A lipase gene of Thermomyces lanuginosus: sequence analysis and high-efficiency expression in Pichia pastoris

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Article

Keywords: Thermomyces lanuginosus, Pichia pastoris, lipase, gene clone, sequence analysis

Posted Date: July 20th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3130261/v1

Abstract

Lipase is a type of enzyme that decomposes and synthesizes triglyceride on hydrophilic and lipophilic interface, which plays an important role in lipid processing. A novel heat-resisting lipase gene (*lip*4) in *Thermomyces lanuginosus* was cloned to the expression vector pPICZαA and then transported into *Pichia pastoris* X33 for high-efficiency expression. The structure of this lipase (Lip4) was analyzed by multiple bioinformatics software. Results showed that the recombinant yeast cell concentration reached the peak at 144h and the lipase activity reached the maximum (3900U/mL) at 168h in the induction. S168, D223 and H280 form the triplet structure of the Lip4 and S168 is also involved in the pentapeptide structure of G166-H167-S168-L169-G170. Furthermore, S168 also constituted the active center of Lip4 with seven other conserved amino acids, such as G104/288, S105, A195, P196, V225, and I287. Specifically, the two α-helices of the lid structure outside the active pocket control the entry of the substrate. Therefore, the eukaryotic system was constructed to express Lip4 efficiently, and the amino acid sites related to the catalytic efficiency of the Lip4 were clarified, providing a theoretical basis for its subsequent property research and industrial application.

Introduction

Lipase [E.C. 3.1.1.3], called triacylglycerol acylhydrolase, is a hydrolytic enzyme which acts on carboxylate bond. Lipase is a kind of serine hydrolase and doesn't need any cofactor. It releases diglycerol, monoglycerol, glycerol and fatty acids by catalyzing hydrolysis of ester bonds of triacylglycerol[1,2]. Compared with chemical catalytic pathway, lipase catalysis is environmentally friendly and sustainable, and can be widely used in textile, paper, food, bioenergy, chemical and detergent industries[3]. Lipase comes from animals, plants and microorganism. Traditionally, lipase has been acquired from animal pancreas and used as digestive supplement in the roughing or purification form[4]. Compared with lipases from animals and plants, microbiota secreted lipases have a wide substrate specificity, wider range of operating temperature and pH, and have been used to improve dietary fat digestion in patients with congenital pancreatic triglyceride lipase deficiency. Moreover, they are used to decompose highly diverse racemic mixtures to produce important enantiomer drugs such as (R) - glycinol[5].

The first commercially available recombinant lipase obtained from the fungus *T. lanuginosus* has been expressed in *Aspergillus oryzae* since 1994[6], large-scale lipase production has come from genera including *Bacillus*[7,8], *Pseudomonas*, *Staphylococcus*, etc[9,10]. With the extensive application of lipase, it also faces some shortages. The production cost and enzymatic characteristics of lipase are main constraints against the commercialization of the lipase catalysis technique. Therefore, existing production of lipase cannot meet the continuously increasing growth of the bioindustry. So far, many strategies have been developed to optimize production of lipase in order to meet commercial demands, including improving enzyme production in a new system[11] improving purification efficiency and effect by molecular modification[12], studying the structure-function relations (such as high temperature, pH, and

activity and stability in organic solvent environment) and catalytic mechanism by using the point mutagenesis[13–16].

According to existing reports, lipase from *T. lanuginosus* has good application characteristics[17]. We will obtain the lipase gene (*lip*4) from *T. lanuginosus* by homologous cloning, express lipase (Lip4) efficiently by constructing a eukaryotic expression system, and analyze its protein structure, which lays a foundation for further study on the properties and functions of Lip4 and its molecular modification.

Results and analysis

Construction of recombinant P. pastoris containing lipase gene lip 4

The genomic cDNA of *T. lanuginosus* was used as the template and the designed primers were applied for PCR amplification of lipase gene *lip*4. This target fragment was purified and cloned to the expression vector pPICZαA to get the recombinant plasmid. The linearized plasmid was transformed into the component cell of yeast. Several recombinant strains in good growth conditions were screened by LB nutrition agar media containing high-concentration bleomycin. Lipase gene *lip*4 were verified using the universal primer AOX1 with recombinant genomic DNA as a template (Fig. 1).

Expression, purification and enzymatic activity assay of lipase Lip4

The extracellular lipase activity of pPICZαA-lip4/X33 in shake flask fermentation can reach to 423-500U/mL. An initial fermentation volume of 4L was conducted in a 10L fermentation tank, and the key parameters such as dissolved oxygen and feeding were strictly controlled. The wet weight and extracellular lipase activity of recombinant *P. pastoris* were detection in the fermentation of 192h (Fig. 2). The results showed that the wet weight and extracellular lipase activity of recombinant *P. pastoris* increased with the prolongation of fermentation time; the wet weight of the bacteria reached the maximum, 119.5g/L in fermentation of 144h and the extracellular lipase activity reached a peak of 3900 U/mL in fermentation of 168 h.

Pure lipase Lip4 was acquired from fermentation liquid by ammonium sulfate precipitation and Ni-NTA affinity chromatography in succession, and assayed by SDS-PAGE and western blot (Fig. 3). The results showed molecular weight of recombinant lipase Lip4 expressed after induction was about 35kD, slightly higher than the predicted molecular weight, which may be caused by adding 6×His tag into the recombinant lipase Lip4 or protein modification, such as glycosylation, phosphorylation, etc.

Sequence and structure analysis of lipase Lip4

A phylogenetic tree was constructed by Lip4 and other lipase sequences from microorganisms in GenBank, and lipases showed typical species specificity (Fig. 4). According to multi-sequence alignment,

6 cysteine of lipase Lip4 form three pairs of disulfide bonds, and conserved peptide acid segments are formed at G134-F135, G166-H167-S168-L169-G170 and P196-R197-V198-G199-N200 (Fig. 5).

The three-dimensional structure of lipase Lip4 is predicted to find the key amino acid sites or peptide segments. The results showed S168, D223 and H280 formed the triplet structure of lipase Lip4. The serine and other four peptide acid residues form the pentapeptide structure G166-H167-S168-L169-G170, which plays an important role in the catalysis function of lipase (Fig. 6A). Serine and other seven conserved amino acids (G104/288, S105, A195, P196, V225 and I287) form the active center of lipase Lip4 (Fig. 6B). The lid structure on the surface of the substrate binding pocket is composed of two α-helics, which can control the substrate entering the active pocket and binding to the catalytic site (Fig. 6C).

Discussions

Enzymatic hydrolysis of lipase, with advantage of selective esterification, hydrolysis and transesterification, has become preferred method for industrial process of fatty acids due to the environmental protection, mild reaction conditions and specificity[18]. The lipase from *T. lanuginosus* is a potential thermostability lipase and it can facilitate enzymatic hydrolysis under high temperature, thus decreasing viscosity of substrate and increasing solubility of substrate, diffusion coefficient and reaction rate. Hence, lipase Lip4 is a good potential industrial biocatalyst.

Enzymatic characteristics of lipase, such as enzyme activity, optimum temperature, thermostability, pH stability and substrate selectivity etc., were improved by protein engineering measures[19,20]. Protein engineering measures introducing amino acid site mutations or peptide insertion/deletion involved in directed evolution, which is to simulate the Darwin evolutionary process under laboratory conditions, artificially introduce in abundant mutations through random mutation and recombination, and construct the gene mutation library of target proteins. Ep-PCR and Site-directed mutagenesis were applied to improve properties of lipase. Niu et al[21] reported a lipase mutant from *Rhizopus arrhizus* with substitutions of three amino acids through Ep-PCR, whose thermostability and the optimum temperature were greatly improved. Wang et al. discovered a key area on the leading peptide through molecular MD simulation (Val5-Thr10) and carried out orthogenesis in this area through Ep-PCR and Site-directed mutagenesis, thus the catalysis efficiency of lipase was increased by 7 times after mutation[22]. As multiple mutation methods introduce in enough mutation diversity, the main constraint of orthogenesis experiment turns to screening mutant conforming to the purpose from mutant library. To achieve fast and efficient screening from a huge mutant library, a series of new screening and selection methods have developed, including flow cytometry sorting, high-performance liquid chromatography (HPLC), mass spectrum, and other high-flux automatic screening methods, thus increase opportunities of the target characteristics and save time and cost significantly. Therefore, orthogenesis becomes an effective research method in enzyme engineering and extensively applied to modification of industrial enzyme[23–26].

Lipase Lip4 is going to be modified according to its structural characteristics. The hydrophobicity and electrostatic area, where substrate combines the active pocket, will be changed to improve catalytic activity. Binding affinity of lipase Lip4 and substrate will be strengthened by replacing amino acid residues with stronger hydrophobicity and different charges in the following research. According to its structure and molecular docking results with different fatty acids, the relationship between charge changes and substrate bonding is disclosed. The improvement of thermostability observes to the principle of increasing rigidity of local peptide fragment and the thermostability of the whole lipase Lip4 is improved by introducing in disulfide bond and tempering secondary structure. Moreover, existing technical means are going to be improved by combining with catalytic characteristics of lipase Lip4. Attentions will be paid to develop and optimize more efficient library construction and screening method.

Conclusions

The heat-resisting lipase gene (*lip*4) from *T. lanuginosus* is cloned to the expression vector pPICZαA and then transformed into *P. pastoris* X33 for high-efficiency expression. The yeast cell concentration reaches the peak at 144h of induction culture and the lipase activity reaches the peak (3900U/mL) at 168h. Moreover, amino acid sequences and characterization structures revealed S168, D223 and H280 formed the triplet structure of the Lip4. Specifically, serine and other four residues formed the pentapeptide structure G166-H167-S168-L169-G170, playing an important role in the catalysis function of Lip4. Moreover, serine forms the active pocket with seven conserved amino acids, including G104/288, S105, A195, P196, V225 and I287. The lid structure on the surface of active pocket can prevent the binding of substrate with catalytic sites by keeping it out of the active pocket.

Materials and methods

Strain and reagent

T. lanuginosus was screened and preserved by functional fiber team of Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences. The expression vector pPICZαA, *P. pastoris* X33, methyl alcohol, Speedy Cut SacI, Ni-NTA and IgG were bought from Sangon (Shanghai, China). *E. coli* TOP10, Ultra HiFidelity PCR Kit, RNAsimple total RNA extraction kit, TIANScript RT Kit, agarose gel DNA recycle kit and plasmid extraction kit were bought from Tiangen (Beijing, China). The primer synthesis and DNA sequencing were completed by Tsingke Biotechnology Co., Ltd (Changsha, China). Other pure biochemical reagents for analysis were purchased from Sinopharm Group (Shanghai, China).

Extraction of genome DNA from T. lanuginosus

A small amount of mycelium was inoculated into the 50mL LB liquid media. It was cultured overnight under 30℃ and 150r/min, and then centrifuged to collect bacterial cells. The total RNA was extracted according to instruction of RNAsimple total RNA extraction kit, and then transformed into cDNA through

reverse transcription according to the instruction of TIANScript RT Kit. The cDNA was kept under − 20℃ for use.

Cloning of lipase gene lip 4

Primers were designed according to lipase gene *lip*4 of *T. lanuginosus* in the NCBI database (GenBank accession number: OP222029). The upstream primer F: 5'-CGAATTCGAGAAAAGAAGTCCTATTCGTCGAGAGGT-3' (including the restriction enzyme sites of *Eco*R I and Kex2). The downstream primer R: 5'-CGCGGCCGCGAATTCGTGGTGGTGGTGGTGGTGGTCGACCCCTGTACAGAAATC-3' (including the restriction enzyme sites of *Not* I and *Sac* I and His tag). PCR amplification was performed using cDNA of *T. lanuginosus* as the template. The PCR reaction system was composed of 1μL (about 50ng) of cDNA, 2μL upstream and downstream primers, respectively, 2×Pfu Master Mix 50μL, and supplement ddH_2O to the total volume of 100μL. PCR reaction conditions included 5min of predegeneration under 94℃, 50s of degeneration under 94℃, 50s of annealing under 58℃ and 1min of extension under 72℃, 32 cycles; 10min of extension under 72℃. PCR products were successively identified and purified by 1% agarose gel electrophoresis, and then preserved under − 20℃.

Construction of the recombinant vector pPICZαA-lip4

The purified pPICZαA plasmid and the lipase gene *lip*4, digested by *Eco*R I *and Not* I, were connected by T_4 DNA ligase overnight under 16℃, so recombinant plasmids were obtained. Next, the recombinant plasmids were transformed to *E. coli* Top10 through heat-shock method and coated onto the LB nutrition agar containing 25μg/mL Zeocin, culturing overnight. Positive clones were screened and cultured in LB broth overnight to extract recombinant plasmids for PCR identification. The positive plasmids were submitted to Tsingke Biotechnology Co., Ltd for sequencing.

Construction and induced expression of recombinant P. pastoris

Accurate recombinant plasmids were linearized with *Sac* and mixed with the competent cells of *P. pastoris* X33 at the volume ratio of 1:8. The mixture was transformed into a pre-cooled electric cup for 5min of ice bath, followed by 5ms of electric shock. Next, the pre-cooled sorbitol was added immediately. Additionally, the vector without lipase gene *lip*4 was transformed to *P. pastoris* X33, as a negative control group. The solution after electroporation was incubated in a constant-temperature (30℃) for 2h and then centrifuged. The bacterial solution was spread onto the YPDS nutrient agar medium (containing 100μg/mL Zeocin) and cultured in 30℃. Next, the monoclone was detected by PCR, and the PCR primer was AOX-1. The positive clone was inoculated into BMGY nutrient medium, and cultured under 28.5℃ until the OD_{600} reached to 2–6. The BMMY nutrient medium was applied for induction by supplementing 1% methyl alcohol, and then cultured at 28.5℃ for 168h. Extracellular lipase activity in the shaker was determined.

To further determine its lipase-producing ability, 10L fermenter was used for extended fermentation. The initial fermentation volume was 4L, and after sterilization, it was cooled and maintained at 28℃and the pH value was adjusted to 6.0 with ammonia water. The seeds were inoculated with 10% (400mL) of YPD for 24h. After fermentation for 24h, the glycerol in the basal medium was basically exhausted, and the glycerol fed-batch phase began. After 8-12h incubation, when the OD_{600} was about 150, the glycerol in the fermentation broth was almost exhausted when glycerol was stopped. The amount of dissolved oxygen to be increased to more than 50%. Feed was added in the ratio of 100% methanol to 12ml micronutrient, and the methanol induction phase was initiated. During the fermentation process, the dissolved oxygen is ensured to exceed 20%. Under this condition, the fermentation time was 192h, and the wet weight and lipase activity were measured by samples taking every 24h. The total fermentation time was 192h.

Lipase activity detection

The fermentation liquor of engineering strain pPICZαA-lip4/X33 with introduction was collected and centrifuged at the rate of 12000r/min for 5min. Supernate (500mL) was collected and purified by ammonium sulfate precipitation and Ni-NTA affinity chromatography sequentially. 20μL 5×Loading Buffer was added to 80μL purified Lip4, boiling in water bath for 10min. The protein secretory expression and purification was tested by SDS-PAGE and western blot. The fermented liquid of engineering strain pPICZαA-lip4/X33 without introduction was treated by the same way and used as the negative control group.

Lipase activity was detected according to the *p*-nitrophenol (*p*-NP) method[27]. The *p*-nitrophenol phosphate was dissolved in isopropanol to prepare 1mM of the substrate solution A. The enzyme solution was diluted to the solution B with appropriate concentration by using pH8.8 100mM Tris-Cl buffer. The solution A and solution B were mixed uniformly after pre-heating under 37℃ and then reacted for 5min under 37℃. After finishing the reaction, 100μL 10%SDS was added into samples immediately to terminate the enzymatic reaction. The lipase activity unit was calculated according to the *p*-NP standard curve by testing the absorbance OD_{405}. The unit enzyme activity (U) is defined as the enzyme volume needed to release 1 μmol *p*-NP per minute.

Bioinformatics analysis

The basic physical and chemical properties of Lip4 were analyzed by the bioinformatics website Expasy (https://web.expasy.org/protparam/), the signal peptides were predicted by SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/), and the domains of Lip4 were analyzed by Pfam (http://Pfam.xfam.org/search/sequence).

Cluster analysis of lipase amino acid sequences was performed using the bioinformatics software Mega 7.0. The secondary structure of Lip4 was predicted by computer online software PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/); Homologous modeling was conducted by the Swiss Bioinformatics

Center online tool SWISS-MODEL (https://swissmodel.expasy.org/) for Lip4, VMD was used for mapping analysis, and Verify-3D was used to score the model.

Declarations

Acknowledgments

We are grateful for the financial support by the Natural Science Foundation of Hunan Province (2023JJ50315 and 2023JJ30621), Natural Science Foundation of Changsha City (kq2208247 and kq2208250), the Science and Technology Innovation Program of Hunan Province (2022RC3059), Agriculture Research System of China (CARS-19-E22), Chinese Agricultural Science and Technology Innovation Project (ASTIP-IBFC-05), Yuelu Youth Funds of IBFC (IBFC-YLQN-202102), Regional Collaborative Innovation Special Project of Xinjiang Uygur Autonomous Region (2022E02105), Central Public-interest Scientific Institution Basal Research Fund (Y2021YJ25 and 1610242023005).

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Lipase gene lip4 of T. lanuginosus has been deposited in the NCBI database. Repository: GenBank. Accession number: OP222029. Link: https://www.ncbi.nlm.nih.gov/nuccore/OP222029.

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Figures

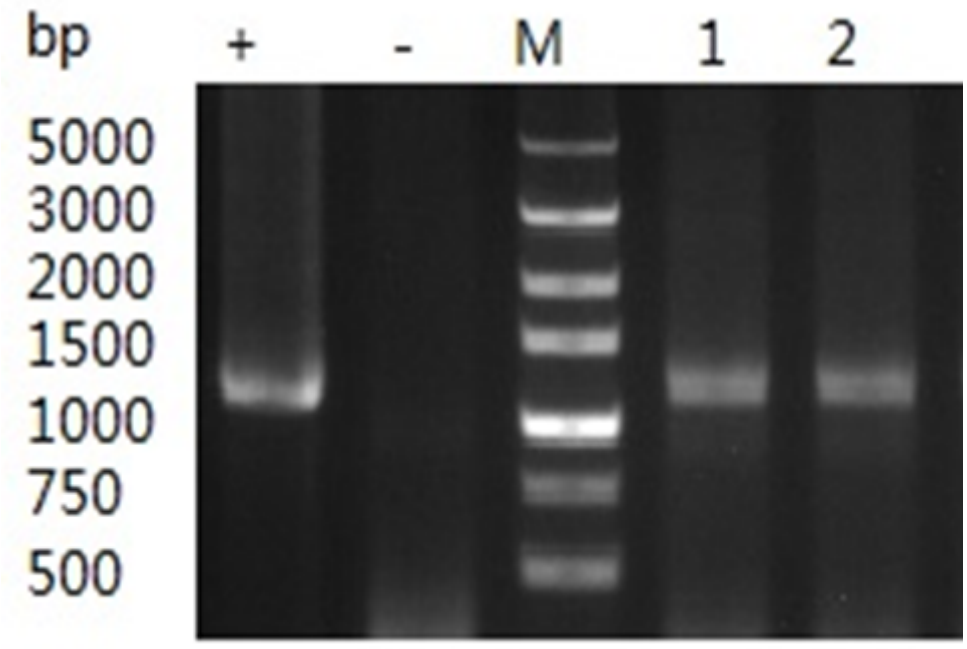

Figure 1

PCR amplification of lipase gene *lip*4

M Marker 1-2 Lipase genes *lip*4 amplified using recombinants as templates; -: Negative control (ddH_2O as template); +: Positive control (a known DNA fragment matched by the kit as template).

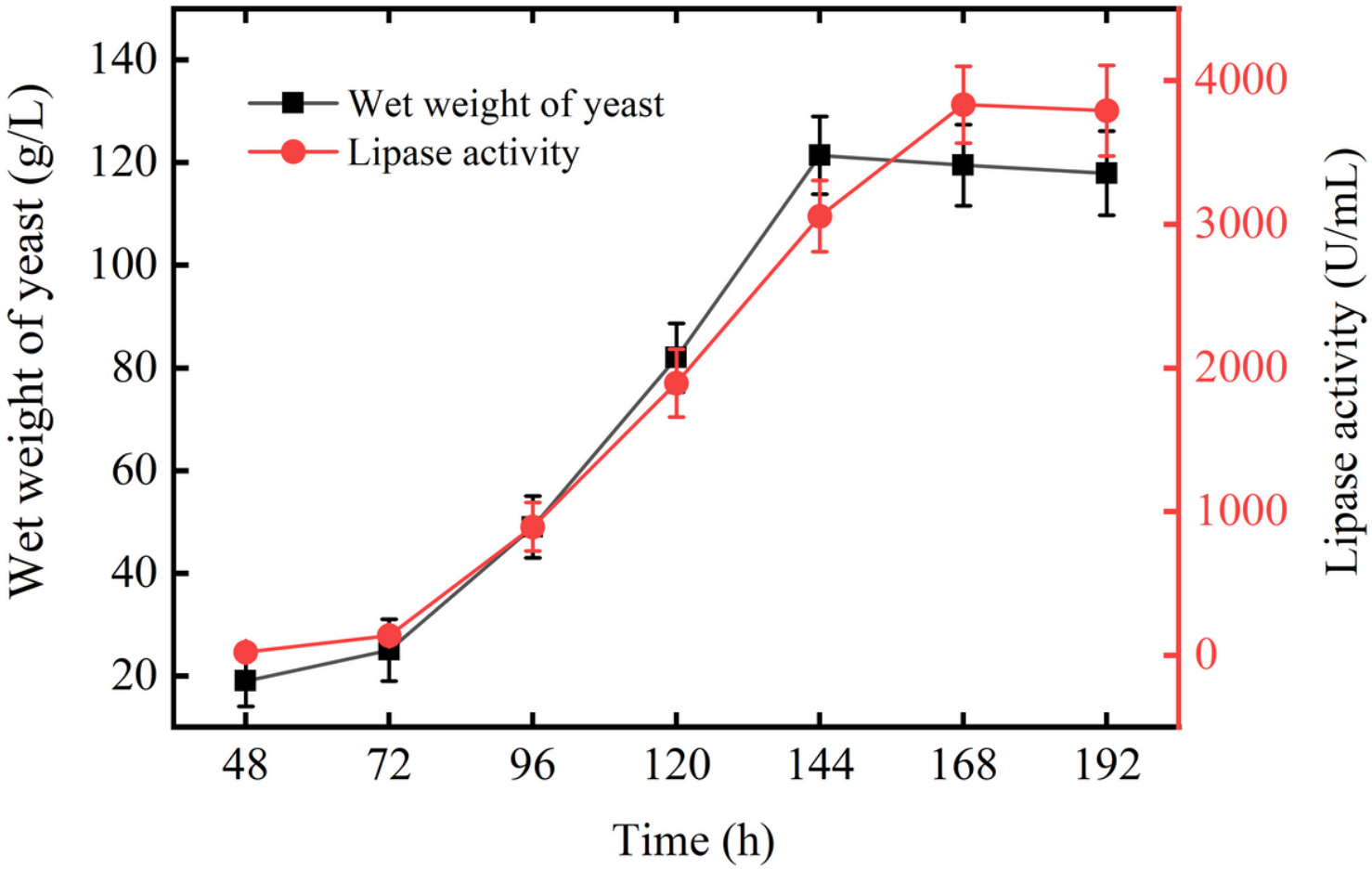

Figure 2

Growth and enzyme production patterns of recombinant yeast

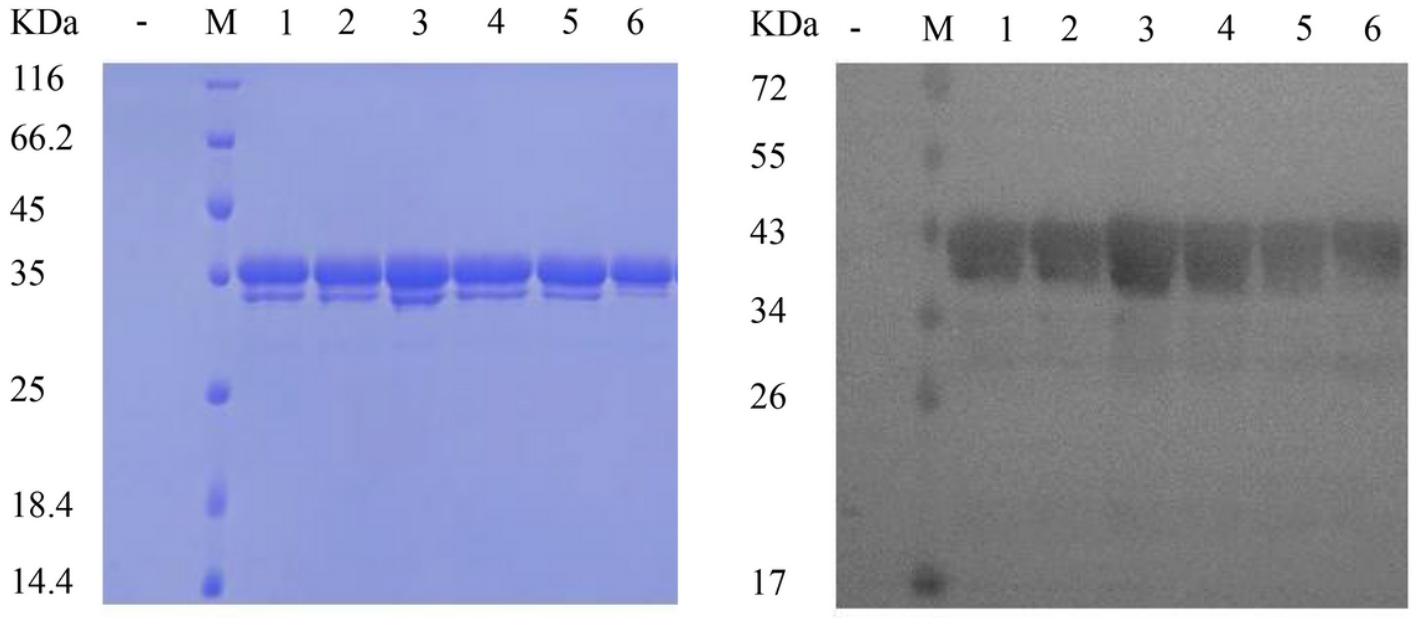

Figure 3

Analysis of lipase Lip4 secretory expression and purification by SDS-PAGE and Western blot

M: Protein molecular weight marker; 1-10: Pure lipase Lip4 by ammonium sulfate precipitation and Ni-NTA affinity chromatography from the fermented liquid of engineering strain pPICZαA-*lip*4/X33 (induced); -: Negative control (not induced).

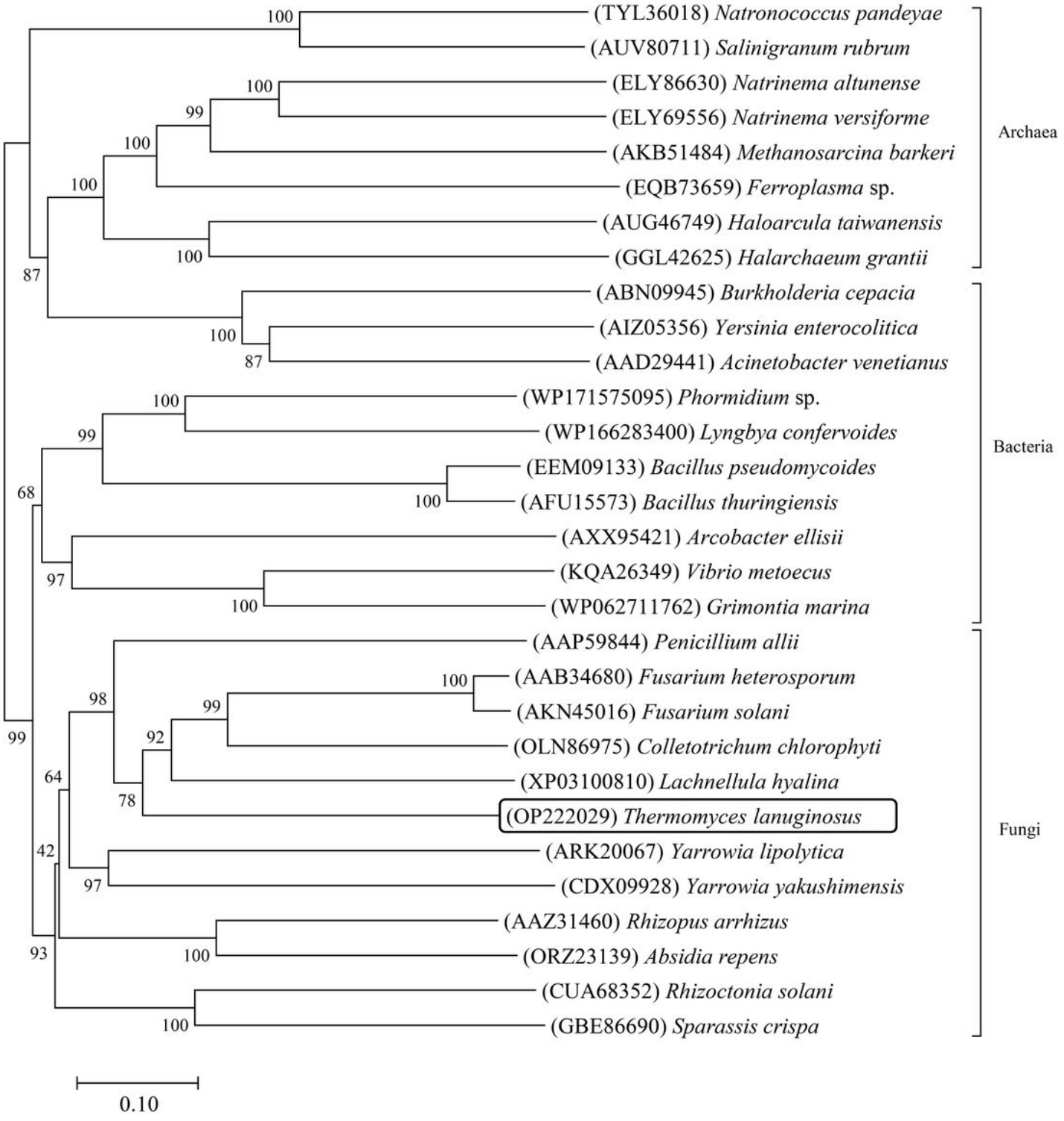

Figure 4

Neighbor-Joining tree constructed showing phylogenetic relationships among Lip4 and other lipases from microbiology

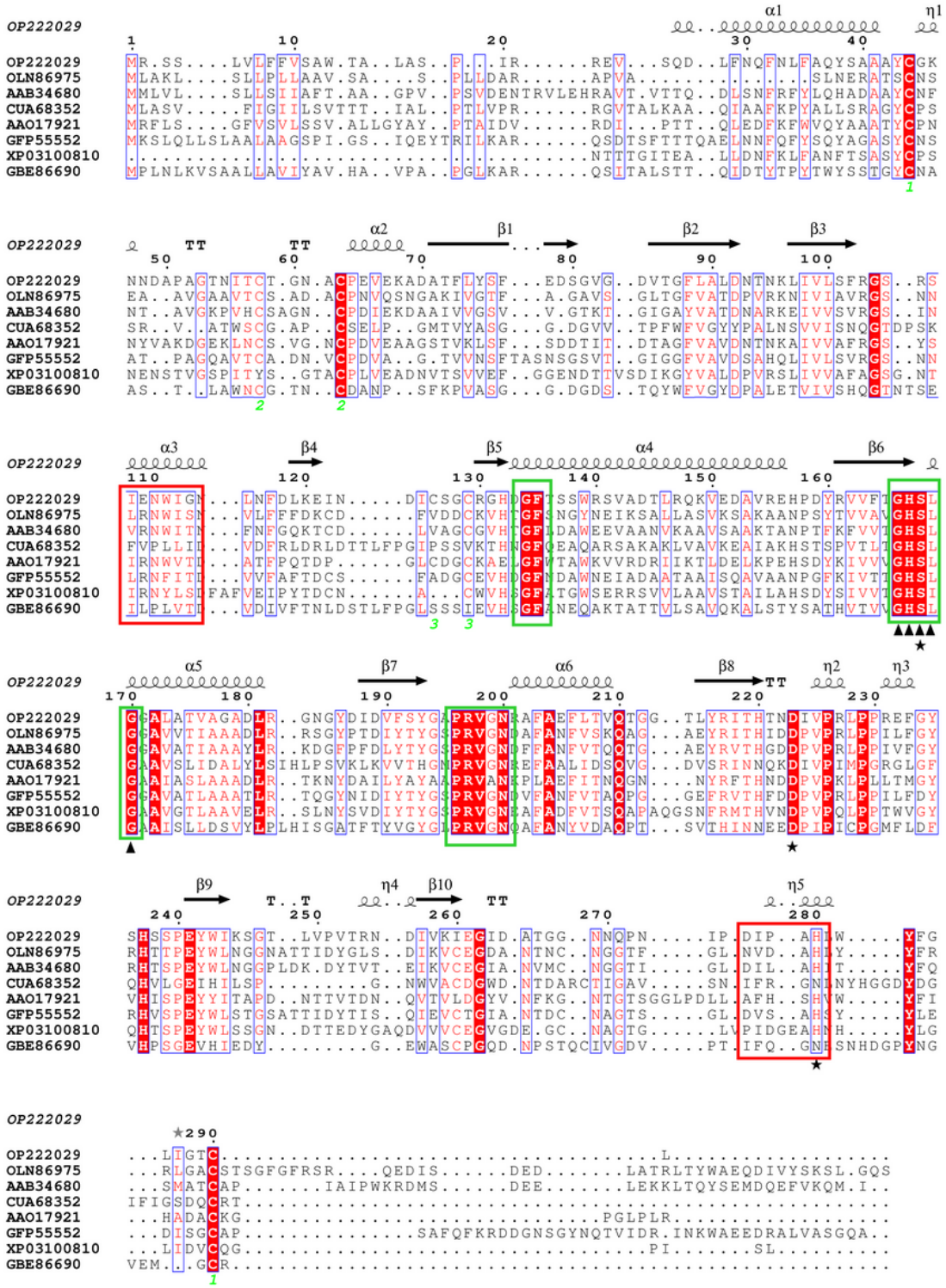

Figure 5

Homologous sequence alignment of Lip4 with lipases from other bacterial sources

Note: Pentagram is marked as a triplet structure site. The triangle is the pentapeptide sequence at the active center. The red box is labeled with the sequence of lipase lid structures. The green boxes are

marked as conservative sequences. The green numbers are labeled as cysteine pairs involved in forming disulfide bonds.

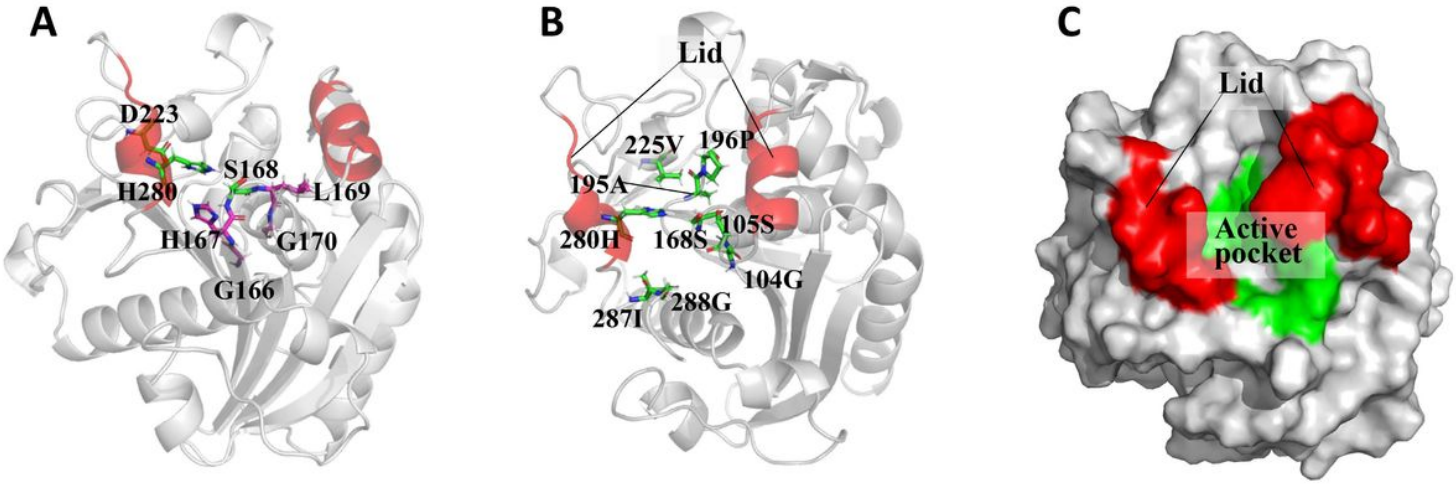

Figure 6

Key amino acid sites and structures of lipase Lip4

A. Triplet and pentapeptide structure; B. Active center; C. “Pocket” and “Lid” structure.

Supplementary Files

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