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Gene Cloning and Characterization of Pcal_0222, α-Amylase from *Pyrobaculum calidifontis*

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ABSTRACT

The gene encoding Pcal_0222 from hyperthermophilic archaeon *Pyrobaculum calidifontis* was cloned and expressed in *Escherichia coli*. Pcal_0222 was composed of 529 amino acids with a theoretical molecular mass of 58 kDa. The amino acid sequence contained the four conserved regions that are a characteristic of GH13 family members. Recombinant Pcal_0222 was purified to apparent homogeneity using cation exchange and gel filtration column chromatographies. Purified Pcal_0222 exhibited optimal α-amylase activity at 85°C and pH 5.5. The activity was not dependent on any metal ion. The hyperthermophilic nature and metal ion independence make it a suitable candidate for both basic as well as applied research.

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Authors' Contribution
MAS designed the study. SA, KN, SA and MAS conducted the experiment work. MAS and NR wrote the paper.

Key words
Hyperthermophile, *Pyrobaculum calidifontis*, α-amylase, glycoside hydrolase family 13

INTRODUCTION

Carbohydrates are most common and widespread biomolecules in nature and among them starch, a polymer composed of repeating subunits of α-D-glucose residues, is the second most abundant homopolysaccharide on our planet after cellulose (Kujawski *et al*., 2002). Green plants are the main source of starch that is utilized by various organisms as energy source (Janeček, 1997). Amylose and amylopectin are two types of α-D-glucose polymers found in starches. Amylose is an unbranched polymer of α-D-glucose monomers which are linked through α-1,4 glycosidic bonds. Due to the presence of α-1,6 glycosidic linkage in addition to α-1,4 glycosidic linkage amylopectin exhibits branched polymer shape (Guzman-Maldonado and Paradez-Lopez, 1995). Starch are mixture of two polymers and the ratio of (amylose to amylopectin) depends on various factors such as the origin, plant species, growth conditions, plants organs and their age etc. (Swinkels, 1985; Hii *et al*., 2012).

Starch hydrolyzing industry converts starch to obtain various useful syrups such as glucose, fructose and dextrose, and sugar alcohols such as erythritol, sorbitol and mannitol. Formerly, industry was using mild acid for the hydrolysis of starch but nowadays, due to the discovery of thermostable and more robust carbohydrates hydrolyzing enzymes, the acid has been replaced by these enzymes Normally four different types of enzymes including endoamylase, exoamylase, transferase and debranching enzyme, are used for the complete hydrolysis of starch. Endoamylases, such as α-amylases, hydrolyze α-1,4 glycosidic linkage of the inner part of starch while exoamylases such as glucoamylases and α-glycosidases hydrolyze α-1,4 and α-1,6 linkages on the outer part of the polymer (Van Der Maarel *et al*., 2002). Enzymes transferases such as amylomaltases and cyclodextrin glycosyltransferases act on donor molecule to cleave α-1,4 glyosidic linkage and transfer part of it to an acceptor molecule to form a new glyosidic linkage. Debranching enzymes such as pullulanases act on α-1,6-glycosidic bond in starch, pullulan, amylopectin and other related oligosaccharides (Van der Maarel *et al*., 2002; Hii *et al*., 2012; Rehman *et al*., 2018).

A large number of wild type and recombinant α-amylases have been purified from all the three domains of life including bacteria, archaea and eukaryotes. *Pyrobaculum calidifontis* is a hypertherthermophilic archaeon isolated from Philippines (Amo *et al*., 2002). In this study we report on cloning, purification and characterization of a highly thermostable α-amylase from this hyperthermophilic archaeon.

MATERIALS AND METHODS

Chemicals, reagents and enzymes

In current report, all the chemicals and reagents such as restriction enzyme (endonuclease), cloning kit for PCR, kit for DNA extraction, Phusion DNA polymerase for

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PCR, DNA ligase, protein and DNA markers used were of analytical grade and bought from Life Sciences Fermentas (USA), New England Biolabs, Inc. (Nebraska, USA), or Sigma (St. Louis, Mo., USA. Chemicals for the Growth of hyperthermophilic archaeon *P. calidifontis* strain were purchased from Nacalai Tesque Inc. (Kyoto).

Plasmids and bacterial strains

Plasmid pET101/D-TOPO from Invitrogen (Thermo Fisher Scientific, California, USA) was used as cloning vector. One Shot chemically competent *E. coli* TOP10 cells were used for the cloning of *amyl* gene and for the expression of cloned gene One Shot™ BL21 Star™ (DE3) cells ware used. All reactions and culture were performed at 37°C.

Culture medium for P. calidifontis *growth*

P. calidifontis cells were cultivated according to previously reported culture medium for the growth of *P. calidifontis* (Amo *et al*., 2002). The medium in distilled water contained tryptone 1%, yeast extract 0.1% and sodium thiosulphate 0.3%. The cells growth temperature was kept at 90°C.

Gene cloning into pET101

For the amplification of *Pcal_0222* gene and insertion of amplified gene in pET101 plasmid, the forward primers Amyl F: 5'- CACCATGTGCGTAGTGGAGAAGTGGAG-3', and a reverse primer Amyl-R: 5'- CTATATAAAGATTTCCAAGGAGCCCCC-3' were designed according to the available sequence of open reading frame, Pcal_0222 and pET101 plasmid as previously published by (Shuman, 1991, 1994). The thermal cycler (Gene Amp PCR System 24000, Perkin Elmer, Foster, Calif) was used for the amplification of gene. Phusion DNA polymerase (New England BioLabs inc.) was used for polymerase chain reaction (PCR), under the following conditions. 2 min at 100°C; 25 s at 95°C, 30 s at 55°C and 55 s at 74°C (32 cycles). The amplified PCR product was gene cleaned, ligated into TOPO pET101 vector to obtain pET101-0222. Commercially available chemically competent *E. coli* cells (One Shot TOP10) were transformed with pET101-AMYL plasmid.

Electrophoretic analyses

DNA samples including PCR amplified *Pcal_0222* gene product were analyzed by performing 1.0% agarose gel electrophoresis. The gels were soaked in ethidium bromide solution for staining and DNA bands were visualizing under the UV light. 0.1% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed for the analysis of protein. Protein samples were stained with Coomassie brilliant blue (CBB).

Production and purification of recombinant Pcal_0222 protein

Recombinant Pcal_0222 protein was produced using *E. coli* BL21 Star™ (DE3) cells. The bacterial cells were transformed with pET101-AMYL plasmid, the cells were grown with shaking at 37 °C for 13 hours in 100 µg/mL ampicillin containing Luria-Bertani broth (LB) culture medium. LB medium containing same amount of ampicillin (100 µg/mL) was freshly prepared. 1% preculture was added into culture medium and cells growth was continued. At 660 nm Optical density (OD) of the culture medium was measured hourly to obtain the required OD 0.5 for the purpose of induction of gene expression. 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used for induction. The incubation was continued at 37°C with shaking and after 6 h of induction, the incubation was stopped and *E. coli* cells were collected by centrifugation (6,000 × g, 08 min). The harvested cells were washed with 50 mM sodium acetate buffer (pH 5.5). The pellet was obtained and resuspended in 50 mM sodium acetate buffer (pH 5.5). The cells were lyzed by sonication on ice and then to obtain the soluble and insoluble fraction, the lyzed cells were centrifuged at 15,000 × g for 20 mins at 4ºC. The gene expression for soluble and insoluble fractions was confirmed by SDS-PAGE. The insoluble fraction was solubilized in sodium acetate buffer (50 mM) and (pH 5.5) containing 20% (v/v) glycerol with continuous stirring at room temperature for 06 hrs. The sample was centrifuged at 15,000 × g for 30 mins. The glycerol-amylase containing supernatant was purified according to modified previously reported method (Rashid *et al*., 2010). The sample was filtered through 0.45 µm pore size containing membrane. The filtrate was loaded on a commercially available (GE Healthcare, UK) prepacked ion exchange chromatography Resource S column. Fast protein liquid chromatography (FPLC) system was used for liquid chromatography. Linear gradient of 50 mM sodium acetate buffer (pH 5.5) containing 1 mol/L NaCl was used to elute the proteins form column at 1 mL/min flow rate. The fractions collected and were checked for amylase activity. The active fractions were combined, concentrated and applied on a gel filtration column Sephadex G 75 column (2.6 x 70.0 cm). 50 mM sodium acetate buffer (pH 5.5) was used as elution buffer containing 50 mM/L NaCl.

Molecular mass determination

The molecular mass of recombinant Pcal_0222 protein was determined by both SDS-PAGE and gel filtration column. For SDS-PAGE, the pre stained protein calibration kit (Invitrogen) containing 12 marker with molecular mass 260–3.5 kDa was used. The gel was stained with Coomassie Blue R-350. Protein calibration kit containing cytochrome c (12.3 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), and bovine albumin (67 kDa) was used for gel filtration column.

Enzyme assay

The α-amylase activity was calculated by the amount of reducing sugar liberated during hydrolysis of soluble starch (1%) in 50mM acetate buffer (pH 5.5). The reaction mixture with substrate was incubated at 85°C for 15 min. Reaction mixture without substrate was used as control experiment. The amount of reducing sugar liberated was calculated by the previously reported dinitrosalicyclic acid (DNS) method (Miller, 1969). The amount of enzyme that released 1 μmol of reducing sugars in one minute was defined as one unit of enzyme activity.

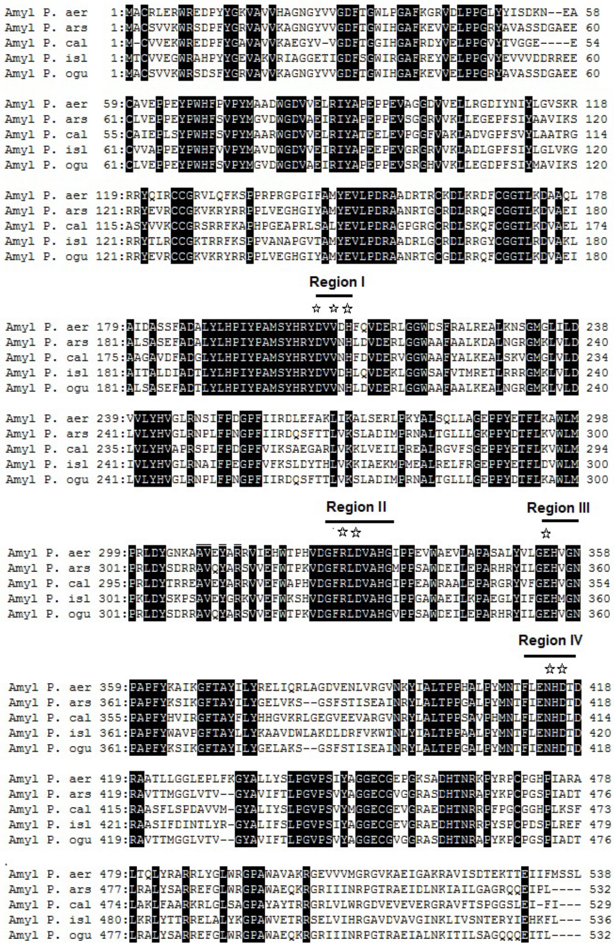
RESULTS AND DISSCUSSION

Sequence analysis of Pcal_0222 gene

The full length of *Pcal_0222* gene (GenBank accession number: Pcal_0222), encodes 529 amino acids containing polypeptide with a calculated molecular mass of 58 kDa exhibiting a theoretical isoelectric point as 7.05. A putative signal polypeptide containing 21 amino acids from Met[1] to Ala[21] was identified using Signal P4.1, indicating the mature protein contains 508 amino acids with calculated molecular mass of 56 kDa. The deduce amino acid sequence of *Pcal_0222* exhibited the highest identities with putative α-amylases from *Pyrobaculum ferrireducin* (NC_016645.1) 62%, and with *Pyrobaculum arsenaticum* (NC_009376.1), *Pyrobaculum oguniense* (CP003316.1) and *Pyrobaculum islandicum* (NC_008701.1) 60% each. It has identities with *Pyrobaculum aerophilum* (NC_003364.1) 59% and with *Thermoproteus tenax* (NC_016070.1) 40%. The deduced amino acid sequence (Fig. 1) has four highly conserved regions indicating *Pcal_0222* belongs to GH family 13 (Van Der Maarel *et al*., 2002). The conserved regions I, II, III and IV contain the amino acids DVVNH, GFRLDVAHG, EHVGN and FLENHDTD, respectively. In region I (DVH), region II (RD), region III (E) and region IV (HD) are the conserved amino acids found in α-amylases.

Expression of Pcal_0222 gene and purification of recombinant protein

The chromosomal DNA was obtained by growing the cells of hyperthermophilic archaeon *P. calidifontis* according to the procedure as described in experimental section. For the amplification of *Pcal_0222* gene, forward and reverse primers were constructed. The amplified *Pcal_0222* gene was ligated in pET101 vector to obtain pET101-0222. To know the exact amplified *Pcal_0222* gene sequence, both strands of the gene were sequenced and no any nucleotide base mutation was observed in both strands. Thus, for the expression of *Pcal_0222* gene the *E. coli* competent BL21 Star™ (DE3) cells were transformed with pET101-0222 plasmid. A number of white colonies were observed on the selection plates. For gene expression a random colony was selected and heterologous gene expression was achieved with induction of 0.1 mM IPTG.



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Amyl P. aer   1:MACRLERWREDPYYGKVAVVHAGNGYVVGDFTGWLPGAFKGRVDLPPGLYYISDKN--EA 58
Amyl P. ars   1:MACSVVKWRSDPFYGRVAVVKAGNGYVVGDFTGWIHGAFKEVVELPPGRYAVASSDGAEE 60
Amyl P. cal   1:M-CVVEKWREDPFYGAVAVVKAEGYV-VGDFTGGIHGAFRDYVELPPGVYTVGGE----E 54
Amyl P. isl   1:MTCVVEGWRAHPYYGEVAKVRIAGGETIGDFSGWIRGAFREYVELPPGVYEVVVDDRREE 60
Amyl P. ogu   1:MACSVVKWRSDSFYGRVAVVKAGNGYVVGDFTGWIHGAFKEVVELPPGRYAVASSDGAEE 60

Amyl P. aer  59:CAVEPPEYPWHFPVPYMAADWGDVVELRIYAPEPPEVAGGDVVELLRGDIYNIYLGVSKR 118
Amyl P. ars  61:CLVEPPEYPWHFSVPYMGVDWGDVAEIRIYAPEPPEVSGGRVVKLLEGEPFSIYAAVIKS 120
Amyl P. cal  55:CAIEPLSYPWHFSVPYMAARWGDVVELRIYATEELEVPGGFVAKLADVGPFSVYLAATRG 114
Amyl P. isl  61:CVVAPPEYPWHFVVPYMAVDWGDVVEIRIYAPEEPEVGRGRVVKLADLGPFSIYLGLVKG 120
Amyl P. ogu  61:CLVEPPEYPWHFSVPYMGVDWGDVAEIRIYAPEPPEVSRGHVVKLLEGDPFSIYMAVIKS 120

Amyl P. aer 119:RRYQIRCCGRVLQFKSPPRPRGPGIFAMYEVLPDRAADRTRCKDLKRDFCGGTLKDAAQL 178
Amyl P. ars 121:RRYEVRCCGKVKRYRRPPLVEGHGIYAMYEVLPDRAANRTGCRDLRRQFCGGTLKDVAEI 180
Amyl P. cal 115:ASYVVKCCGRSRRFKAPHPGEAPRLSALYEVLPDRAGPGRGCRDLSKQFCGGTLKDVAEL 174
Amyl P. isl 121:RRYTLRCCGKTRRFKSPPVANAPGVTAMYEVLPDRAADRLGCRDLRRGYCGGTLRDVAKL 180
Amyl P. ogu 121:RRYEVRCCGKVKRYRRPPLVEGHGIYAMYEVLPDRAANRTGCGDLRRQFCGGTLKDVAEI 180

Amyl P. aer 179:AIDASSFADALYLHPIYPAMSYHRYDVVDHFQVDERLGGWDSFRALREALKNSGMGLILD 238
Amyl P. ars 181:ALSASEFADALYLHPIYPAMSYHRYDVVNHLDVDERLGGWAAFAALKDALNGRGMKIVLD 240
Amyl P. cal 175:AAGAVDFADGLYLHPIYPAMSYHRYDVVNHFDVDERVGGWAAFYALKEALSKVGMGIVLD 234
Amyl P. isl 181:AITALDIADTLYLHPIYPAMSYHRYDVVDHLQVDEKLGGWSAFVTMRETLRRRGMKIVLD 240
Amyl P. ogu 181:ALSASEFADTLYLHPIYPAMSYHRYDVVNHLDVDERLGGWAAFAALKEALNGRGMKIVLD 240

Amyl P. aer 239:VVLYHVGLRNSIFPDGPFIIRDLEFAKLIKALSERLPKYALSQLLAGEPPYETFLKAWLM 298
Amyl P. ars 241:LVLYHVGLRNPLFPNGPFIIRDQSFTTLVKSLADIMPRNALTGLLLGKPPYDTFLKVWLM 300
Amyl P. cal 235:LVLYHVAPRSPLFPDGPFVIKSAEGARLVKKLVEILPREALRGVFSGEPPYETFLKVWLM 294
Amyl P. isl 241:IVLYHVGLRNAIFPEGPFVFKSLDYTHLVKKIAEKMPMEALRELFRGEPPYETFLDVWLM 300
Amyl P. ogu 241:LVLYHVGLRNPLFPNGPFIIRDQSFTTLVKSLADIMPRNALTGLLLGEPPYDTFLKAWLM 300

Amyl P. aer 299:PRLDYGNKAAVEYARRVIEHWTPHVDGFRLDVAHGIPPEVWAEVLAPASALYVLGEHVGN 358
Amyl P. ars 301:PRLDYSDRRAVQYARSVVEFWTPKVDGFRLDVAHGMPPSAWDEILEPARHRYILGEHVGN 360
Amyl P. cal 295:PRLDYTRREAVEYARRVVEFWAPHVDGFRLDVAHGIPPEAWRAALEPARGRYVFGEHVGN 354
Amyl P. isl 301:PKLDYSKPSAVEYGRKVVEFWKSHVDGFRLDVAHGIPPGAWAEILKPAEGLYIFGEHMGN 360
Amyl P. ogu 301:PRLDYSDRRAVQYARSVVEFWTPKVDGFRLDVAHGVPPSAWDEILEPARHRYILGEHVGN 360

Amyl P. aer 359:PAPFYKAIKGFTAYILYRELIQRLAGDVENLVRGVNKYIALTPPHALPYMNTFLENHDTD 418
Amyl P. ars 361:PAPFYKSIKGFTAYILYGELVKS--GSFSTISEAINRYLALTPPGALPYMNTFIENHDTD 418
Amyl P. cal 355:PAPFYHVIRGFTAYFLYHHGVKRLGEGVEEVARGVNRYLALTPPSAVPHMNLFLENHDLD 414
Amyl P. isl 361:PAPFYWAVPGFTAYLLYKAAVDWLAKDLDRFVKWTNLYIALTPPAALPYMNTFLENHDTD 420
Amyl P. ogu 361:PAPFYKSIKGFTAYILYGELAKS--GSFSTISEAINRYLALTPPGALPYMNTFIENHDTD 418

Amyl P. aer 419:RAATLLGGLEPLFKGYALLYSLPGVPSIYAGGECGEPGKSADHTNRKPYRPCPGHPIARA 478
Amyl P. ars 419:RAVTTMGGLVTV--GYAVIFTLPGVPSVYAGGECGVGGRASDHTNRAPYKPCPGSPIADT 476
Amyl P. cal 415:RAASFLSPDAVVM-GYALIYSLPGVPSVYMGGECGEVGRAEDHTNRRPFPGCGGHPLKSF 473
Amyl P. isl 421:RAASIFDINTLYR-GYALIFSLPGVPSIYAGGECGEVGRAEDHTNRRPYSPCPDSPLREF 479
Amyl P. ogu 419:RAVTTMGGLVTV--GYAVIFTLPGVPSVYAGGECGVGGRASDHTNRAPYKPCPGSPIADT 476

Amyl P. aer 479:LTQLYRARRLYGLWRGPAWAVAKRGEVVVMGRGVKAEIGAKRAVISDTEKTTEIIFMSSL 538
Amyl P. ars 477:LRALYSARREFGLWRGPAWAEQKRGRIIINRPGTRAEIDLNKIAILGAGRQQEIPL---- 532
Amyl P. cal 474:LAKLFAARKRLGLSAGPAYAYTRRGRLVLWRGDVEVEVERGRAVFTSPGGSLEI-FI--- 529
Amyl P. isl 480:LKRLYTTRRELALYKGPAWVETRRSELVIHRGAVDVAVGINKLIVSNTERYIEHKFL--- 536
Amyl P. ogu 477:LRALYSARREFGLWRGPAWAEQKRGRIIINRPGTRAEIALNKITILSAGQQQEITL---- 532
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Fig. 1. Multiple sequence alignment of *P. calidifontis* α-amylase with the sequences of other putative amylases from *P. arsenaticum* (NC_009376.1), *P. oguniense* (CP003316.1), *P. islandicum* (NC_008701.1), *P. aerophilum* (NC_003364.1) and with *T. tenax* (NC_016070.1). The identical residues are highlighted with soid black. Four highly conserved regions I, II, III and IV contain the amino acids DVVNH, GFRLDVAHG, EHVGN and FLENHDTD respectively have been shown with bold lines (—). Asterisks (*) indicate the conserved residues of conserved regions among all α-amylases.

The expression of *Pcal_0222* gene in *E. coli* indicated that ≈95% recombinant protein was produced in insoluble protein aggregates.

Usually the insoluble aggregates do not exhibit enzyme activity (Hockney, 1994; Thomas and Baneyx, 1997) but insoluble aggregates of Pcal_0222 exhibited significant (13 U mg^{-1}) α-amylase activity indicating that the protein was not completely misfolded (Linden *et al.*, 2000). Due to complications in using denaturizing agents for solubilization of proteins (Rudolph and Lilie, 1996) or heating up to certain extent (Dong *et al*., 1997) we used the glycerol extraction method (Rashid *et al*., 2010). When the glycerol-Pcal_0222 containing supernatant was applied to ion exchange column (Resource S), Pcal_0222 was eluted from the column with 0.4 mol/L NaCl in 50 mmol/L sodium acetate buffer (pH 5.5). The active fractions after Resource S column were combined, concentrated and further purified by gel filtration column. When purity of the recombinant protein was analyzed by SDS-PAGE, production of 56 kDa recombinant protein was observed (Fig. 2). Approximately 87-fold purification was achieved with a final yield of 290%. The specific activity of purified Pcal_0222 was 1131 U/mg. The purification steps and corresponding activities are summarized in Table I.

Table I. Purification of recombinant α-amylase from *P. calidifontis* expressed in *E. coli.*

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield %	Purification (fold)
Centrifugation	60.0	780	13	100	1.0
Solubilization	3.2	2922	913	374	70
Resource S column	2.3	2413	1050	309	81
Gel filtration	2.0	2262	1131	290	87

Enzymatic properties

The effects of pH and temperature on amylase activity were determined. The buffers used for pH determination were 50 mM sodium acetate; Tris-HCl and potassium phosphate. The soluble starch was used as substrate for the determination of amylase activity at different pH ranges. The enzyme exhibited highest activity at pH 5.5, the rapid decrease in activity was observed above or below 5.5 (Fig. 3A). Highest activity was observed at 85°C when measured at pH 5.5. Pcal_0222 retained more than 50% activity at 65 and 95°C (Fig. 3B).

In order to know the effect of metal ions, the enzyme activity was measured in 50 mM Na-acetate bufferat pH 5.5 and 85°C in the presence of chloride salt of various metals ions at a final concentration of 1 mM. It was noticed that metals ions are not essential for the activity of Pcal_0222 (Table II). In fact, the activity was reduced in the presence of metal ions. Cu^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} exhibited 47, 86, 82 and 73% inhibitory effect on the activity while Hg^{2+} inhibited the activity completely. Other metals ions Ca^{2+}, Co^{2+}, Mg^{2+} and Sr^{2+} did not significant affect the enzyme activity.

Table II. Effect of metal ions on amylase activity.

Metal ion	Relative activity (%)
None	100
Ca^{2+}	109
Co^{2+}	102
Cu^{2+}	47
Hg^{2+}	02
Mg^{2+}	99
Mn^{2+}	86
Ni^{2+}	82
Sr^{2+}	100
Zn^{2+}	73

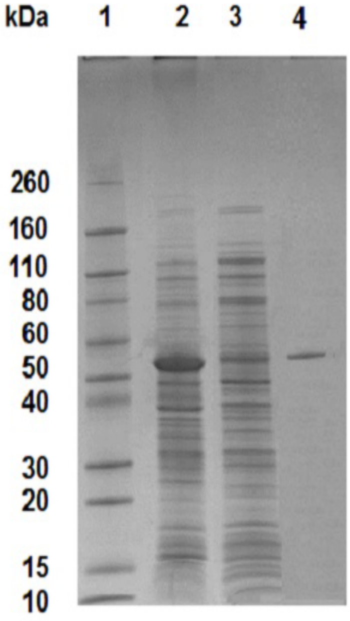

Fig. 2. The 0.1% SDS-15% polyacrylamide gel electrophoresis (SDS-PAGE) demonstrating the purified α-amylase. Lane 1, Novex™ Sharp Pre-stained Protein Standard; lane 2, cells carrying pET101-AMYL plasmid insoluble faction; lane 3, cells carrying pET101-AMYL plasmid soluble faction; lane 4, purified recombinant α-amylase.

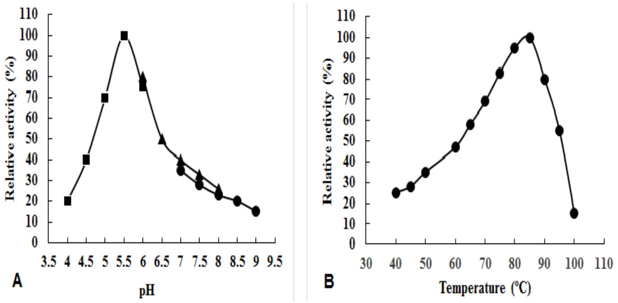

Fig. 3. Enzymatic properties of α-amylase.
(A) Effect of pH on α-amylase activity using (■) 50 mM 50 mM Na-acetate buffer; (▲) 50 mM Phosphate buffer and (●) 50 mM Tris-HCl buffer. (B) Effect of temperature on α-amylase activity.

CONCLUSION

Pcal_222, a homologue of α-amylase from hyperthermophilic archaeon *P. calidifontis,* is a true α-amylase enzyme which exhibits highest activity at 85°C and pH 5.5. Pcal_0222 is a metal ion independent enzyme. These features make it a good candidate for its use in starch industry.

ACKNOWLEDGEMENTS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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