



Research Article

## Three-dimensional structure and functional studies of neopullulanase from *Thermus brockianus*

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### Abstract

The structural guided-rational design has been suggested to meet the demand for pullulanase in starch industries. Neopullulanase (EC 3.2.1.135), a pullulan hydrolase type I belonging to the alpha-amylase family that hydrolysis  $\alpha$ -1,4-glycosidic bonds in pullulan to produce panose is one such enzyme that can be manipulated. Therefore, this study aimed at modeling three 3-dimensional structures of neopullulanase from *Thermus brockianus*, a thermophilic bacteria belonging to the *thermus* genus. The multiple sequence alignment was carried out with Clustal Omega while the domain and 3D structure of this enzyme was predicted with Pfam and four different modeling servers. Model validation was done using SAVES 06 based on the Ramachandran plot and VERIFY3D analysis. YASARA was employed to minimize the energy of the model. PyMOL software was employed to visualize and superimpose the model with the template. The conserved residues and alpha-amylase domain were identified in this enzyme. The RaptorX model was found to be the best with 93.1% residues in the most favoured region based on the Ramachandran plot. The model energy was reduced to -281707.5 kJ/mol with 95.03% of the residues having an average score of 3D-1D score  $\geq 0.2$  in the VERIFY3D analysis. TIM barrel active site fold and the three important catalytic residues; Asp329, Glu360 and Asp431 were also identified. This structure prediction will be helpful in the improvement of neopullulanase for large-scale industrial use.

**Keywords:** Pullulanase, Neopullulanase, *Thermus Brockianus*, Thermophilic Bacteria, Three-Dimensional Structure

### Introduction

Pullulanase (EC.3.2.1.41) is an essential debranching enzyme that cleave the  $\alpha$ -1,6 glycosidic linkages in pullulan, amylopectin, starch, and associated oligosaccharides. In saccharification, these enzymes produce small fermentable sugars from the complete degradation of branched polysaccharides (Hii et al., 2012). Pullulanase and the majority of starch-hydrolyzing enzymes are classified as glycosyl hydrolase family13 (GH13) based on sequence classification (Janeček et al., 2014), while according to their substrate reaction end products and specificity, they are grouped into either pullulanase type I and II or pullulan hydrolase type I, II, and III. pullulanase type I cleave  $\alpha$ -1,6 glycosidic linkages but

cannot cleave  $\alpha$ -1,4 glycosidic linkages whereas pullulanase type II (amylopullulanase) hydrolyze both  $\alpha$ -1,4 and  $\alpha$ -1,6 linkages in pullulan, starch, and associated oligosaccharides.

Pullulan hydrolase type I and II cleaves  $\alpha$ -1,4 glycosidic linkages in pullulan but cannot cleave  $\alpha$ -1,6 glycosidic linkages in this substrate. These hydrolases produce panose and isopanose as end products respectively. Pullulan hydrolase type III is unique as it can cleave both  $\alpha$ -1,4 and  $\alpha$ -1,6 linkages in pullulan and produces a mixture of panose, maltose, and maltotriose (Hii et al., 2012; Niehaus et al., 2000; Nisha & Satyanarayana, 2013; Nisha, 2016).

Neopullulanase (EC 3.2.1.135) (pullulan 4-D-glucanohydrolase) is a pullulan hydrolase type I belonging to the alpha-amylase family. This enzyme hydrolysis  $\alpha$  -1,4-glucosidic bonds in pullulan to form panose (6-alpha-D-glucosylmaltose) (Imanaka & Kuriki, 1989; Roy et al., 2003).

Thermophiles are organisms that thrive at high temperatures between 41°C and 122°C having most species with 80°C as their optimal growth temperature (Madigan et al., 2006). They have been isolated from different environments with high temperatures such as deep-sea hot vents, hot springs, volcanic eruptions, deep-sea hot vents etcetera and produce thermozymes, enzymes with superior conformational structure and adaptive tools due to the presence of a small number of extra hydrogen bonds, hydrophobic interactions, and electrostatic interactions. These enzymes play a significant role in biotechnological, chemical, food, pharmaceutical, pulp, paper and waste-treatment industries (Bruins et al., 2001; Kumar et al., 2019; Sharma et al., 2019; Schäfers et al., 2017). Researchers have isolated and described the activities and structures of neopullulanases from thermophilic bacteria such as *Geobacillus stearothermophilus* TRS40 (Kuriki et al., 1992), *Bacillus stearothermophilus* (Ece et al., 2015; Hondoh et al., 2003; Kamasaka et al., 2002; Kuriki et al., 1988). and *Desulfurococcus mucosus* DSM (Jafari et al., 2022). However, just few of their three-dimensional structure have been reported and none have been reported for *Thermus brockianus*. This organism is also a thermophilic, aerobic, gram-negative bacteria belonging to the *thermus* genus (Williams et al., 1995).

Recently, there is an increased application of pullulanase in starch industries particularly in the production of glucose and the natural microbial pullulanase production is not sufficient to meet this demand (Hii et al., 2012). Structural guided-rational design is one of the genetic modification techniques proved to effectively enhance enzyme yield, catalytic efficiency and thermostability. Enzyme structural information is a requirement for successive structure-guided rational design (Han et al., 2019). In this study, we reported the 3D structure of neopullulanase of *Thermus brockianus* which will be helpful in the improvement of neopullulanase for large-scale industrial use.

**Methods and material**

**Sequence retrieval, analysis and domain prediction**

The amino acid sequence of Neopullulanase from *Thermus brockianus* was retrieved from Uniprot (Boutet et al., 2016) and BLAST against Protein Data Bank (PDB) (Altschul et al., 1997). Multiple sequence alignment was carried out between the query sequence and other sequences in the PDB using Clustal Omega (Sievers et al., 2011). The sequence was further submitted to the Pfam server (Bateman et al., 2004) for domain prediction.

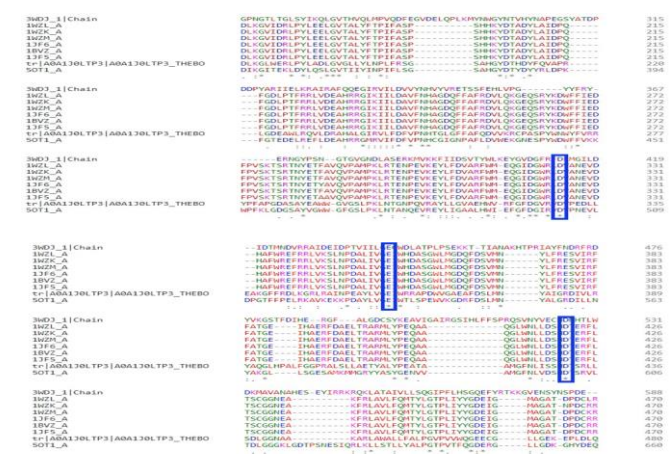
**Homology modelling, validation and visualization**

The three-dimensional structure of the Neopullulanase was modelled employing SWISS-MODEL, RAPTOR X, PHYRE2 and I-TASSER modelling server (Arnold et al., 2006; Källberg et al., 2012; Kelley et al., 2015; Zhang, 2008). The Protein Data Bank (PDB) files generated from the modelling serves were submitted to PROCHECK (Laskowski et al., 2006) for the Ramachandran plot. The best model from the Ramachandran plot analysis was submitted to YASARA server energy minimization (Krieger et al., 2009) and further analyzed using VERIFY3D (Eisenberg et al., 1997). The model visualization, superimposition with the template (PDB ID: 5OT1) and catalytic residues identification was carried out with PyMOL software (DeLano, 2002).

**Results and Discussion**

**Sequence retrieval, analysis and domain prediction**

The blast of the sequence against the protein data bank showed the highest identity of 46.25% with the pullulanase structures in the gene bank. The multiple sequence alignment of the query sequence and eight other sequences (PDB ID: 3WDJ, 1WZL, 1WZK, 1WZM, 1JF6, BVZ, 1JF5 and 5OT1) selected from the blast result is shown in figure 1. Some residues are absolutely conserved (\*), some are conserved (:), and some are semi-conserved (.) Residues are probably conserved in a protein family as they are concerned with structural stability or protein function (Rebehmed et al., 2013). The absolutely conserved residues D, E & D (highlighted in blue color) have been identified as the active site residues in the previous studies of this class of enzymes (Guo et al., 2018; Xu et al., 2014).



**Fig. 1 Multiple sequence alignment of the query sequence of *Thermus bronkianus* with eight other sequences from the blast result. Symbols: absolute conserved (\*), conserved (:), and semi-conserved (.). The active site residues are highlighted (blue).**

Fig. 2 shows the Alpha-amylase domain found in this enzyme which starts from residue 175 and ends at residue 476 (in green color). This result confirmed that this enzyme belongs to the Alpha-amylase family. This family also known as glycosyl hydrolase family 13 is made up of a large number of starch hydrolases and associated enzymes. This family is and consists of about twenty different enzyme specificities sharing numerous common features. They are widespread having bacteria, fungi and plants as the major sources. Examples of these enzymes include Alpha-glucosidase, Neopullulanase, Trehalose-6-phosphate hydrolase, Cyclomaltodextrinase, Alpha-amylase and etcetera (Janecek et al., 1997; Kuriki & Imanaka, 1999; Reddy et al., 2003).



Fig. 2 Alpha-amylase domain (green) found in Neopullulanase of *Thermus brockianus*

Homology modeling, validation and visualization

The stereochemical quality of the models was checked through Ramachandran plot. Ramachandran plot results of the models from RaptorX, Swiss-Model, Phyre2 and I-Tasser have 93.1%, 87.6%, 86.0% and 78.0% residues in the most favored regions respectively. RaptorX model emerge the best as it has > 90% residues in the most favored region (Fig. 3).

YASARA energy minimization server reduced the energy of the initial model from 2.65966911963981e+20 kJ/mol to -281707.5 kJ/mol., naturally, flexibility and function of most proteins is associated with their lower energy profile (Hendlich et al., 1990).

Further validation of this end model from the YASARA with the VERIFY3D server confirmed that 95.03% of the residues

have an average score of 3D-1D score  $\geq 0.2$  (Fig. 4). This high score result indicates the accuracy of the model.

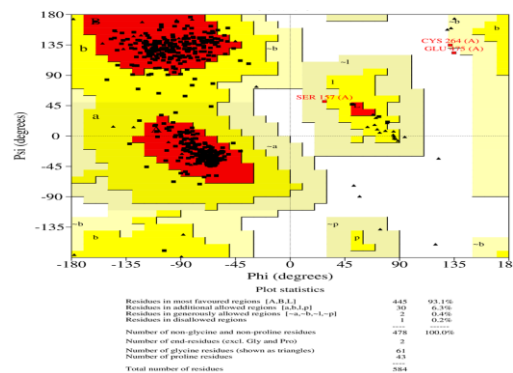


Fig. 3 Ramachandran plot showing the number of residues in the favored, allowed and outlier regions.

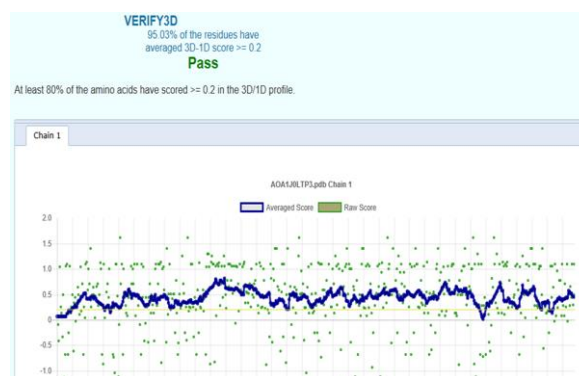


Fig. 4 VERIFY3D result confirming the quality of the model with over 90% of the residue scoring  $\geq 0.2$  in the 3D/1D profile.

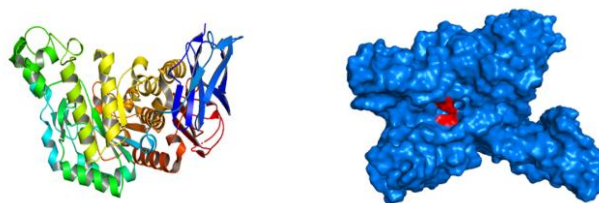


Fig. 5a Cartoon view of the model (b) Surface view of the model with catalytic residues in (red).

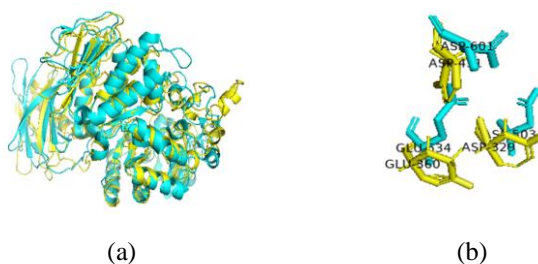


Fig. 6 (a) 3D structure of the model (yellow) superimposed with the template (cyan) (b) Superimposition of catalytic residues of the model and template

Fig. 5a displays the cartoon view of the three-dimensional structure of *Thermus brokianus* Neopullulanase. It consists of mostly alpha-helices, TIM-barrel fold, some  $\beta$ -strands and loops. The catalytic domain of pullulanase have been reported to adopt a TIM-barrel structure (Guo et al., 2018; Xu et al., 2014). The proteins of the  $\alpha$ -amylase family have been mostly observed to have a catalytic ( $\beta/\alpha$ )<sub>8</sub> barrel (Janecek et al., 1997; Kuriki & Imanaka, 1999; Reddy et al., 2003).

Generally,  $\alpha$ -helices have been reported to improve protein structure stability and rigidity more than  $\beta$ -strands and loops. In thermozymes, helices favor arginine and avoid cysteine and histidine in comparison with mesophilic enzymes (Kumar et al., 2000). Arginine guanidinium side chain can form salt bridges that stabilize proteins (Russell et al., 1997).

The surface view of the model with Pymol is shown in Fig. 5b having the catalytic residues highlighted in red color. The superimposition of the model with the template as well as their catalytic residues is shown in Fig. 6a, 6b respectively. The template with PDB Id: 5OT1 was selected in this study due to its appearance in all the modelling servers as template. This template was found to be well aligned with our model having the catalytic residues Asp503, Glu534 and Asp601 (Guo et al., 2018) aligned with Asp329, Glu360 and Asp431 of our model (Fig. 6b). Xu et al. (2014) also found Asp413, Glu442 and Asp526 as catalytic residues in pullulanase of *Anoxybacillus sp. LM18-11*. These three residues have been discovered to catalyze pullulan hydrolysis to panose in the active site of neopullulanase. The first protonation of glycosidic oxygen is done by the glutamate residue carboxyl group via generic acid catalysis. The nucleophilic aspartate residue then attacks the C1 of pullulan while the second aspartate residue carboxylate group deprotonates an adjacent H<sub>2</sub>O molecule to produce a hydroxide ion that hydroxylates at the C1. Total loss of enzymatic activity was observed when these three amino acid residues were mutated in neopullulanase (Svensson, 1994). These three particular residues are important in neopullulanases.

## Conclusion

The conserved residues and alpha-amylase domain identified in this enzyme prove the belonging of this enzyme to the alpha-amylase family. The model from RaptorX is the best model in this study as proved by the model validation tools. The TIM barrel active site fold and the three important catalytic residues in this class of enzymes; two aspartates and a glutamate were also identified. This structure of neopullulanase from *Thermus brokianus* is one of the few predicted so far that can be used in structural guided-rational design to enhance the enzyme's yield, catalytic efficiency and thermostability which will in turn improve this class of enzymes for a large-scale industrial use.

## Conflict of Interest

The author hereby declares no conflict of interest.

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## References

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25(17), 3389–3402.
- Arnold, K., Bordoli, L., Kopp, J., & Schwede, T. (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics*, 22(2), 195–201.
- Bateman, A., Coin, L., Durbin, R., Finn, R. D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E. L. L., Studholme D. J., Yeats, C. & Eddy, S. R. (2004). The Pfam protein families database. *Nucleic Acids Research*, 32(suppl\_1), D138–D141.
- Boutet, E., Lieberherr, D., Tognolli, M., Schneider, M., Bansal, P., Bridge, A. J., Poux, S., Bougueleret, L., & Xenarios, I. (2016). UniProtKB/Swiss-Prot, the manually annotated section of the UniProt KnowledgeBase: how to use the entry view. In *Plant Bioinformatics* (pp. 23–54). Springer.
- Bruins, M. E., Janssen, A. E. M., & Boom, R. M. (2001). Thermozymes and their applications. *Applied Biochemistry and Biotechnology*, 90(2), 155–186.
- DeLano, W. L. (2002). Pymol: An open-source molecular graphics tool. *CCP4 Newsl. Protein Crystallogr.*, 40(1), 82–92.
- Ece, S., Evran, S., Janda, J.-O., Merkl, R., & Sterner, R. (2015). Improving thermal and detergent stability of *Bacillus stearothermophilus* neopullulanase by rational enzyme design. *Protein Engineering, Design and Selection*, 28(6), 147–151.
- Eisenberg, D., Lüthy, R., & Bowie, J. U. (1997). [20] VERIFY3D: assessment of protein models with three-dimensional profiles. In *Methods in enzymology* (Vol. 277, pp. 396–404). Elsevier.
- Guo, J., Coker, A. R., Wood, S. P., Cooper, J. B., Keegan, R. M., Ahmad, N., Muhammad, M. A., Rashid, N., & Akhtar, M. (2018). Structure and function of the type III pullulan hydrolase from *Thermococcus kodakarensis*. *Acta Crystallographica Section D: Structural Biology*, 74(4), 305–314.
- Han, H., Ling, Z., Khan, A., Virk, A. K., Kulshrestha, S., & Li, X. (2019). Improvements of thermophilic enzymes: From genetic modifications to applications. *Bioresource Technology*, 279, 350–361.
- Hendlich, M., Lackner, P., Weitckus, S., Floeckner, H., Froschauer, R., Gottsbacher, K., Casari, G., & Sippl, M. J. (1990). Identification of native protein folds amongst a large number of incorrect models: the calculation of low energy conformations from potentials of mean force. *Journal of Molecular Biology*, 216(1), 167–180.
- Hii, S. L., Tan, J. S., Ling, T. C., & Ariff, A. Bin. (2012). Pullulanase: role in starch hydrolysis and potential industrial applications. *Enzyme Research*, 2012, 1–14.
- Hondoh, H., Kuriki, T., & Matsuura, Y. (2003). Three-dimensional structure and substrate binding of *Bacillus stearothermophilus* neopullulanase. *Journal of Molecular Biology*, 326(1), 177–188.
- Imanaka, T., & Kuriki, T. (1989). Pattern of action of *Bacillus stearothermophilus* neopullulanase on pullulan. *Journal of Bacteriology*, 171(1), 369–374.
- Jafari, F., Kiani-Ghaleh, F., Eftekhari, S., Razlighi, M. R., Nazari, N., Hajirajabi, M., Sarvestani, F. M., & Sharafieh, G. (2022) Cloning, overexpression, and structural characterization of a novel archaeal thermostable neopullulanase from *Desulfurococcus mucosus* DSM 2162. *Prep. Biochem. Biotechnol.* 1-12
- Janecek, S., Svensson, B., & Henrissat, B. (1997). Domain evolution in the  $\alpha$ -amylase family. *Journal of Molecular Evolution*, 45(3), 322–331.
- Janeček, Š., Svensson, B., & MacGregor, E. (2014).  $\alpha$ -Amylase: an enzyme specificity found in various families of glycoside hydrolases. *Cellular and Molecular Life Sciences*, 71(7), 1149–1170.
- Källberg, M., Wang, H., Wang, S., Peng, J., Wang, Z., Lu, H., & Xu, J. (2012). Template-based protein structure modeling using the RaptorX web server. *Nature Protocols*, 7(8), 1511–1522.

- Kamasaka, H., Sugimoto, K., Takata, H., Nishimura, T., & Kuriki, T. (2002). *Bacillus stearothermophilus* neopullulanase selective hydrolysis of to maltose in the presence of amylopectin. *Applied and Environmental Microbiology*, 68(4), 1658–1664.
- Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. E. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols*, 10(6), 845–858.
- Krieger, E., Joo, K., Lee, J., Lee, J., Raman, S., Thompson, J., Tyka, M., Baker, D., & Karplus, K. (2009). Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: Four approaches that performed well in CASP8. *Proteins: Structure, Function, and Bioinformatics*, 77(S9), 114–122.
- Kumar, Sandeep, Tsai, C.-J., & Nussinov, R. (2000). Factors enhancing protein thermostability. *Protein Engineering*, 13(3), 179–191.
- Kumar, Sumit, Dangi, A. K., Shukla, P., Baishya, D., & Khare, S. K. (2019). Thermozymes: adaptive strategies and tools for their biotechnological applications. *Bioresour Technol*, 278, 372–382.
- Kuriki, T., & Imanaka, T. (1999). The concept of the  $\alpha$ -amylase family: structural similarity and common catalytic mechanism. *Journal of Bioscience and Bioengineering*, 87(5), 557–565.
- Kuriki, T., Okada, S., & Imanaka, T. (1988). New type of pullulanase from *Bacillus stearothermophilus* and molecular cloning and expression of the gene in *Bacillus subtilis*. *Journal of Bacteriology*, 170(4), 1554–1559.
- Kuriki, T., Tsuda, M., & Imanaka, T. (1992). Continuous production of panose by immobilized neopullulanase. *Journal of Fermentation and Bioengineering*, 73(3), 198–202.
- Laskowski, R. A., MacArthur, M. W., & Thornton, J. M. (2006). PROCHECK: validation of protein-structure coordinates.
- Madigan, M. T., Martinko, J. M., & Parker, J. (2006). *Brock biology of microorganisms* (Vol. 11). Pearson Prentice Hall Upper Saddle River, NJ.
- Niehaus, F., Peters, A., Groudieva, T., & Antranikian, G. (2000). Cloning, expression and biochemical characterisation of a unique thermostable pullulan-hydrolysing enzyme from the hyperthermophilic archaeon *Thermococcus aggregans*. *FEMS Microbiology Letters*, 190(2), 223–229.
- Nisha, M., & Satyanarayana, T. (2013). Characterization of recombinant amylopullulanase (gt-apu) and truncated amylopullulanase (gt-apuT) of the extreme thermophile *Geobacillus thermoleovorans* NP33 and their action in starch saccharification. *Applied Microbiology and Biotechnology*, 97(14), 6279–6292.
- Nisha, M., & Satyanarayana, T. (2016). Characteristics, protein engineering and applications of microbial thermostable pullulanases and pullulan hydrolases. *Applied Microbiology and Biotechnology*, 100(13), 5661–5679.
- amylose
- Rebehmed, J., Alphand, V., De Berardinis, V., & de Brevern, A. G. (2013). Evolution study of the Baeyer–Villiger monooxygenases enzyme family: Functional importance of the highly conserved residues. *Biochimie*, 95(7), 1394–1402.
- Reddy, N. S., Nimmagadda, A., & Rao, K. R. S. S. (2003). An overview of the microbial  $\alpha$ -amylase family. *African Journal of Biotechnology*, 2(12), 645–648.
- Roy, A., Messaoud, E. Ben, & Bejar, S. (2003). Isolation and purification of an acidic pullulanase type II from newly isolated *Bacillus* sp. US149. *Enzyme and Microbial Technology*, 33(5), 720–724.
- Russell, R. J. M., Ferguson, J. M. C., Hough, D. W., Danson, M. J., & Taylor, G. L. (1997). The crystal structure of citrate synthase from the hyperthermophilic archaeon *Pyrococcus furiosus* at 1.9 Å resolution. *Biochemistry*, 36(33), 9983–9994.
- Schäfers, C., Blank, S., Wiebusch, S., Elleuche, S., & Antranikian, G. (2017). Complete genome sequence of *Thermus brockianus* GE-1 reveals key enzymes of xylan/xylose metabolism. *Standards in Genomic Sciences*, 12(1), 1–9.
- Sharma, S. Vaid, S. Bhat, B. Singh, S., & Bajaj, B. K. (2019) Thermostable enzymes for industrial biotechnology, in: advances in enzyme technology, Elsevier, pp. 469–495.
- Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., & Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, 7(1), 539.
- Svensson, B. (1994). Protein engineering in the  $\alpha$ -amylase family: catalytic mechanism, substrate specificity, and stability. *Plant Molecular Biology*, 25(2), 141–157.
- Williams, R. A. D., Smith, K. E., Welch, S. G., Micallef, J., & Sharp, R. J. (1995). DNA relatedness of *Thermus* strains, description of *Thermus brockianus* sp. nov., and proposal to reestablish *Thermus thermophilus* (Oshima and Imahori). *International Journal of Systematic and Evolutionary Microbiology*, 45(3), 495–499.
- Xu, J., Ren, F., Huang, C.-H., Zheng, Y., Zhen, J., Sun, H., Ko, T.-P., He, M., Chen, C.-C., Chan, H.-C., Guo, R. T., Song, H., & Ma, Y. (2014). Functional and structural studies of pullulanase from *Anoxybacillus* sp. LM18-11. *Proteins: Structure, Function, and Bioinformatics*, 82(9), 1685–1693.
- Zhang, Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics*, 9(1), 1–8.

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