



ABSTRACT

The present research work is focused on the cloning, expression and characterization of thermostable α -amylase from *Thermotoga petrophila*. From NCBI genomic data of *T. petrophila* (Reference Sequence: NC_009486.1) 1.668 kb gene sequence was retrieved. The sequence was used as query for the NCBI nucleotide BLAST and results revealed that the Tpet AmyA shares homology with α -amylase catalytic domain or part of it from a variety of bacterial species.

The Tpet Amy A was cloned first in to TA cloning vector (pTZ57R/T) followed by cloning in pET21a(+) vector. The recombinant vector was transformed and expressed in *E. coli* BL21 Codon Plus. SDS-PAGE analysis showed the expression of about 64 kDa protein, however, the protein expression was very low. Thus, cultural conditions were optimized to enhance the expression of Tpet AmyA gene. The gene expression was optimum in LB medium when induction was carried out with 0.8 mM IPTG at 0.8 cell density ($OD_{600\text{ nm}}$) of *E. coli* followed by heat shock. Whereas, optimum post induction period was 24 h for the Tpet AmyA expression in LB medium at 30°C. After optimization, an increase of 2.63 fold in the enzyme activity was obtained.

Lactose was also tested as an alternative inducer to enhance the expression of recombinant α -amylase. Recombinant protein was expressed in nine different media (LB, ZB, 4xZB, ZBM, ZYB, 3xZYB, M9, ZYBM9 and 3xZYBM9) by using IPTG and lactose as the individual inducers. Lactose was found to be the better inducer in all the media with maximum expression in the ZBM medium. The protein expression in the ZBM medium was optimum when the host cells ($OD_{600\text{ nm}} 0.8$) were given heat shock after the induction with 200 mM lactose followed by incubation at 37°C for 48 h. After optimization, 1.5 fold increase in the intracellular α -amylase activity in ZBM medium was observed.

Albeit, there was an increase in the protein expression after the optimization, but it was still not enough to purify the recombinant protein to homogeneity level.



Therefore, to enhance the expression and facilitate the purification by affinity chromatography, catalytic domain of Tpet AmyA gene was subcloned into pHis Parallel I (pHis-P1) expression vector and expressed in *E. coli* BL 21 Codon Plus using 0.8 mM IPTG in LB medium. SDS-PAGE analysis showed that the Tpet AmyA protein with N-terminus His₆ tag was successfully overexpressed.

The purification of the recombinant α -amylase was carried out by heat treatment (at 80°C for 1 h) followed by affinity chromatography. After tag cleavage with rTEV (Tobacco Etch Viral) protease, sample was purified to homogeneity level by exchange chromatography. The molecular mass of protein was 62720 Dalton as determined by Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS). Product of soluble starch hydrolysis by the recombinant enzyme was dextrin as determined by HPLC.

Purified α -amylase was characterized to find out the optimum temperature (98°C), incubation time (3 min), pH (8.5), buffer (100 mM Tris-Cl), substrate concentration (1%) and Ca²⁺ ions concentration (0.8 mM). Among the other metal ions, Li¹⁺ and Na¹⁺ did not inhibit the enzyme activity. K¹⁺ and Rb¹⁺ ions enhanced the activity by 16 and 10%, respectively. Whereas, Cu²⁺, Mn²⁺, Ni²⁺, Fe²⁺, Pb²⁺, and Fe³⁺ completely inhibited the activity of recombinant α -amylase. Tween 80, SDS and β -mercaptoethanol appeared as moderate inhibitors of the enzyme. The α -amylase was sensitive to inhibitor acarbose, which reduced the enzyme activity to 56.4% when present at a concentration as low as 0.02 μ M in the reaction mixture.

Thermostability of the enzyme was also explored by pre-incubation of purified protein from 65-100°C for 2 h. The α -amylase was quite stable from 65 to 75°C. However, enzyme lost 16 and 19% of its activity after 2 h pre-incubation at 80 and 85°C, respectively. Whereas, after 2 h pre-incubation at 90, 95 and 98°C, the enzyme retained 62.9, 55.1 and 48.8% of its activity, respectively. However, at 100°C it lost 55% activity in 40 min. Alpha amylase from *T. petrophila* was stable at room temperature (27 \pm 1°C) for three weeks, and in 4th and 5th week the loss in activity was only 6% and 13%, respectively.



ABSTRACT

The K_m and V_{max} values for amylose, amylopectin, soluble starch and raw starch were 0.57, 1.095, 2.985 and 3.63 mg and 1.398, 2.325, 2.674 and 2.105 mg/min, respectively. Activation energy (E_a) for the starch hydrolysis was 42.97 kJ mol⁻¹. Whereas, ΔG , ΔH and ΔS were 74.141 kJ mol⁻¹, 39.883 kJ mol⁻¹ and -92.304 J mol⁻¹ K⁻¹, respectively for the hydrolysis of soluble starch at 98°C. While, ΔG_{E-S} and ΔG_{E-T} were 3.375 kJ mol⁻¹ and -14.059 kJ mol⁻¹, respectively. The half-life of α -amylase at 98°C was 1.7 h. Activation energy E_{aD} for α -amylase denaturation was 108.36 kJ mol⁻¹. Other thermodynamic parameter for enzyme inactivation i.e. ΔG^* (106.80 kJ mol⁻¹), and ΔH^* (105.28 kJ mol⁻¹) and ΔS^* (-4.094 J mol⁻¹ K⁻¹) were also calculated.

Complete desizing of the sized cotton fabric with 0.01 μ M enzyme was achieved after 25 min at 98°C that was 9 according to the rating on TEGAWA scale. The x-ray crystallography of the α -amylase cloned from *T. petrophila* showed the typical 3D structure with domain A, B and C. Residues Glu253, Asp213 and Asp305 constitute the catalytic triad with 5.5 Å distance between Glu253 (nucleophile) and Asp305 (proton donor) indicating the retaining mechanism for the substrate hydrolysis.