that this is a very important issue for industrial processes that require efficient catalysis under extreme conditions. The investigation of pullulanase from *T. hydrothermalis* GKE 08 showed significant results. Optimal conditions for enzyme production were determined, with peak activity observed in the presence of 1.5% soluble pullulan and 0.5% peptone. The study delved into the pH and temperature dynamics, identifying an optimal pH of 7.0 and a temperature of 55°C. Notably, the neopullulanase exhibited time-dependent stability, retaining 72% activity after 1 hour but declining to 50% after 2 hours. Purified pullulanase from *T. hydrothermalis* GKE 08 displayed optimal activity at pH 7.0, with a subsequent time-dependent decline observed during incubation at this pH: retaining 72% activity after 1 hour, approximately 50% after 2 hours, and a significant 77% loss after one day. Furthermore, the enzyme displayed remarkable thermostability at 60°C, with 88% activity after 30 minutes. Metal ion studies indicated susceptibility to inhibition by Cu^{2+}, Mg^{2+}, and Zn^{2+}, while Ca^{2+} stimulated activity up to 138% at higher concentrations. The enzyme's response to specific reagents revealed sensitivity to SDS and EDTA, while urea surprisingly enhanced activity to 85%. The study enhances understanding of pullulanase behavior, offering valuable insights for biotechnological and industrial applications.

Keywords: *Neopullulanase; Thermomonas hydrothermalis; thermophiles; thermozyme*

1. Introduction

Starch, a pivotal biomolecule, serves as a versatile material extensively employed not just in the food processing industry but in many different industries. Traditionally acknowledged for its applications in food, recent technological advancements have expanded its utility in diverse fields, including health, medicine, textiles, paper production, fine chemicals, agriculture and, both petroleum and construction engineering (Egharevba, 2019; Miao and BeMiller, 2023). Within the realm of starch debranching enzymes, pullulanase (EC 3.2.1.41) emerges as a well-known catalyst capable of hydrolyzing not only starch but also other polysaccharides such as pullulan, glycogen, and amylopectin (Boersma, 2024). The vital hydrolytic activity of pullulanase plays a crucial role in applications within the food and pharmaceutical sectors (Hii et al., 2012; Møller et al., 2016; de Souza et al., 2023). Pullulanase (EC.3.2.1.41) plays an essential role as a debranching enzyme, catalyzing the hydrolysis of α-1,6 glucosidic linkages within pullulan, amylopectin, starch, and associated oligosaccharides (Kim et al., 2024). During saccharification processes, this class of enzymes is essential for producing small fermentable sugars by thoroughly breaking down branched polysaccharides (Das and Kayastha 2023).

Pullulanases' unique ability to target α-1,6 linkages is highly beneficial in various settings. In the food industry, it plays

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a role in improving the digestibility and nutritional content of starch-based products like bread and beer (Naik et al., 2023). Furthermore, in pharmaceuticals, pullulanase aids in converting starch into glucose, a step in producing glucose syrups used in medicinal products. Its impact also extends to applications by assisting in the breakdown of starch-based pollutants during bioremediation processes (de Souza, 2010). Additionally, in the textile industry, pullulanase helps efficiently remove starch sizing agents from fabrics during desizing operations, reducing harm (Araujo et al., 2008) The versatility of pullulanase emphasizes its importance across sectors due to its enzymatic functions that support effective processes and sustainable practices. As ongoing research uncovers new uses and improves its properties, pullulanase continues to play a role in advancing industries dependent on starch and its derivatives. The multifaceted contributions of this enzyme underscore its position, in driving technological progress and addressing current industrial challenges.

The pullulanase family exhibits diverse substrate specificity and product profiles, leading to its categorization into five distinct types: (1) Type I, denoted as true pullulanases (EC 3.2.1.41), primarily hydrolyzing α-(1,6) glycosidic bonds and yielding maltotriose; (2) Type II, referred to as amylopullulanases (EC 3.2.1.41), showcasing activity on both α-(1,6) glycosidic bonds in pullulan and α-(1,4) glycosidic bonds in starch, resulting in the production of maltotriose and a mixture of glucose, maltose, and maltotriose, respectively; (3) pullulan hydrolase Type I, recognized as neopullulanase (EC 3.2.1.135), displaying high hydrolytic activity on α-(1,4) and α-(1,6) glycosidic linkages, particularly on pullulan and cyclodextrins, generating panose; (4) pullulan hydrolase II, identified as isopullulanase (EC 3.2.1.57), exhibiting α-(1,4) glycosidic bond hydrolysis on cyclodextrins and yielding isopanose; and finally, (5) pullulan hydrolase III, with a versatile activity spectrum capable of degrading both α-(1,4) and α-(1,6) glycosidic linkages, resulting in a mixture of panose, glucose, maltose, and maltotriose (Kłosowski et al., 2010). Neopullulanase (EC 3.2.1.135), also known as pullulan 4-D-glucanohydrolase, falls within the category of pullulan hydrolase type I and is affiliated with the alpha-amylase family. This enzyme facilitates the breakdown of α-1,4 glycosidic linkages in pullulan, resulting in the formation of panose (6-alpha-D-glucosylmaltose) (Imanaka and Kuriki, 1989; Roy et al., 2003; Bajpai, 2023; Park et al., 2023).

Thermophiles, organisms thriving in elevated temperatures reaching from 41°C to 122°C, predominantly exhibit optimal growth conditions at 80°C (Madigan et al., 1997). These extremophiles are sourced from diverse high-temperature environments, including hydrothermal vents, thermal hot springs, volcanic eruptions, and more, where they produce thermozymes. Thermozymes exhibit enhanced conformational structures, and their adaptability is attributed to the minimal occurrence of additional hydrogen bonds, hydrophobic interactions, and electrostatic interactions (Hussian and Leong, 2023). They have a pivotal role across various industries, including biotechnology, chemistry, food processing, pharmaceuticals, pulp and paper, and the treatment of waste (Bruins et al., 2001; Schäfers et al., 2017; Kumar et al., 2019; Sharma et al., 2019).

Thermozymes have characteristics that are greatly appreciated in industries working with temperatures and difficult environments. In biotechnology, these enzymes are highly valued for their stability and efficiency, in settings that facilitate processes such as DNA amplification (PCR) and protein manipulation. Their resilience is also advantageous in the chemical sector, enabling the catalysis of reactions that would otherwise demand specific conditions or hazardous materials (Giordano, 2010).

Many studies have been conducted to comprehensively study neopullulanases derived from thermophiles for instance *Geobacillus stearothermophilus* TRS40 (Kuriki et al., 1992), *Bacillus stearothermophilus* (Kuriki et al., 1988; Kamasaka et al., 2002; Hondoh et al., 2003; Ece et al., 2015), *Desulfurococcus mucosus* DSM (Jafari et al., 2022), and *Thermotoga maritime* MSB8 (Zhao et al., 2023), as well as hyperthermophiles like *Rhodothermus marinus* (Gomes et al., 2003) and *Thermococcus siculi* HJ21 (Wu et al., 2023). The identification and utilization of pullulanase enzyme have initiated a transformative epoch in various sectors, tackling existing challenges and offering resolutions that contribute to increased efficiency, sustainability, and the production of superior-quality products and processes. This enzyme is poised to outperform in various food processing sectors, heralding a potential reduction in product costs (Naik et al., 2023).

This study is driven by the overarching goal of addressing the crucial role pullulanase plays in biotechnology and the starch industry, particularly in saccharification processes. Great potential of thermophilic pullulanases was investigated for industrial use. Pullulanases, crucial enzymes in the industrial applications, have optimal activity at temperatures exceeding 60°C, making their thermal stability paramount for effective catalysis (Nair et al., 2006).

Given the escalating demand for pullulanase, the primary goal of this study is to identify a novel thermophilic bacterial strain with the ability to produce elevated amounts of this enzyme at high temperatures. The results of this study will contribute to the industry's needs by uncovering a bacterial strain that can meet the growing demand for pullulanase, emphasizing the significance of thermal stability to ensure efficacy in diverse industrial applications.

The genome project of two different strains of *Thermomonas hydrothermalis* is deposited at DDBJ/EMBL/GenBank with the accession number FQUK00000000.1 (isolate: DSM 14834) and JAILZK000000000.1 (isolate: HOT.CON.106). A previous study focused on examining the genomic content of two isolates to identify the presence of glycoside hydrolase enzymes (Yasar Yildiz, 2024). Both isolates were annotated, and the analysis revealed that the neopullulanase (EC 3.2.1.135) enzyme is present in the genome of both strains, serving as the starting point for the present study. Furthermore, within the broader context of this study, the main focus is on isolating neopullulanase from the thermophilic bacterium *T. hydrothermalis*, as there is currently no published information on neopullulanase production from this bacterial strain. The introduction underscores the pivotal role of starch as a widely utilized biomolecule across various industries, underscoring the importance of pullulanase, specifically neopullulanase, in the hydrolysis of starch and related polysaccharides. The growing need, for neopullulanase in starch industries particularly in glucose production, highlights the importance of comprehending its properties and structural features. This study seeks to fill this gap by examining the extraction and analysis of neopullulanase from *T. hydrothermalis* GKE 08 aiming to expand the understanding of neopullulanases found in thermophilic bacteria for use, in various industries.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Bacterial strain *Thermomonas hydrothermalis* GKE 08 was isolated from Golan hot spring, Karakocan, Elazig, Türkiye (Yasar Yildiz, 2024). The 16S rDNA sequence of the bacterial strain was deposited in GenBank with the accession number OQ940533. The strain was incubated on pullulan agar (g/l; pullulan 2; agar 18) and fermented for a duration of 24 hours at a temperature of 55°C. Pullulan degradation was detected through the observation of halo-zones surrounding colonies by covering the agar plate with 99% (v/v) ethanol and letting it stand for 3 hours (Morgan et al., 1979). *T. hydrothermalis* GKE 08 was grown on a basal medium (BM) with the following composition (g/l): peptone 5, yeast extract 2, $MgSO_4$ 0.5, NaCl 0.5, $CaCl_2$ 0.1, glucose 5. Before the medium was sterilized, its pH was adjusted to 7.0. The carbon source was sterilized separately for 3 min at 121°C and mixed with growth media under an aseptic condition (Lee et al., 2001).

2.2. Growth conditions for pullulan production

The pullulan production basal medium (PPBM) contains the same ingredients as BM, except for pullulanase, which is added at a concentration of 5 g/l to replace the presence of glucose. The pH of the PPBM was adjusted to 7.0. *T. hydrothermalis* GKE 08 was cultured in BM for 18 hours under controlled conditions at 55°C, accompanied by agitation at 180 rpm. Subsequently, 1% (v/v) concentration of the BM culture was used to inoculate PPBM. The culture was incubated at 55°C for 48 hours at 180 rpm, following the methods described in previous studies (Nair et al., 2007; Ling et al., 2009).

Growth quantification was achieved through the measurement of optical density at a wavelength of 660 nm in the culture medium utilizing a spectrophotometer (PhotoLab 7100 VIS, Weilheim, Germany) as outlined by Brunswick et al. (1999). This analytical approach facilitated the assessment of microbial proliferation by evaluating the absorbance characteristics indicative of cellular density. Samples obtained periodically were subjected to centrifugation at 5000 rpm for 15 minutes at a temperature of 4°C. The obtained supernatant, devoid of cells, was served as a crude enzyme source for subsequent analyses.

2.3. Enhancing neopullulanase production through culture condition optimization

The impact of variable sugar concentration on production of extracellular enzyme was examined by introducing variable concentrations of soluble pullulan into PPBM. Diverse nitrogen sources, including beef extract, tryptone, peptone, and yeast extract, were individually introduced to assess their effects. The impact of pH and temperature on enzyme production was examined separately, varying the pH and temperature of the production media. Neopullulanase production in the fermentation media was monitored at two-hour intervals until a decline in enzyme activity was observed.

2.4. Pullulanase activity

The procedure established by Kanno and Tomimura (1985) for the determination of pullulanase activity has been modified and conducted at 55°C. The measurement was based on the quantity of enzyme necessary to generate reduced sugars from pullulan, following the protocol of the abovementioned researchers. A 150 µl of supernatant (devoid of cells) was mixed with an equal amount of 50 mM sodium phosphate buffer at pH 7.0, which contained 1% (w/v) pullulan. The same procedures were used to prepare a blank, without the presence of pullulan. The mixtures obtained were incubated at 55°C for one hour, followed by termination of the reaction through the addition of 150 µl of DNS reagent (DNS reagent preparation: 1 g of 3,5-dinitrosalicylic acid dissolved in 20 ml of 2M NaOH, with the gradual addition of 30 g sodium potassium tartrate, and final dilution to 100 ml using distilled water).

After being diluted four times with distilled water, the mixture was boiled for five minutes. Prior to the introduction of the crude enzyme, a blank solution consisting solely of DNS reagent was prepared. Furthermore, a control was established by introducing the DNS reagent before the substrate. The absorbance at 540 nm was used to measure the enzyme activity, following the methods described by Miller (1959) and Kim et al. (2008). The quantity of pullulanase needed to yield-reducing sugar equal to one µmol of glucose per minute under assay conditions was specified as one unit or U/ml (Nair et al., 2007).

2.5. Purification of neopullulanase

The cell-free supernatant obtained from the cultivation broth underwent a process of partial purification using an acetone precipitation method with slight modifications (Obi and Odibo, 1984). To achieve this, 4 volumes of cold acetone were slowly added to 1 volume of the extract, and the resulting mixture was allowed to precipitate overnight at -20°C. After the centrifugation step at 10,000 rpm for a duration of 10 minutes, a resulting pellet was obtained. This pellet was subsequently dissolved in a minimal volume of 0.2 M Tris-HCl buffer with a pH of 8.5.

The subsequent purification steps included anion exchange chromatography, wherein the dissolved sample underwent column chromatography utilizing DEAE cellulose. The fractions eluted from the chromatographic column were meticulously analyzed for enzyme activity. Then all fractions obtained from column chromatography were lyophilized and used for characterization.

2.6. Characterization of pullulan hydrolysis products

Thin-layer chromatography (TLC- TLC Silica gel 60F254, Merck Co, Germany) was used to recognize products that were obtained after the hydrolysis of pullulan. This method provides analysis of hydrolysis products by separating individual components on the chromatographic plates. A 5 µl sample was applied for each spot. A modified version of the method by Raha et al. (1992) was used. An eluent was used which has a mixture of butanol, ethanol, and water in a ratio of 3:1:1 (v/v/v).

The hydrolysis products were observed by spraying the plates with 20% H_2SO_4 and subjecting them to charring for approximately 10 minutes at 100°C. Incubation of the supernatant (devoid of cells) with 1% (w/v) pullulan for 1 hour at 55°C provided reaction products. Along with standard compounds such as glucose, maltose, maltotriose, maltotetraose, maltopentaose, and panose, reaction hydrolysates were applied as spot inoculations onto TLC sheets.

2.7. Biochemical characterization of neopullulanase

The optimal conditions were obtained by investigating influence of varying temperature and pH values on the activity of enzyme. 0.1 mM sodium phosphate buffer was used for the reaction and the reaction mixture was incubated for 1 hour (Duffner et al., 2000; Tang et al., 2008). The pH range of 3.0 to 11.0 was investigated using 0.2 M strength buffers.

In the next stage, time-dependent changes in enzyme activity were observed by investigating the effect of varying pH on enzyme stability. The variation in enzyme stability was observed at temperatures ranging from 0 to 100°C using the standard test protocol at the optimum pH. Additionally, the effect of metal ions on enzyme activity was evaluated. For this purpose, selected metal ions such as Cu^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+} were added to the enzyme at two different concentrations (0.2 mM and 2 mM) and examined with a pre-incubation of 10 minutes. Enzyme activity after this incubation was evaluated separately. Enzyme activity is affected not only by metal ions but also by variable group-specific reagents such as ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and urea. To determine the effect of reagents on enzyme activity, these three different reagents were applied at varying concentrations. The enzyme was individually exposed to each reagent, and subjected to pre-incubation for 10 minutes, and the reaction was initiated by adding the substrate. The resulting activity was then evaluated as previously mentioned. A control was employed using a reaction mixture that lacked metal ions and reagents.

2.8. Statistical analysis

The experimental design employed a fully randomized structure to minimize bias and ensure reliability. Each treatment or sample was evaluated with 3 biological replicates, and the mean ± standard deviation (SD) represents the results that are shown.

The GraphPad Prism was used to analyze the data. The statistical significance of the differences between groups was evaluated using one-way analysis of variance (ANOVA) and independent samples t-tests. Duncan's multiple range test was used at a 95% confidence level for multiple mean comparisons, ensuring that the differences identified were statistically significant with a *p*-value less than 0.05. This rigorous approach to statistical analysis underlines the robustness and credibility of the study's findings.

3. Results and discussion

3.1. Optimization of culture conditions for maximum neopullulanase production

The highest production of pullulanase was achieved in the existence of soluble pullulan at a concentration of 1.5% (Fig. 1) and with 0.5% peptone (Fig. 2). Previous research on *B. cereus* FDTA-13 has also reported an increase in pullulanase synthesis in the presence of pullulan (Nair et al., 2007). However, substrate repression was reported. When pullulan concentration was elevated to higher levels a decrease in enzyme production was observed. Similar results were also reported for *Bacillus halodurans* (Asha et al., 2013), peak pullulanase activity was observed at a concentration of 1.5%, followed by a decline in enzyme production beyond that concentration. These findings

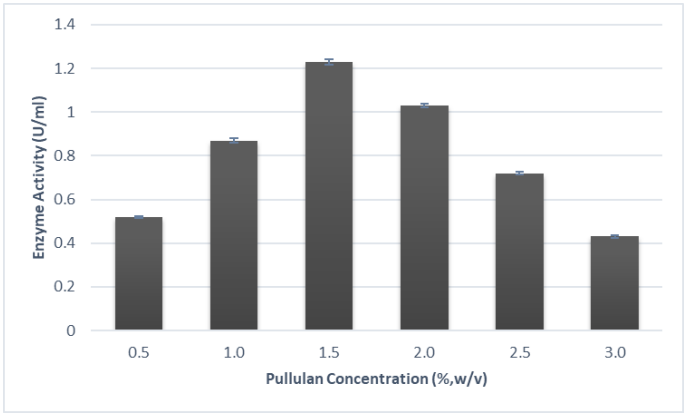

Fig. 1. Effect of different concentrations of soluble pullulan on *T. hydrothermalis* GKE 08 pullulanase production.

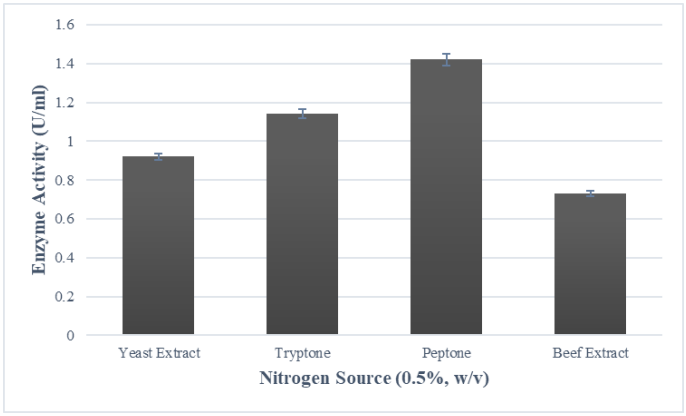

Fig. 2. Effect of different nitrogen sources on *T. hydrothermalis* GKE 08 pullulanase production.

indicate that pullulanase production in microbial systems is not always similar and it shows complex regulation.

Among the various nitrogen sources examined, peptone proved to be the preferred choice for extracellular pullulanase production. Similar trends were observed in *Clostridium thermosulfurogenes* SV918 (Swamy and Seenayya, 1996) and *B. halodurans* (Asha et al., 2013). The results of these studies are similar to the findings of this study. It was found that the strain, composition of the fermentation medium, and growth conditions have an impact on pullulanase yields. The neopullulanase derived from the *Thermomonas hydrothermalis* GKE 08 strain in this investigation exhibited optimal activity at pH 7.0 (Fig. 3) and 55°C (Fig. 4), which is crucial for the application of the enzyme in saccharification processes. Interestingly, Tang et al. (2008) conducted enzyme screening on soil sediments from a hot spring located in Thailand and reported a novel neopullulanase that is thermally stable. In their study, the neopullulanase displayed the highest activity at 75°C and a pH of 7.0.

Similarly, *Geobacillus stearothermophilus* ADM-11 demonstrated optimal pullulanase activity under identical conditions (Bukhari et al., 2024). *Bacillus amyloliquefaciens* (Castro et al. 1993) showed peak production of pullulanase at a temperature of 44°C and pH level of 5.6. The research conducted by Swamy and Seenayya in 1996 on extracellular pullulanase production by *C. thermosulfurogenes* SV9 revealed the conditions for this process. They identified that the favorable pH range was around pH 7.0 while the best temperature for production was determined to be 60°C. These findings provide insights into enhancing the efficiency of extracellular pullulan-

Table 1
Data of purification process for pullulanase from *T. hydrothermalis* GKE 08.

Fraction	Volume (ml)	Total Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)	Purification Fold	Recovery (%)
Cell free extraction	200	493.64	28.42	17.37	1	100
Acetone precipitation	20	189.43	3.87	48.95	2.61	38.31
Anion exchange chromatography	10	78.21	1.02	76.68	6.31	15.85

ase production by *C. thermosulfurogenes* SV9 making it more suitable for industrial applications. Notably a significant amount of the enzyme totaling 82% was produced at a temperature of 55°C indicating efficiency at this temperature for enzyme production and its potential importance in applications requiring enzymatic activity. Additionally, a study investigated the incubation of *B. cereus* SDK2 under conditions, in the presence of pullulan followed by an assessment of the bacterium's extracellular enzyme activity to understand how varying conditions affect *B. cereus* SDK2 and its enzymatic capabilities when exposed to pullulan (Davaeifar et al., 2015). The unique microbial strain showed healthy growth at a range of pH levels from 5.0 to 9.0 and different temperature conditions, between 30 and 50°C. Its ability to grow vigorously under varying pH and temperature conditions indicates its flexibility and strength. The peak growth performance was particularly impressive at a pH of 7.0 and a temperature of 37°C. This adaptability in thriving under pH and temperature ranges underscores the resilience and versatility of the strain suggesting its potential for use, in environmental and industrial settings.

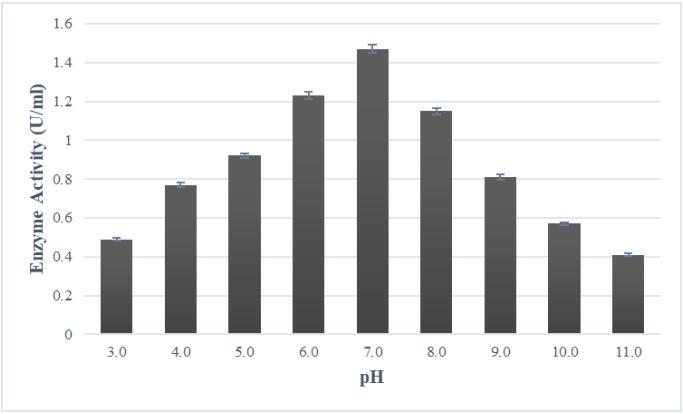

Fig. 3. Effect of different initial pH values on *T. hydrothermalis* GKE 08 pullulanase production.

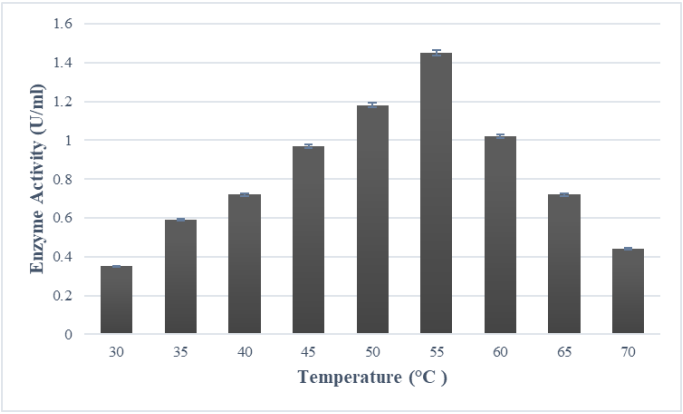

Fig. 4. Effect of temperature on *T. hydrothermalis* GKE 08 pullulanase production.

The isolate was studied under optimal conditions, and different fermentation durations, ranging from 6 to 72 hours, were explored and the 48-hour mark was identified as the time when the highest enzyme production occurs (Fig. 5).

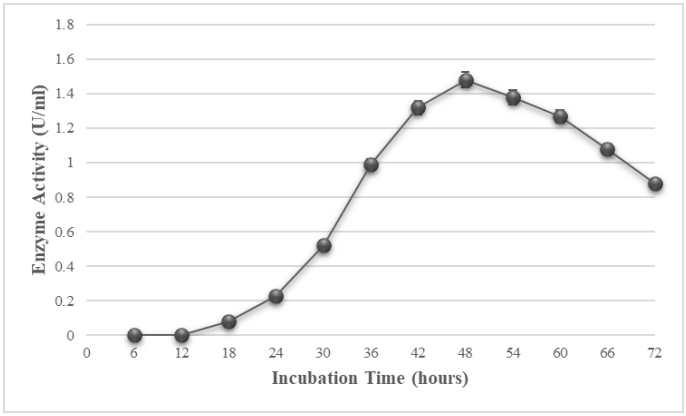

Fig. 5. Time course of *T. hydrothermalis* GKE 08 pullulanase production.

3.2. Purification of neopullulanase

Throughout each stage of the purification process, both the enzymes' specific activity and purification fold consistently showed an increase as outlined in Table 1. This steady progression, during purification highlights how effectively the process enhances the enzymes' activity and overall yield. The systematic improvement in these parameters further confirms the refinement of the enzyme at each purification step demonstrating the reliability and efficiency of the purification method. This affirms that acetone precipitation followed by anion exchange chromatography are techniques for pullulanase enzyme purification. These findings are consistent with research on *Thermus caldophilus* GK-24, where a similar purification process achieved homogeneity in pullulanase resulting in a specific activity of 86.2 U/mg protein and a recovery rate of 13.2% (Kim et al., 1996). The objective of this purification process aimed to extract and enhance pullulanase to a level indicated by the enzyme activity and recovery rate data provided shedding light on the efficiency of the purification method. Similarly, in another study, on *B. halodurans* it was observed to exhibit an activity level of 87.64 U/mg protein with an 8.87% recovery rate (Asha et al., 2013). Another study on pullulanase, from *Bacillus cereus* H1.5 revealed an activity level of 8.987 U/mg, a purification factor of 18.4, and a yield of 10.9% as documented by Ling et al. in 2009.

3.3. Characterization of pullulan hydrolysis products

To verify the identity of neopullulanase, TLC analysis was conducted following an enzymatic reaction with pullulan as the substrate under optimal pH and temperature conditions. For this

purpose, the enzymatic substrate reaction's hydrolyzed products were analyzed through TLC using standard sugar solutions. The TLC analysis revealed that panose was the final byproduct when pullulan was hydrolyzed by *T. hydrothermalis* GKE 08, thereby confirming that the enzyme synthesized extracellularly by *T. hydrothermalis* GKE 08 was indeed a neopullulanase (Fig. 6). The designation of the catalyzing enzyme as a neopullulanase is warranted when panose is the exclusive product recovered from the reaction, as established by previous studies (Labes et al., 2008; Hii et al., 2012; Davaeifar et al., 2015; Balolong et al., 2016).

Panose shows promising potential, as a prebiotic as highlighted in a study by Mäkeläinen et al. (2009). It can also act as a sweetener that helps prevent tooth decay in food products due to its qualities. To begin with, it offers a sweetness making it an appealing substitute for sugars. Moreover, panose resists fermentation by mouth bacteria, which contributes to maintaining health. It hinders the production of glucans from sucrose a process associated with plaque formation. Consequently, panose plays a role in preventing cavities as shown in research by Tsunehiro et al. in 1997. Additionally, a diet rich in carbohydrates, like panose, combined with bacteria can positively impact the human gut system. This duo can improve gut health support a microbiome and potentially enhance digestive function. Findings supported by studies led by Andersen et al. (2012) and Choi et al. (2014).

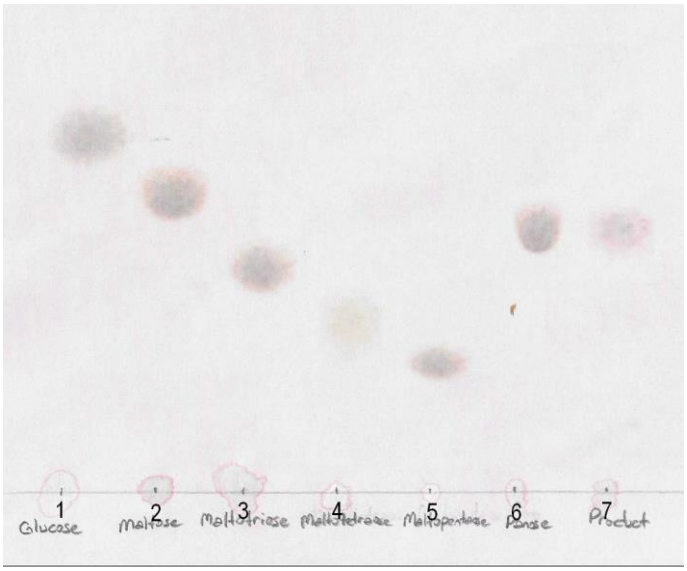

Fig. 6. Pullulan-degrading enzyme profile of *T. hydrothermalis* GKE 08 as determined by TLC: (1) Glucose, (2) Maltose, (3) Maltotriose, (4) Maltotetraose, (5) Maltopentaose, (6), Panose. (7) Product from *Thermomonas hydrothermalis* GKE 08.

3.4. Biochemical characterization of neopullulanase

The investigation into the purified pullulanase, isolated from *T. hydrothermalis* GKE 08, revealed an optimum pH of 7.0, as illustrated in Fig. 7. Further exploration involved subjecting the enzyme to incubation at pH 7.0 for varying durations. After 1 hour, a substantial 72% of the enzyme activity was retained. However, as the incubation period extended to 2 hours, nearly 50% of the enzyme activity was lost. The decline continued with a 77% loss of activity observed after a one-day incubation period, indicating a time-dependent impact on enzyme stability, as represented in Fig. 8.

Examining the temperature influence on the purified pullulanase, the investigation identified a peak activity at 60°C, showcasing the enzyme's thermostable nature, as revealed in Fig. 9. Further characterization of thermostability involved incubating the enzyme at 60°C for an extended duration. After 30 minutes of incubation, an impressive 88% enzyme activity was recorded. The residual activity persisted over time, with 69% observed after the 1st hour, 47% after the 2nd hour, and 21% after the 24th hour, highlighting the enzyme's resilience under prolonged exposure to elevated temperatures (Fig. 10). These findings underscore the importance of understanding the pH and temperature dynamics governing the stability and functionality of the isolated pullulanase, offering valuable insights for potential industrial applications.

Table 2
Analysis of the effect of the differences in metal ions effects on neopullulanase activity of *T. hydrothermalis* GKE 08.

	Relative Activity (%) at two different concentrations	
Reagent	**0.2 mM**	**2 mM**
Cu^{2+}	16	12
Fe^{2+}	75	41
Ca^{2+}	105	138
Mg^{2+}	46	35
Zn^{2+}	51	39
Mn^{2+}	82	57
Control*	100	100

*The control exhibited 100% activity and contained no metal ions. In each case, *p*-values less than 0.05 ($p<0.05$) demonstrated statistical significance.

The activity of the pullulanase enzyme was found to be affected by the presence of metal ions, like Cu^{2+}, Mg^{2+}, and Zn^{2+}. Both Mn^{2+} and Fe^{2+} showed an effect on the enzyme indicating a subtle sensitivity to these specific ions. Interestingly, Ca^{2+} had a stimulating impact on the enzyme activity as shown in Table 2. The pullulanase activity decreased noticeably with concentrations of ions revealing a dose-dependent influence on enzymatic function.

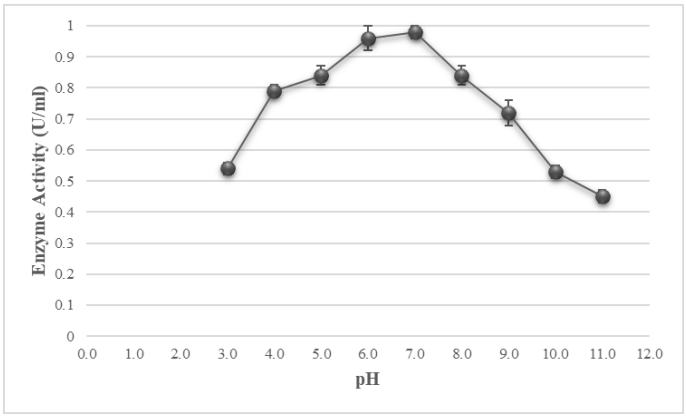

Fig. 7. Effect of pH on *T. hydrothermalis* GKE 08 neopullulanase activity.

Conversely, the introduction of Ca^{2+} ions led to an increase in pullulanase activity highlighting a connection between ion levels and enzyme response. Of interest is the finding that the effect of Ca^{2+} on enzyme activity rose by up to 138% when Ca^{2+} ion concentration was increased tenfold. This indicates an adjustment in pullulanase activity based on the varying levels of Ca^{2+} emphasizing the intricate relationship, between metal ions

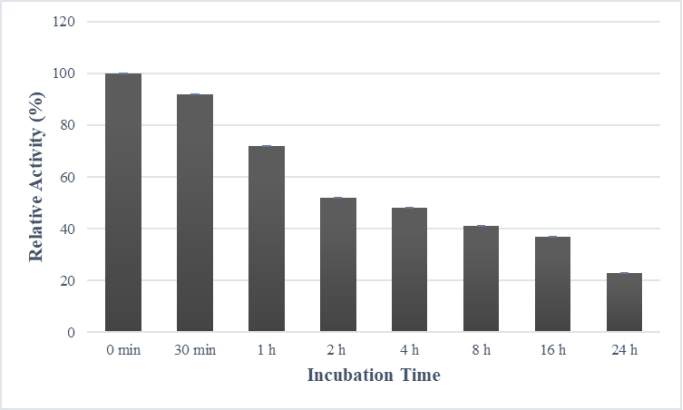

Fig. 8. pH stability of *T. hydrothermalis* GKE 08 neopullulanase at pH 7.0.

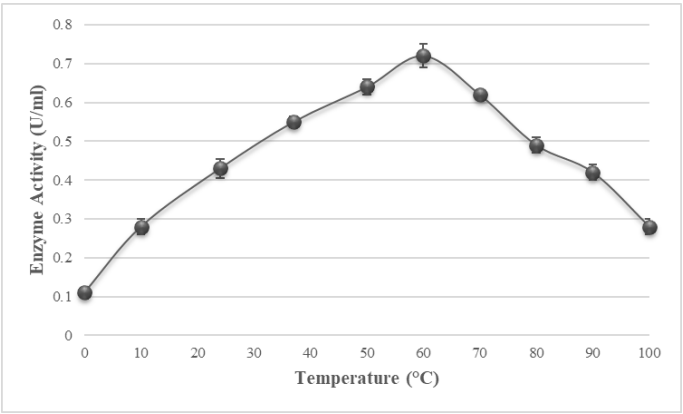

Fig. 9. Effect of temperature on *T. hydrothermalis* GKE 08 neopullulanase activity.

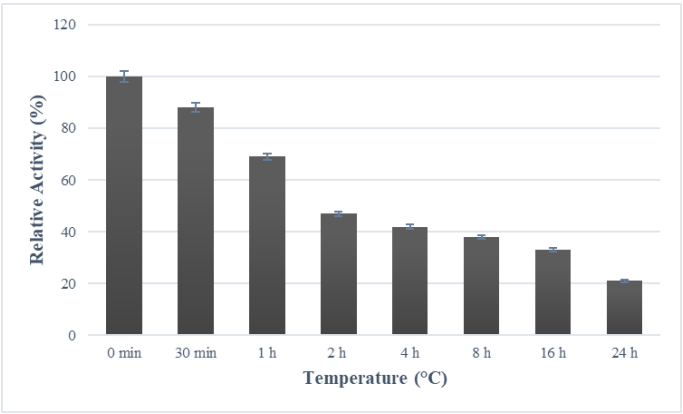

Fig. 10. Temperature stability of *T. hydrothermalis* GKE 08 neopullulanase at 60°C.

and the catalytic efficiency of pullulanase. These findings provide insights into understanding how different metal ions regulate the enzymes' function.

The investigation focused on studying how certain reagents affect the enzymes' performance using pullulan as the material being processed (Table 3). It was observed that when SDS was present, there was a halt in enzyme activity, indicating the inhibitory impact of this substance. On the other hand, when EDTA, a compound known for its ability to bind metal ions was introduced there was a 65% decrease in enzyme activity. This demonstrates the enzymes' susceptibility, to metal ions in the presence of EDTA. However, an interesting discovery was made when urea was introduced. The enzyme exhibited activity levels of around 85%. Urea is commonly used in studies involving changes. Seemed to have a positive impact on enzyme activity in these specific circumstances. The unexpected resilience and enhanced activity raise questions about the stability of the enzyme's structure and its response to substances that modify it. These experiments reveal the detailed nature of interactions between enzymes and specific substances providing insights into their functions. This understanding enhances the comprehension of how enzymes adapt to varying environments opening up applications, in biotechnology and industry.

Table 3
Analysis of the effect of the differences in reagent effects on neopullulanase activity of *T. hydrothermalis* GKE 08.

	Concentration	Relative Activity (%)
EDTA	10 mM	35
SDS	1 mM	0
Urea	0.1 mM	85
Control*	-	100

*The control exhibited 100% activity and contained no reagents. In each case, *p*-values less than 0.05 ($p<0.05$) demonstrated statistical significance.

In a recent study explored the stability and activity of pullulanase derived from *G. stearothermophilus* ADM 11. They focused on how factors like temperature, pH levels, and culture conditions affected the enzymes' performance using pullulan as the substrate. The purified pullulanase exhibited efficiency at 70°C and pH 7.0. Interestingly it remained stable at temperatures up to 90°C showing only a slight 10% decrease in activity at 100°C. The researchers also examined the impact of metal ions on the enzyme activity noting that Ca^{+2} enhanced its function while Sr^{+2} and Ni^{+2} caused deactivation. Mg^{+2} and Mn^{+2} also influenced pullulanase activity to some extent. Additionally, inhibition tests were conducted using substances. The findings revealed that cyclodextrins inhibited the enzyme activity while EDTA and PMSF showed no effect, on its function according to Bukhari et al. (2024).

The study of *B. cereus* strain SDK2 discovered that the presence of calcium ions, Ca^{+2} resulted in an increase, in pullulanase activity within the SDK2 strain (Davaeifar et al., 2015). The inclusion of calcium ions affected how efficiently pullulanase worked in this strain highlighting the role of Ca^{+2} in regulating enzyme performance. This discovery underscores how metal ions interact with enzymes and sheds light on their impact on pullulanase activity in the SDK2 strain. These findings are consistent with a study by Ling et al. (2009) which demonstrated that EDTA hindered pullulanase activity by not forming chelates with divalent cations for enzyme stability and functionality under certain conditions. In their study, they explored how divalent metal ions impact the pullulanase activity produced by *B. cereus* H1.5. The results revealed that, except for Ca^{+2} and Mn^{+2}, the remaining divalent metal ions significantly repressed the activity of the enzyme. Understanding these interactions between EDTA and pullulanase provides insights, for research and practical applications. Similar to this, research findings they noted increased enzyme activity (up to 170%) when Ca^{+2} ions were present leading to improved thermos-activity and thermos-stability. The optimal conditions identified by Ling and colleagues (temperature of 55°C and pH of 6.0) matched with the observations of this study on *T. hydrothermalis* GKE 08 where neopullulanase exhibited peak activity at 60 °C in the existence of Ca^{+2} ions.

The continuous search for effective enzymes in starch

processing, as well as in the food, animal feed, detergent, leather, pulp and paper, textile, medical, and pharmaceutical industries, has prompted extensive investigations into thermophilic or hyper-thermophilic amylolytic microorganisms. The importance of amylolytic enzymes with high thermal stability in the hydrolysis of starch cannot be overstated. The integration of a thermostable neopullulanase, combined with other debranching enzymes and α-glucosidase, presents notable benefits for the starch processing industries. Utilizing collaboration brings benefits improving the efficiency and effectiveness of starch processing. The use of heat, along with neopullulanase enzymes enhances the modification of starch showing promise in transforming practices in the starch processing industry. This advancement is remarkable as it enhances the efficiency, stability, and overall performance of starch hydrolysis processes playing a role in advancing the capabilities and productivity of starch-related sectors. Neopullulanase variants resistant to alkali are utilized in detergent manufacturing.

Furthermore, producing panose with neopullulanase offers an advantage. This is particularly significant as panose is widely recognized as an option in applications. Generating panose with neopullulanase not only increases its versatility but also underscores its impact on gut health and overall, well-being. The association with prebiotic properties positions neopullulanase as a tool for progress in food and nutritional sciences. Diets enriched with bacteria and prebiotic carbohydrates like panose have demonstrated effects on digestive health. Panose, known for its taste possesses qualities that make it an excellent non-cariogenic sweetener for various food applications. Its notable feature lies in its ability to resist fermentation in the mouth leading to health advantages. Panose aids in preventing the formation of sucrose, which helps inhibit bacteria adherence, to tooth surfaces. This versatile feature does not enhance its sweetness. Also highlights its potential as a tooth-friendly sweetener making it an attractive and advantageous option, for various food products. The varied characteristics of panose position it as a choice, for developing tooth sweetener substitutes in the food sector.

4. Conclusion

This study explores the analysis of neopullulanase, from *Thermomonas hydrothermalis* GKE 08 focusing on its production, purification, and enzymatic characteristics. It emphasizes the practicality of this enzyme in applications in processes that involve sugar degradation at elevated temperatures.

The substantial production of pullulanase was successfully accomplished using 1.5% pullulan and 0.5% peptone indicating the regulation of pullulanase production in systems. Among the nitrogen sources investigated peptone was identified as the choice for extracellular pullulanase production. The enzyme from *T. hydrothermalis* GKE 08 exhibited performance at pH 7.0 and a temperature of 55°C aligning with findings from studies on strains like *B. cereus* FDTA 13 (Nair et al., 2007) and *Bacillus halodurans* (Asha et al., 2013). Moreover, this strain displayed growth across a range of pH values (5.0 to 9.0) and temperatures (30 to 50°C) with peak activity observed at pH 7.0 and a temperature of 37°C.

The purification methods employed, such, as acetone precipitation and anion exchange chromatography demonstrated effectiveness in enhancing the purity and activity of neopullulanase. The activity and purification efficiency of the enzyme consistently improved throughout the purification process validating the efficacy of these techniques. The behavior of the enzyme was assessed under conditions including pH levels, time sensitivity and thermal stability. It showed its effectiveness at a temperature of 60°C. It continued to work when subjected to high temperatures for an extended period. The enzyme activity was enhanced in the presence of Ca^{2+} ions whereas ions such, as Cu^{2+}, $Mg^{2+,}$ and Zn^{2+} had varying effects. Additionally, the enzyme reacted differently to substances like urea (which stimulated its activity). SDS/EDTA (which inhibited it) revealing insights, into its characteristics.

Analysis using TLC confirmed that panose was produced as the end product when pullulan was broken down by *T. hydrothermalis* GKE 08 confirming the enzyme as a neopullulanase. Panose's applications as an anticariogenic sweetener highlight the importance of this enzyme in food-related settings.

Future directions may involve genetic engineering strategies to improve neopullulanase production, stability and specificity. Exploring synergies with enzymes or applications in bioremediation processes could open up new avenues for research. This study paves the way for the exploration of neopullulanase offering opportunities for its use, across various biotechnological and industrial fields.

The incorporation of a heat-resistant neopullulanase enzyme, in the processing of starch and various industries could potentially transform the effectiveness and output leading to advancements, in enzyme utilization and industrial biotechnology.

This study sets the stage for the exploration of neopullulanase presenting opportunities for its application across various biotechnological and industrial sectors. Incorporating a heat-resistant neopullulanase enzyme in starch processing and other industries could potentially enhance effectiveness and production output resulting in advancements in enzyme utilization and industrial biotechnology. By leveraging the characteristics of *T. hydrothermalis* GKE 08 neopullulanase industries could achieve sustainable and efficient production processes ultimately driving innovations, in biotechnology and industrial applications.

Conflict of interest: The author declares that she has no conflict of interest.

Informed consent: The author declares that this manuscript did not involve human or animal participants and informed consent was not collected.

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