**Running title:** Studies on the gene of Pullulanase in Nelumbo nucifera and the properties of Pullulanase in Nelumbo nucifera

**Gene cloning and characterization of** **Pullulanase (*PUL*) in** **Nelumbo nucifera**

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**Novelty statement:** In this study, the gene full length, the amino acid sequence and structural characteristics of lotus pullulanase were studied for the first time. We found lotus pullulanase gene expression is higher at the peak period of material accumulation in seeds, and reached its highest level at about 21 days. Lotus pullulanase showed the best enzyme activity at a temperature of 50 °C, which was lower than other reported PULs. Lotus pullulanase's optimal pH is 5.8, which is between the optimum pH of most industrial pullulanase (5.0-7.0).

**Abstract**

Pullulanase is a significant debranching enzyme, has been widely utilized to hydrolyse the α-1,6 glucosidic bonds by converting the branched polysaccharides into small fermentable sugars in the complicated saccharification process in starch, amylopectin, pullulan, and related oligosaccharides. Pullulanase has huge potential on industrial application. The full-length ORF of *NnPUL* is 3232 bp and encoding a protein of 958 amino acids based on the obtained cDNA in this study. Phylogenetic relationship analysis showed the nucleotide sequence of *NnPUL* was highly similar to grape (80%) and oak (79.7%), respectively. Pfam domain analysis showed that the NnPUL protein had four conserved domains. The expression pattern of *NnPUL* was higher at the peak period of material accumulation in seeds, and reached its highest level at about 21 days. The highest activity of pullulanase in lotus was detected at the temperature of 50℃, the pH of 5.8 and substrate concentration of 4.0 mg/ml. The NnPUL as one of starch debranching enzyme genes was identified in this study, and perhaps provided basic information for further research on the regulation of lotus starch content and application directions for the industrial development of lotus pullulanase.

**Keywords:** *NnPUL,* lotus, clone, expression, enzyme activity

**Introduction**

Pullulanase is a significant debranching enzyme, has been widely utilized to hydrolyse the α-1,6 glucosidic bonds in starch, amylopectin, pullulan, and related oligosaccharides by converting the branched polysaccharides into small fermentable sugars in the complicated saccharification process(Hii et al. 2012). To date, five groups of pullulanase enzymes include pullulanase type I, amylopullulanase, neopullulanase, isopullulanase, and pullulan hydrolase type III have been reported(Hii et al. 2012). Recently, *PUL* genes from some microorganism had been cloned and the corresponding enzyme activity were studied, such as *Anoxybacillus* sp. WB42(J. Wang, Liu, and Zhou 2018), *Exiguobacterium acetylicum*(Qiao et al. 2015), *Anaerobranca gottschalkii*(Bertoldo et al. 2004), Geobacillus sp. LM14-3(Sun et al. 2011). However, there are a few studies about *PUL* genes in plants. For example, the PUL gene ZPU1 had been purified in maize in 1999(Beatty et al. 1999), and an *OsPUL* gene in rice during seed development and germination had been reported in 2009(Q.-F. Li et al. 2009). Only one PUL gene has been identified as type I pullulanase in plants, belonging to the glycoside hydrolase 13 family (GHF13), also known as the alpha amylase family. The previously study reported that both isoamylase and pullulanase are involved in the determination of amylopectin structure and belong to debranching enzyme(DBE), and that the loss or reduction in these debranching enzymes affect the phytoglycogen production of amylopectin synthesis(Kubo et al. 1999). The PUL deletion mutants reduced the chain with a DP of 13 to 29, while the short chain with a DP of 12 increased(Fujita et al. 2009). The structure and composition of the endosperm starch of the PUL mutant in maize were not significantly different from the wild type, but the endosperm of the ISA/PUL double mutant accumulated a large amount of plant glycogen. The PUL gene was expressed in the ISA deletion type, which can produce plant glycogen not found in the type, PUL not directly participated in the synthesis of amylopectin, but also showed a partial complementarity between PUL and ISA(Dinges et al. 2003). Different from isoamylase, pullulanase has high industrial value and can work synergistically with other amylases to decompose the smallest unit of amylopectin in the processing industry, so as to maximize the use raw materials of starch and improve starch the utilization rate and industrial production efficiency.

Lotus (Nelumbo nucifera), an important aquatic vegetable, has been cultivated for more than 2000 years in China(Guo 2009). Actually, lotus is considered as a multi-used plant not only used as vegetable and food but also as tea, medicine and so on. Its different parts contain different bioactive substances. The glycoside rich in lotus pollen has strong antioxidant activity and the flavonoids and alkaloids mainly contained in lotus leaves can eliminate free radicals. Its extended products can play a certain role in reducing blood fat, treating cardiovascular diseases and even cancer treatment(Chen, Zhu, and Guo 2019).

The rhizome and seed, the main edible parts of lotus, are rich in starch, protein, vitamins and secondary metabolites(Mukherjee et al. 2010), so it has great potential in the development of functional foods and drugs(Zhang et al. 2015). Lotus seed starch content is very high and changes significantly during growth. It is a good research object for studying starch synthesis and its regulation in plants. Although there are many reports on starch biosynthetic pathways in lotus through analysis of transcriptome, genome, polymorphic markers, and gene identification(J. Li et al. 2018; L. Wang et al. 2016; Cheng et al. 2020; Zhu et al. 2022), little is known about *PUL* gene in lotus.

In this study, the full-length cDNA of the lotus pullulanase gene was obtained and the expression patterns and functions on lotus seed starch synthesis were analyzed. In addition, the [thermostability](javascript:;), pH stability and substrate suitableness of lotus pullulanase was tested. It is the first report on a pullulanase from lotus, and the results can provide more helpful information for the further research and industrial application on pullulanases in the future.

**Method and materials**

*Plant material*

The lotus cultivar “Nelumbo nucifera cv. Taikonglian 36” was used in this study. Seeds were soaking in water for germination for 3~5 days, and then planted in a bowl. The first DAP was the day of planting. Functional leaves, petioles, rhizomes and roots were separately collected in the 8th week, 10th week and 12th week. The different developmental stages of lotus seeds were harvested at August in the Lotus Germplasm Garden of Wuhan University. The seeds were collected at 12 DAF, 16 DAF, 20 DAF, 24 DAF and 28 DAF(days after fertilization) with a four days interval. Various materials from lotus were quickly-frozen in liquid nitrogen and the lotus seeds were immediatly wrapped with tin foil, then all materials were stored in the refrigerator at -80℃ for next manipulation.

*Total RNA extraction and cDNA synthesis*

The total RNA of Lotus was extracted with the total RNA extraction kit (DP441) (TIANGEN BIOTECH CO., LTD. Beijing, China). DNase I was used to remove gDNA contamination. The RNA extraction quality was detected by 1.2% agarose gel electrophoresis. The synthesis of the first strand of cDNA was based on FastKing cDNA kit (de-genomic) (KR116) (TIANGEN BIOTECH CO., LTD. Beijing, China) with a superiority of gDNase digestion prior before reverse transcription and it was beneficial to the follow-up assay such as qRT-PCR and other experiments. The obtained cDNA was stored at -20℃ for later use.

PCR was carried out with KOD-Plus-Neo DNA Polymerase (TOYOBO CO., LTD, Osaka, Japan) with cDNAs as templates. PCR primer (NnPUL forward-5′ CCGTTGCTCTGCTTTAATCTTTC 3′, NnPUL reverse-5′ CGCTCACCAGGAGTCTTTACAAT 3′) was designed from reference sequences’ data in the NCBI. The reaction mixture of cDNA synthesis contained 5 μl of 2 mM dNTPs, 5 μl of 10 × PCR Buffer, 3 μl of 25 mM MgSO4, 2 μl of 10 mM a primer pair, 2 μl of cDNA template, 1 μl of KOD-Plus-Neo (1.0 U/μl) and the last supplementary 31μl of dd H2O with a total volume of 50 μl. Amplification conditions followed the following amplification procedure: 94°C for 2min, 36 cycles of 94 °C for 15s, (Tm)°C for 30s, and 68°C for 2min, finally 68°C for 10min. We used T4 ligase to PCR products clone in pGEM-T vectors (Promega), then transformed to *E. coli* DH5α competent cells (TransGen, Beijing, China). Ten positive clones of E. coli on culture dish were selected for sequencing. The plasmids with *NnPUL* gene were isolated using a plasmid extract kit (Tiangen) and stored at - 20 °C for subsequent application in other experiments.

*Sequence analysis and phylogenetic tree construction*

The protein sequences used in this study were all downloaded and filtered in NCBI database. We used the online program Gene Structure the Display Server 2.0 (http://gsds.cbi.pku.edu.cn/) for genetic structure drawing(Hu et al. 2015). The conserved domain of the target gene was predicted and analyzed using the Pfam Sever online prediction site (http://pfam.xfam.org/search/sequence;) (Finn et al. 2016). Cluxal- X and DNAman were used for sequence alignment(Thompson 1997). The restriction enzyme cutting sites analysis of the target gene was used Bioedit. Phylogenetic Neighbour-joining trees were produced using MEGA6.0 with the Bootstrap value set to 1000(Tamura et al. 2013).

*Expression profile analysis*

The consumables used in the reaction were all RNase free and carried out in a super clean platform. The Tiangen SuperReal fluorescence quantitative premix kit was used in this experiment. The qRT-PCR primer for amplification of *NnPUL* gene was designed from conservative region (forward-5′ ATGGTGGTTATCTTGCTTTC 3′, reverse-5′ CATATCGGTGTCTACGTGCTT 3′). The fluorescence quantitative PCR reaction system with a final volume of 20 μl inclued 10 μl 2×SuperReal PreMix Plus (with) SYBR Green I), 2 μl 50×ROX Reference Dye, 0.6 ul +0.6 ul upstream and downstream primer, 2ul of cDNA template and 4.8ul RNase-free ddH2O for supplementary. *18sRNA* (XM-010264930.1) was selected as reference gene (forward-5′ AGTATCTCGGCAGTTCAGTG 3′, reverse-5′ GCAAATCCAGCACGCATA 3′) for qRT-PCR analysis. The diluted cDNA was used as a template for qRT-PCR reaction on Step One real-time PCR System, and was repeated for 3 techniques. *C*T value of different concentration cDNA was used for standard curve. The amplification efficiency is ((−k) √10 − 1) × 100% (*k* was the slope of standard curve). The reaction procedure was as following: 95℃ for 15min, 40 cycles of 95℃ for 15s and 60℃ for 1min. Three biological repeats along with three technical repeats were conducted in this study. StepOne™ Software v2.3 (Applied Biosystems) was used to resolve the experimental data. The relative expression pattern files of *NnPUL* was calculated by qRT-PCR using the comparative *C*T (2-ΔΔCT) method(Schmittgen and Livak 2008).

*Prokaryotic expression analysis*

NnPUL protein expression assay was selected pET-30a as expression vector with BamHⅠ and SacⅠ restriction enzyme site in the primers (PUL-B1-forward 5′ CGGGATCCATGTCAATTACATCCCCCCT 3′, PUL-S1-reverse 5′ ATGCGAGCTCTCAAATGTCACGACTTTCCAC 3′ the underlined sequences were the restriction enzyme sites). An expression vector of pET-28a-NnPUL was established and BL21 was applied as the expression strain. Monoclonal colonies were selected and inoculated into LB liquid medium containing kanamycin (50 mg/ml), incubated until OD 600 was between 0.6 and 1.0 with the parameter of 200 rpm and 37℃ in shaking table. IPTG with a final concentration of 3mM was added to induce expression at 180rpm and 18℃.

*Enzyme activity and characteristic of NnPUL*

The activity of pullulanase was determined by the DNS method, which measured the content of reducing sugar produced by the pullulanase hydrolyzing the pullulan. The detailed experimental steps represented as following. The bacterial solution with the light absorbance value of about 3.0 at OD 600 was used as the reaction enzyme solution, then we washed them twice with PBS and sucked out the residual liquid as much as possible. Finally, put them in liquid nitrogen and store it at - 80℃ for standby application. The bacteria were suspended in HAC-NaAC (0.05 M, pH=5.8) at a ratio of 2:1, after fully blending, put them quickly frozen in liquid nitrogen, following dissolved at room temperature. Adding the lysozyme with the concentration of 1 mg/ml, then Triton-x-100 was added until the final concentration was 1% at 4℃ for 30 min as reaction enzyme solution. Using HAC-NaAC buffer to prepare 50 mg/ml pullulanose solution as the substrate solution. We get 475 μl reaction enzyme solution (the reaction enzyme solution boiled at high temperature was used as the control) and added 25 μl of the substrate solution for reacting at 50℃ for 30 min. After that, 0.5 ml DNS solution was added and mixed into boiling water for 5 min to conduct color development, then cooling it to room temperature on ice. The absorbance was then measured at OD 540. The requirement of enzyme hydrolyzed pullulanose to produce 1umol glucose per minute was defined as one enzyme activity unit. The glucose standard curve was plotted by the later steps. First of all, we added 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ml 1.0 mg/ml glucose into eight EP tubes respectively, then added ddH2O to 1 ml system. Secondly, 1ml DNS solution was added and mixed uniformly, then the color was developed by boiling water bath for 5min, after cooling to room temperature, the absorbance was measured at wavelengths of 520nm and 540nm. At last, a standard curve with glucose content as the x-coordinate and light absorption value as the y-coordinate was generated. The [computational](javascript:;) [formula](javascript:;): Enzyme activity (U) (A is the absorption value of the test sample; b is the curve intercept; k is the curve slope; T is the reaction time; 180 is the relative molecular weight of glucose;1000 is the conversion rate of mmol to μmol).

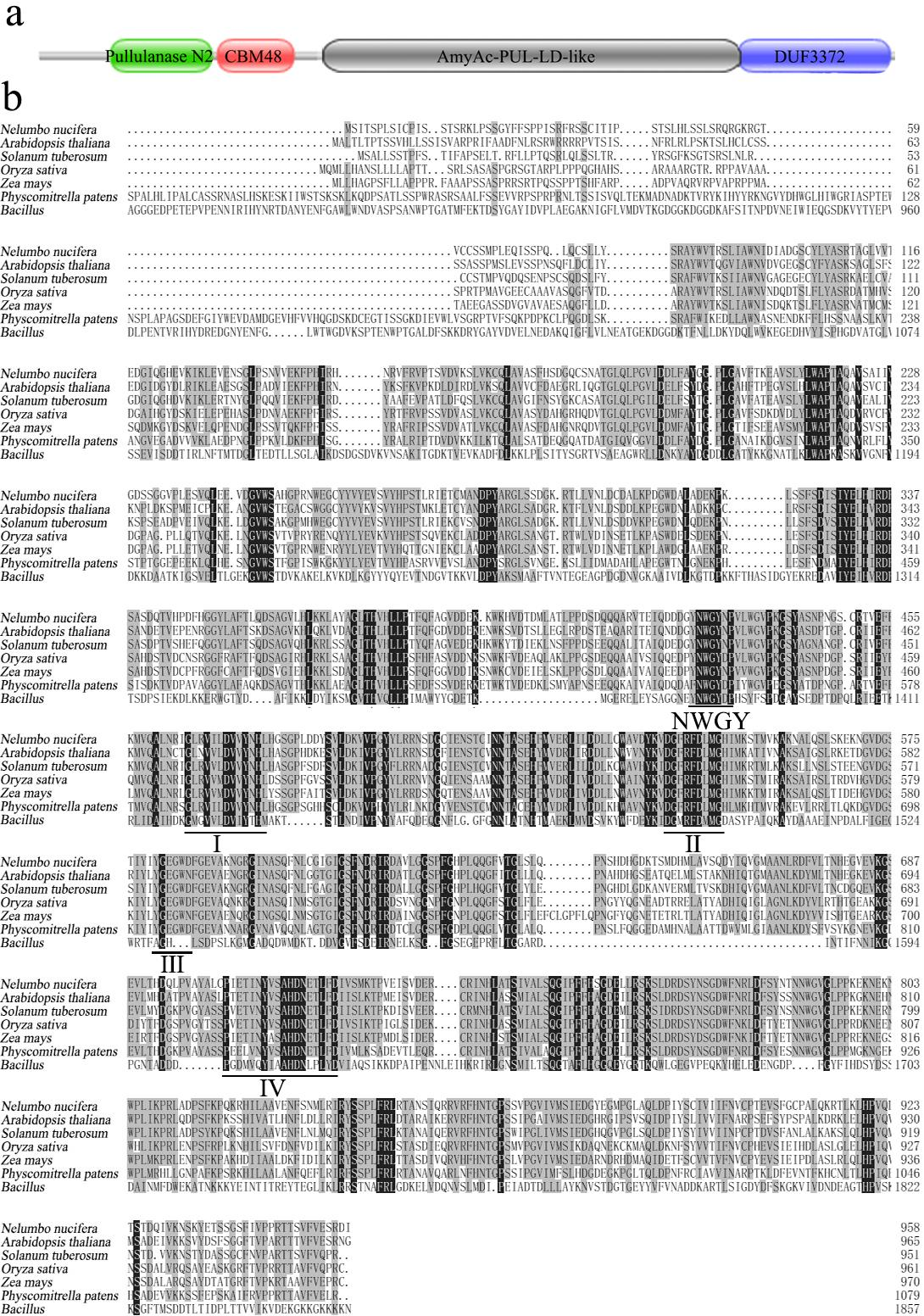
The activity of pullulanase was affected by different pH, reaction temperature and substrate concentration. Therefore, we control variables determine the enzymatic character of PUL. The optimum pH and temperature, as well as Km were aiming to determine. Determination of optimum pH value: different pH values (3.6, 4.2, 4.8, 5.2, 5.6, 5.8, 6.0, 6.4, 6.8, 7.4) buffer solution was used for the preparation of enzyme solution and substrate solution, the reaction was carried out at 50℃. Optimum temperature determination: the substrate concentration was selected 2.5 mg/ml with pH 5.8 at 25, 30, 35, 40, 45, 50, 55, 60, 65, 70℃ for reaction. Substrate concentration reaction determination: the substrate concentrations were 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 mg/ml with pH 5.8 and then reaction at 50℃. The same concentration of substrate solution was used as control, and the Km was calculated by double reciprocal plotting.

**Results**

*Gene clone and sequence analysis*

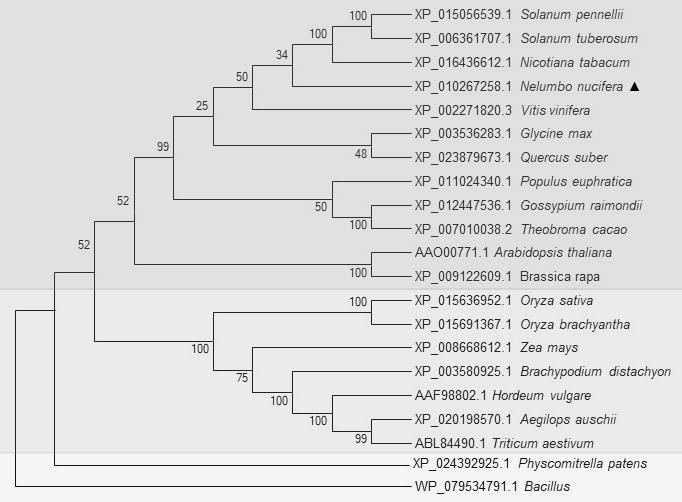
The full-length ORF of NnPUL with a full length of 3232 bp which encodes 958 amino acids based on the obtained cDNA. The nucleotide sequence of *NnPUL* was highly similar to grape (80%) and oak (79.7%), respectively, and the similarity is between 75% and 80%. The similarity of NnPUL protein sequence to grape and oak was 77.7% and 77.6%, and the similarity in the most dicotyledons was ranged from 70% to77.7%.

Pfam domain analysis showed that the NnPUL protein had four conserved domains (Fig. 1a), including the pullulanase N2 domain at the N-terminal (81~194; PUBMED:16650854), the carbohydrate binding activity of CBM48 domain of (201~286;CL0369), amylase catalytic domain of AmyAc - Pullulanase - LD - like (326 ~ 789;CDD:200480) and a domain DUF3372 (786-954;CL0369) with unknown functionality. DNAman was utilized to NnPUL amino acid sequence align with [dicotyledon](javascript:;) (soybean, XP\_003536283.1; Arabidopsis, AAO00771.1), monocotyledons (rice, Xp\_015636952.1; maize, XP\_008668612.1), bryophytes (*Physcomitrium patens*, Xp\_024392925.1) and Bacillus (WP\_079534791.1). The results showed that the PUL proteins were highly conserved among several plants, as well as the motifs and domains had high similarities (Fig. 1b). Seven amino acid compositions (YNWDYNP) were also found in NnPUL and other most Type Ⅰ pullulanase, which was associated with substrate binding and enzyme catalysis. In addition, four highly conserved sequence (I ~ Ⅳ) as a catalytic domain included regional Ⅰ (GLRVILDVVYNHL) associated with the substrate combination of sequence, regional Ⅱ (DGFRFDLMGH), region Ⅲ (YGEGWD) as the enzyme catalysis related sequence and regional Ⅳ (PIETINYVSAHDNETLFD) as the substrate binding and enzymatic catalysis sequences(Domań-Pytka and Bardowski 2004). These regions were identified as the characteristic sequences of catalytic domain and substrate binding domain.



**Figure 1** The functional domains(a) and multiple sequence alignment of PULs.

In order to analyze the classification and evolutionary relationship of NnPUL, we filtered 20 PUL protein sequences from NCBI database to construct the phylogenetic tree, as shown in Fig 2. Evolutionary analysis shows that PUL proteins were divided into three main large branches, including monocotyledons, dicotyledons and other species. *Nelumbo nucifera* was a dicotyledonous plant and NnPUL is closed to the PULs of *Nicotiana tabacum* and *Vitis vinifera*.



**Figure 2** Phylogenetic analysis of the PUL protein. The dendrogram was constructed using

MEGA6.0 software with the neighbor-joining method. Different color regions represent three

subclades of monocotyledons, dicotyledons and others. The NnPUL were labeled with “▲”.

*Expression pattern of NnPUL at seed different developmental stages of fertilization period*

The analysis of the expression pattern during seed development showed that NnPUL gene expression was higher at the peak period of material accumulation in seeds, and reached its highest level at about 21 days (Fig 3).

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**Figure 3** The relative expression of NnPUL in different development stage of seeds (p<0.05).

*Pullulanase characteristic analysis*

The standard curve was yielded by using DNS solution and reducing sugar chromogenic principle, shown in Fig. 4a. The absorbance value increased linearly with the increase of glucose concentration. The regression equation y = 0.336x - 0.0746 was obtained by calculation. The determination coefficient R2 = 0.9936, the regression equation was considered to match the requirements, and could be used to calculate the content of reducing sugar.

The ultimate direction for the pullulanase industrial production mainly covered three aspects including high activity, good thermal stability and pH stability. In this experiment, the NnPUL enzyme activity was different at different temperature, when at 50℃, the enzyme activity was the highest, and the optimum enzyme activity reached 50% at 25℃. However, the enzyme activity decreased sharply with the increase of temperature, the enzyme activity is only 25% at 60℃ as before (Fig. 4b). The pH value of NnPUL optimum enzyme activity was analyzed, and the NnPUL enzyme activity variated in the range of 4.5-7.5. NnPUL was detected to have an optimal pH scope of 6.3-7.5. There was no activity detected basically when the pH was lower than 5.2. The activity could reach to 50% of the highest activity when the pH was reached to 5.8 (Fig. 4c).

The variation of substrate concentration has a huge influence on the enzyme reaction efficiency, and maybe emerged saturation phenomenon. The NnPUL enzyme activity was analyzed in the range of substrate concentration of 0.5-4.5 mg/ml (Fig. 4d). With the increase of substrate concentration, the activity of NnPUL enzyme increased continuously. The fitting curve showed a logarithmic growth pattern. The plateau stage was found at 4 mg/ml of substrate concentration. The fitting curve equation was calculated y=0.7021ln(x) + 0.7619, and the R2 was 0.9878. The Km is the concentration of substrate (S) when the enzymatic reaction reaches half of the maximum velocity (Vm), is an extremely important kinetic parameter. Using double reciprocal plotting method, namely Lineweaver Burk (Lin-Bayes), Km value was determined by equation ( as the x-coordinate and as the y-coordinate), resulted to Lin-Bayes equation y=0.7837x+3871, R2=0.9863 via calculation, which was significantly consistent (Fig. 4e). Finally, the Km value was 2.02, which was consistent with the results in Fig.4d.

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**Figure 4 Characteristic of NnPUL.** (A) Standard curve of glucose. (B) The enzyme activity in different temperatures. (C) The enzyme activity in different pH. (D) Enzymatic reaction rate and substrate concentration. (E) Map of Lineweaver Burk equation.

**Discussion**

Pullulanase is a branching enzyme that is commonly applied to specifically hydrolyze the - 1,6 glycosidic linkages in amylopectin in order to increase the usage of the compound, which is thought to have a large market and many potential uses in starch.

The NnPUL gene that was successfully cloned in this study has high similarity (between 70% and 80%) to other PUL genes. A phylogenetic tree revealed that NnPUL had close genetic relationship with that of other dicotyledons, indicating PUL was conserved among dicotyledons. The amino acid sequence of NnPUL demonstrates that it has a type I pullulanase conserved sequence domain (I, II, III and IV). It takes part in the active site structure of the catalytic domain of glycosyl hydrolase family 13 along with the other four conserved sequence domains. The conservatism of NnPUL is demonstrated by the presence of essential amino acid residues that bind to the substrate and are involved in catalysis, such as Asp and Glu, in its sequence; for the PUL gene in bacteria, Asp and His in the sequence are conservative(Xu et al. 2021).

Because lotus seeds are high in protein and starch and the content changes with the lotus' life cycle, they serve as a useful materials for researching expression pattern of NnPUL. Although the lotus root is rich in amylopectin, the lotus seed is rich in high amylose, but in the total starch, the content of lotus amylopectin accounts for 33.21% of the total starch content, and during the 20-25DAP growth of lotus see, starch and protein are continuously stored in large quantities(L. Wang et al. 2016). The highest expression level of NnPUL in lotus seeds at the 21st DAP may be the accumulation of amylopectin.

The stability and activity of pullulanase are typically regarded as essential features in industrial production. Numerous studies have demonstrated that between 70 and 80 °C is the ideal reaction temperature range for the production of pullulanase genes from the majority of sources in genetic engineering technology(Domań-Pytka and Bardowski 2004). We found NnPUL showed the best enzyme activity at a temperature of 50 °C, which was lower than that of other reported PULs. Lotus is a relic plant of the ice age, and this low reaction temperature of NnPUL might be one of the adaption way for her survival in the cold climate. The fields of detergen, biofuels, and food may have high requirements for this low-temperature, high-activity pullulanase(Thakur et al. 2021). Moreover, for the pullulanase with low reaction temperature, it can save energy in the application process. It needs to improve the thermal stability through modification to increase the reaction time and easy to store. Many low-temperature pullulanase from microorganisms improve their thermal stability and catalytic efficiency through site-directed mutation, which also provides ideas for the transformation of NnPUL(X. Wang, Nie, and Xu 2019). The pullulanase gene's optimal reaction pH, as expressed by genetic engineering technology from a variety of sources, is between 5.0 and 7.0(Xu et al. 2021); NnPUL's optimal pH is 5.8, so it is within the optimal reaction pH range. Due to the requirements of industry, pullulanase also needs to have the property of acid and alkali resistance, so it is also necessary to carry out these modifications on NnPUL. In Li's research(X. Li et al. 2018) on an acidophilic and hyperthermophilic amylopullulanase from *Thermofilum pendens* and in Huang's research(Huang et al. 2020) on *Alcaliberium sp. SL3* pullulanase, the acid and alkali resistance of the pullulanases may be due to the change of conformation caused by the content of acid-base amino acids in the enzyme and the charge of the groups in the protein, forming a highly active structure. For the modification of NnPUL, the acid resistance or alkali resistance can be improved according to the industrial demand and the ideal reaction temperature, so as to produce the required industrial products.

Lotus has been a traditional aquatic plant in China for thousands of years. The primary organs for storing starch are the subterranean stems and seeds. A multitude of enzymes must work together and be controlled for starch to develop. An essential enzyme for enhancing starch storage crops is NnPUL starch debranching enzyme. The starch debranching enzyme gene NnPUL was identified in this study, and the expression mode and function of lotus root starch, as well as preliminary research on the thermal stability, pH stability, and substrate adaptability of pullulanase in lotus, were all described. These findings provided the foundational knowledge for further investigation into the amount of lotus root starch, the transformation of NnPUL enzyme, and its industrial application.

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**Author contribution** Lingling Zhao and Liangbo Yang designed and performed the experiments, Fenglin Zhu drafted the manuscript and conducted the experiment. Teng Cheng, Ye Li and Xingwen Zheng processed the data. Ying Diao, Lingling Zhao and Mengyang Li revised the manuscript.

**Conflict of interest** I declare that the authors have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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