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Cloning and characterization of thermo-alkalistable and surfactant stable endoglucanase from Puga hot spring metagenome of Ladakh (J&K)

Puneet Gupta [a], Arjun K. Mishra [b], Jyoti Vakhlu [a,*]

[a] School of Biotechnology, University of Jammu, Jammu, India
[b] Regional Centre for Biotechnology, Faridabad, Haryana, India

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ABSTRACT

A thermo-alkalistable and surfactant stable endoglucanase (PHS) gene consisting of 554 amino acids was identified from metagenomic library of Puga hot spring using functional screening. PHS gene was overexpressed and purified to homogeneity using affinity chromatography The purified PHS protein presented a single band of 60 kDa on the SDS-PAGE gel and zymogram. The recombinant PHS exhibited activity over a broad range of pH and temperature with optima at pH 8.0 and 65 °C, respectively and having optimum stability at 60 °C and pH 8.0, respectively. The recombinant PHS showed highest substrate specificity using CMC (218.4 U/mg) as compared with Barley β-glucan (89.2 U/mg) and Avicel (0.8U/mg). The K_m and V_{max} of recombinant PHS for CMC were 3.85 mg/ml and 370.37 μmolmin^{-1}mg^{-1}, respectively. The activity of the recombinant PHS was enhanced by treatment with 10 mM non-ionic detergents such as Tween 20, Tween 40, Tween 80, Triton X- 100 and PEG and was inhibited by CTAB, SDS. Its functionality was stable in the presence of Fe^{3+} but inhibited by Cu^{2+}, Hg^{2+}, Mn^{2+} and Zn^{2+}. These properties make PHS endoglucanase a potential candidate for use in laundry, textile,paper and pulp industries

1. Introduction

Cellulose represents the most abundant renewable biopolymer of plant origin available on earth which is commonly hydrolyzed by cellulases. Cellulases are responsible for catalyzing the hydrolysis of the β-1,4 glucosidic bonds in cellulose. The complete hydrolysis of cellulose requires the synergistic action of three major components; endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Currently, cellulases occupy the third position in world enzyme market and are projected to become the most invaluable enzyme for the generation of ethanol [1]. Now a days, cellulases are commonly employed in detergents causing color brightening and softening, in the color extractions of juices [2], in the pretreatment of biomass that contains cellulose to improve nutritional quality of forage [3], in the biostoning of jeans [4], in the pretreatment of industrial wastes [5] and in bioethanol production [6]. Cellulases with specific properties are required according to their application in particular industrial processes. Thermostable cellulases active at high temperature and alkaline pH are required for paper recycling in paper industry, saccharification of lignocellulosic biomass for bioethanol production and in detergent industry

Although several cellulases have been reported from a plethora of microorganisms, most of them do not have requisite thermostability and alkalistability for their application in relatively hostile conditions of industrial processes such as paper industry, biofuel production and in detergent industry. Metagenomics has provided access to novel enzymes and biocatalysts that are not achievable by conventional cultivable approaches. Discovery of novel biocatalysts from extreme environment i.e hot springs [7], glaciers [8], hydrothermal vent [9] is illustrated by the fact that extremophiles produce enzymes that are functional under extreme conditions. A variety of enzymes with novel characteristics have been isolated from extreme environments through metagenomics such as lipases [10], cellulases [11], esterases [12,13] and chitinases [14], however there are few reports on discovery of novel enzymes such as β-glucosidase [15], lipases [16], cyclomaltodextrinase [17] from hot springs through metagenomics. Considering the requirement of thermo-alkalistable endoglucanases in various industrial processes, Puga hot spring sediment was selected as the source of metagenome as it has high temperature amidst cold dessert and has not been explored using metagenomics so far. The aim

* Corresponding author.
E-mail address: jyotimetagenomic@gmail.com (J. Vakhlu).

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of the present study was to identify thermostable cellulase gene from Puga hot spring sediment using metagenomic approach. The function-based screening of the metagenomic library resulted in isolation of endoglucanase gene (PHS). The recombinant PHS was overexpressed and characterized for process suitable applications. To the best of our knowledge, this is the first report on retrieving thermo-alkalistable and surfactant stable endoglucanase by metagenomic approach.

2. Material and methods

2.1. Sample collection

Sediment samples were collected from Puga hot spring (33°13′N, 78°18′W) of Ladakh, having outlet temperature up to 84 °C. Multiple sediment samples were collected from side of spring with sterile spatulas and were placed into 50 ml polypropylene tube. Temperature and pH were recorded in situ using a Hach pH meter equipped with a pH and temperature probe. All sediment samples were immediately frozen in liquid nitrogen, stored on dry ice during transportation for 48 h and stored at −20 °C in the laboratory.

2.2. Strains and plasmids for DNA manipulations

BamH1 digested/dephosphorylated pUC19 (Fermentas) and pET28a(+) (Novagen) were used for cloning and expression of endoglucanase gene, respectively. *E.coli* DH10 B and *E.coli* BL21 (DE3) were used for the propagation of the plasmid. *E.coli* was grown at 37 °C on Luria–Bertani (LB) medium supplemented with appropriate antibiotics [18]. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (UK).

2.3. Construction and functional screening of metagenomic library

Metagenomic DNA from Puga hot spring sediment was extracted according to Gupta et al. [19] and was further purified from 1% low melting agarose gel using QIAquick Gel Extraction Kit (Cat 28704). Purified metagenomic DNA was partially digested using *BamH1* restriction enzyme. DNA fragments ranging from 2 Kb to 10 Kb were excised and purified using QIAquick gel extraction kit (Qiagen). Purified metagenomic DNA was ligated using T4 DNA ligase into *BamH1* digested/dephosphorylated pUC19 vector (fermentas) incubated overnight at 16 °C. The ligation mixture was electroporated into competent *E.coli* DH10 B cells (200×, 25 lF and 2.5 kV) using Gene Pulser (Bio-Rad,USA). Transformants were grown on LB agar plates containing ampicillin (100 μg/ml), X-gal (20 μg/ml) and IPTG (50 μg/ml) with overnight incubation at 37 °C and recombinant clones were selected based on blue-white screening. Plasmid DNA from recombinant clones were digested with *BamHI* and average insert size was confirmed using agarose gel electrophoresis. Recombinant clones were cultured on LB agar plates supplemented with ampicillin (100 μg/ml), IPTG (50 μg/ml) and 1% CMC at 37 °C for 48 h −72 h. Endoglucanase activity was detected by yellow halo formation against red background using congo red staining [20]. The plasmid DNA from positive clone was isolated using Qiagen plasmid isolation kit (Qiagen, USA) and retransformed into *E.coli* DH10 B for endoglucanase activity confirmation.

2.4. Sequencing and analysis of endoglucanase gene

DNA inserts of positive clone was sequenced using ABI prism big dye terminator protocol at the Scignome Labs, Cochin (India) using primer walking sequencing approach. Sequence similarity searches were carried out with the NCBI BLASTp program. Phylogenetic tree was constructed using the neighbor-joining method with MEGA 6.06. Signal peptides were predicted with the SignalP 4.1 server. Open reading frames (ORFs) in the inserts were analyzed with BLASTp against the GenBank non-redundant database. The alternative structures of recombinant endoglucanase were generated using SWISS-MODEL. Models generated were scored for various parameters by NIH SAVES server and the best model was selected. Three dimensional structure was visualized and quality image was generated with PyMOL software

2.5. Cloning, over expression and purification of the recombinant endoglucanase

The putative endoglucanase gene was amplified from positive clone (PHS) by PCR using specific primers PHS Forward 5′-GGG GGA TCC ATG GCT GCG GAA AAA GTA TTT TCG AAA AAT − 3′ and PHS Reverse 5′-AAA GCG GCC ATG TCG CGA TTC CAG TTT GTT CTG CTC −3′ (*BamH1* and *Not1* restriction enzyme sites underlined). PCR product was digested with *BamH1* and *Not1* restriction enzyme and ligated into *BamH1*/*Not1* digested pET-28a(+) plasmid and transformed into *E.coli* BL21(DE3) cells (Novagen). The *E.coli* BL21 (DE3) harboring pET21a (+) PHS was grown at 37 °C in 100 ml of LB broth with kanamycin (50 μg/ml) until the optical density of the cell reached 0.6 at 600 nm.The cultures were induced by the addition of 1 mM IPTG and further grown for 12 h at 30 °C. Cells were then collected by centrifugation (6000 g for 20 min at 4 °C), resuspended in buffer A (50 mM Tris-Cl pH 7.5, 200 mM NaCl and 10 mM Imidazole), and lysed by sonication. The cell lysate was centrifuged at 14,000 rpm for 20 min at 4 °C to remove the cell debris. The supernatant was loaded on a Ni^{2+} −NTA column (Amersham) pre-equilibriated with Buffer A (50 mM Tris-Cl pH 7.5, 200 mM NaCl, 10 mM Imidazole). The column was subsequently washed with buffer B (50 mM Tris-Cl pH 7.5, 200 mM NaCl and 50 mM Imidazole). The protein was eluted using elution buffer containing 50 mM Tris-Cl pH 7.5, 200 mM NaCl and 500 mM.

2.6. Protein content quantification and endoglucanase assay

Protein concentration was determined by Bradford method using bovine serum albumin (BSA) as a standard. Endoglucanase activity was measured by adding 50 μl of diluted purified protein (0.02 mg/ml) to 1.45 ml of 50 mM phosphate buffer (pH 7) containing 1% (w/v) CMC. The reducing sugars so released were measured using 3, 5- dinitrosalicylic acid (DNSA) reagent [21]. One unit (U) of endoglucanase activity was defined as the amount of endoglucanase required to release 1 μmol of glucose equivalents per minute under the assay conditions.

2.7. SDS PAGE and Zymogram analysis

The molecular mass and purity of the recombinant PHS protein were determined using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions as previously described [22]. For Zymogram analysis, purified protein was resolved on SDS PAGE containing 0.1% CMC incorporated directly into the resolving gel at 4 °C, as described previously by lee et al. [23]. After electrophoresis, gel was kept in renaturation buffer (50 mM sodium phosphate buffer, pH 7.0, 2.5% Triton X-100) under shaking condition for 30 min. The gel was washed thrice and incubated in the renaturation buffer at 40 °C for 1 h. Congo-red (1%) staining of the gel was performed; destaining with 1 M NaCl was done afterwards to visualize the clear zone formation against the red background of the polyacrylamide gel.

2.8. Biochemical characterization of recombinant endoglucanase

The characteristics of the recombinant endoglucanase like the effect of pH, temperature, metal ions, inhibitors and surfactants on enzyme activity, thermostability as well as substrate specificity have been studied. Optimal temperature of recombinant PHS was determined by performing assay in a temperature ranging from 30 °C–80 °C at pH 7 using 50 mM of the phosphate buffer. Similarily for optimal pH determination, endoglucanase assay was conducted at different pH from 4.0 and 10.0 at 50 °C using buffers of different pH *viz*. citrate phosphate buffer (pH 4.0–5.0), sodium phosphate buffer (pH 6.0–8.0), and glycine-NaOH buffer (pH 9.0–10.0). For thermostability, the relative endoglucanase activity was measured after incubating the recombinant PHS at different temperatures from 30 °C–80 °C for 30 min. The pH stability was investigated by the addition of recombinant PHS using buffers of different pH followed by incubation at 4 °C for 24 h and the relative activity was measured. The effect of various metal ions ($FeCl_3$, $CoCl_2$, $CuSo_4$ and EDTA) and chemicals (SDS, Tween 20 Tween 40 Tween 80, Trixon X, CTAB, PEG) was assayed by adding them into the reaction mixture followed by measurement of relative endoglucanase activity.

Substrate specificities of the recombinant PHS was investigated using different kinds of substrates (oat gum, barley b-glucan, carboxymethyl cellulose, lichenan, oat spelt xylan, methyl cellulose, birchwood xylan, avicel and filter paper). The kinetic parameters, K_m and V_{max}, were determined by carrying the reaction under optimal condition using CMC of different concentrations, ranging from 1 to 10 mg/ml. After that K_m and V_{max} values were calculated by directly fitting the data to the Lineweaver-Burk double reciprocal plots. The substrate specificity was quantitated by assaying the recombinant PHS activity on various substrates (xylan, like starch, cellulose, chitin and pullulan in glycine- NaOH buffer). All the enzyme activity assays above were performed in triplicates and the standard deviations were calculated.

2.9. Nucleotide sequence accession number

The nucleotide sequence of the PHS endoglucanase, was deposited in the GenBank database under the accession number KU352745.

3. Results and discussion

Ladakh being cold arctic desert of Northwestern Himalayas is unique because of high altitude and extremely cold and dry weather. Some unexplored hot springs are also present in such extremely cold environments. Puga hot springs of ladakh are boron-sulphur rich, high elevation hot springs (33°13′N, 78°18′W) but there are handful of reports for exploration of novel enzymes from such distinctive extreme environment using functional metagenomics. Samples were collected from Puga hot spring of Ladakh for the isolation of thermo-alkalistable endoglucanase using metagenomic approach.

3.1. Construction of metagenomic DNA library from puga hot spring sediments

Metagenomic DNA library from Puga hot spring sediment was constructed using vector pUC19 in *E.coli* DH10B, with insert size ranging from 1.5 to 5.0 kb. Average insert size of metagenomic library was found to be ~3Kb, which was confirmed by restriction analysis of inserts isolated from 20 randomly selected clones. Metagenomic libraries have been constructed in high capacity vectors such as BAC [24,25], Cosmid [26] and Fosmid [27] and low capacity vectors such as plasmid [11,28,29]. High capacity vectors

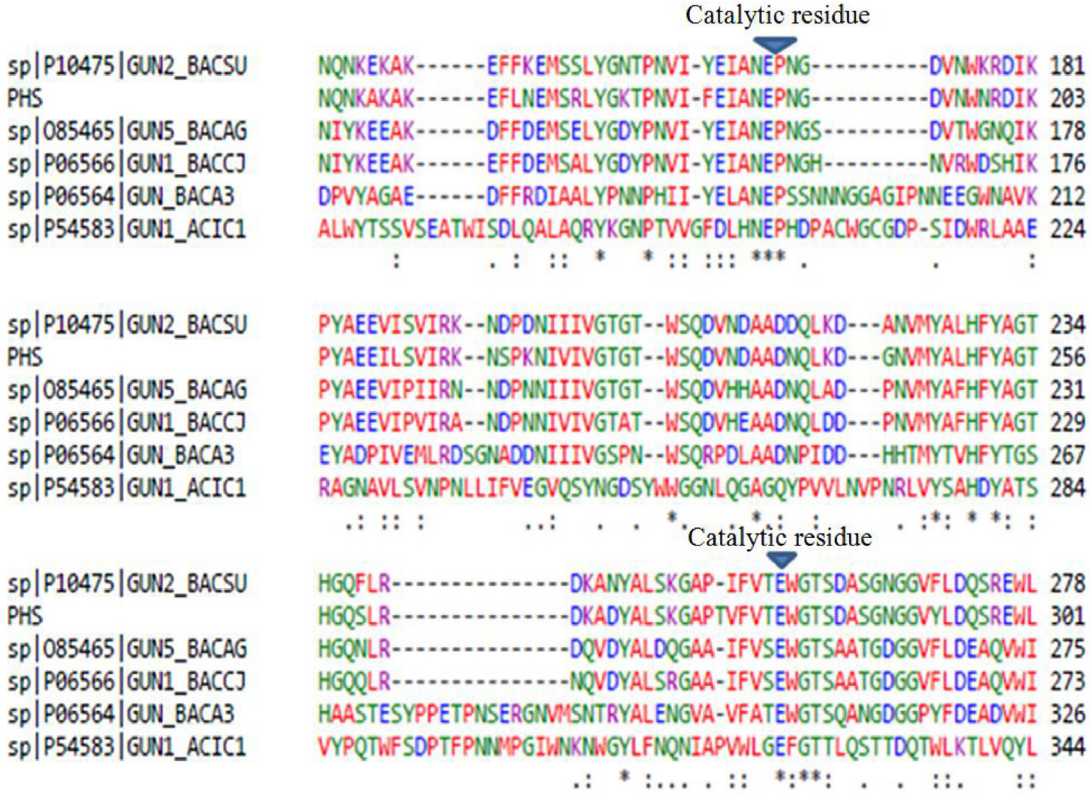

Fig. 1. Multiple sequence alignment of the deduced amino acids of recombinant PHS cellulase and five homologous endoglucanases from GH5 family using ClustalW showing conserved catalytic residues at Glu-191, Glu-280.

offer the possibility of including entire operons and gene clusters as well as achieving a better sequence coverage with fewer clones. Plasmids are typically easy to handle and are suitable for single gene products such as most enzymes, and transformation efficiencies are high. Moreover, it will be easy to sequence DNA fragment cloned in pUC19 using universal primers once positive clone is identified.

3.2. Screening of metagenomic DNA library for endoglucanase gene

The metagenomic DNA library constructed from Puga hot spring metagenome was subjected to function based screening. After screening of ~60,000 clones on CMC agar plates, one clone (PHS) showing endo-β-1,4-glucanase (endoglucanase) activity was selected. The recombinant plasmid pUC PHS was retransformed into *E.coli* DH10B to confirm the endoglucanase activity of the clone. All the three type of cellulases i.e endoglucanses [24,28,11,30,23], exoglucanase [31] and β-glucosidase [32,33] have been retrieved using function based screening of diverse metagemomes.

3.3. Molecular analysis of the PHS endoglucanase

Sequence analysis confirmed PHS clone insert size to be ~2.2 Kb, harboring 7 ORF'S including complete endoglucanase ORF of 1665 bp with 97% similarity to endo-β-1,4-glucanase gene from *Bacillus licheniformis* by BLASTn analysis (Fig. 1) In silico analysis predicted that PHS encods a protein of 554 amino acids with molecular mass of 61 kDa and a signal peptide of 39 amino acids. The size of PHS has been found comparable to endoglucanases derived

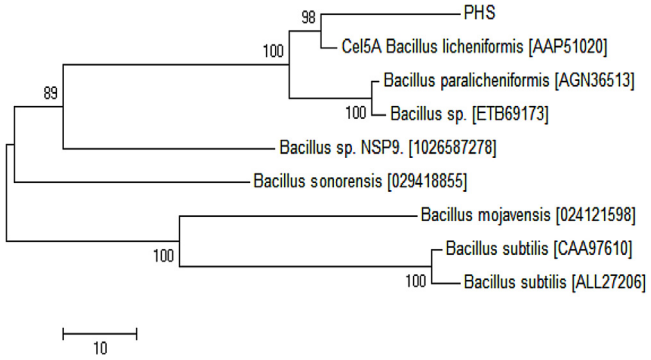

Fig. 2. Phylogenetic tree construction of PHS cellulase with its closest neighbor using the neighbor joining (NJ) method. The tree was constructed according to Tamura et al. (2007).

from *bacillus* or *bacillus* related species ranging from 52 kDa to 62 kDa where as in case of metagenomic derived endoglucanases size varies from 42 kDa to 72 kDa.

PHS conserved domain analysis revealed GH5 catalytic module along with presence of carbohydrate binding domain. Multiple sequence alignment of PHS with five homologous endoglucanases from GH5 family exhibited Glu-191 and Glu-280 as catalytic residue which acts as nucleophile and proton donor, respectively, during substrate hydrolysis (Fig. 2) Six conserved residues such as Arg-113, His-153, Asn-190, His-251, Tyr-253, Trp-337 along with Glu-191 and Glu-280 as catalytic residues were found in PHS (Fig. 2). Six

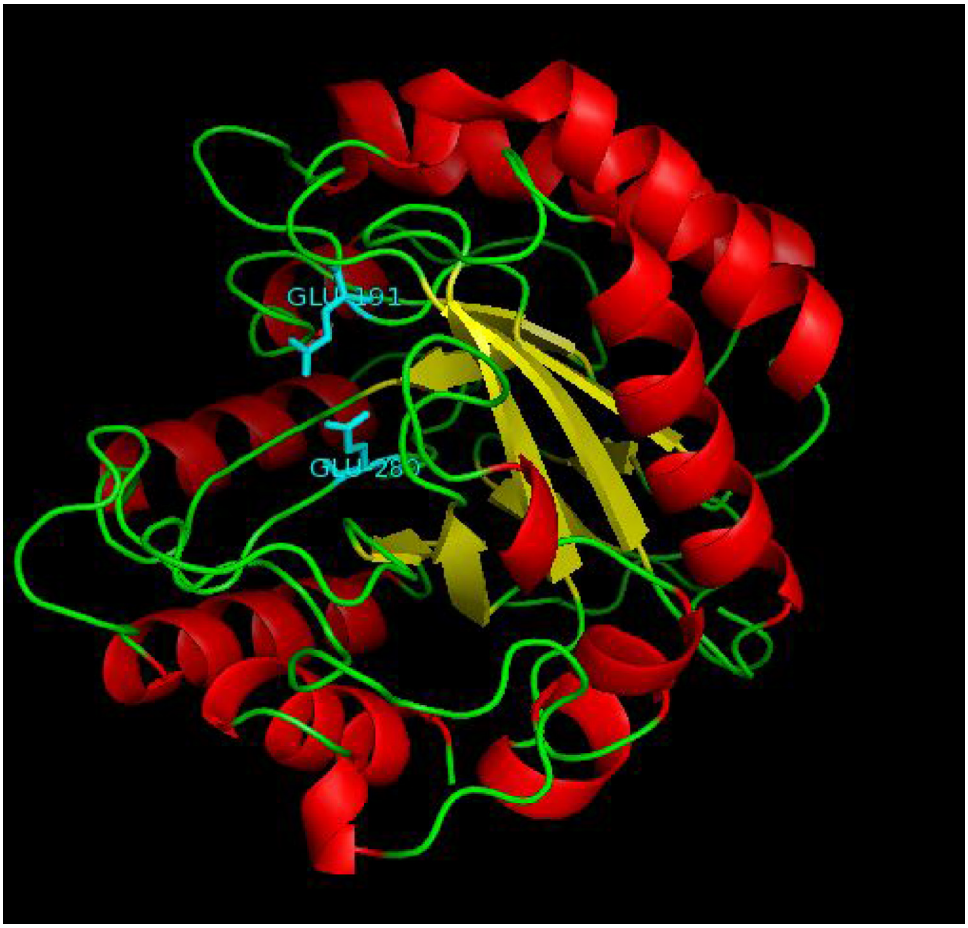

Fig. 3. Insilico structure of recombinant PHS cellulase showing the catalytic residues Glu-191 (Blue), Glu-280 (Blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

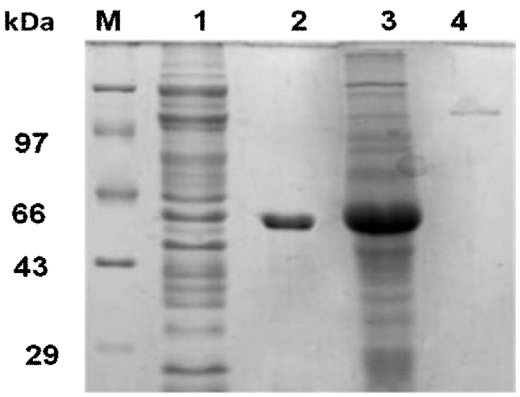

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of recombinant PHS protein stained with Coomassie blue. Lane M Molecualr Weight Marker Lane 1 Total cellular extracts before induction Lane 2 Purified recombinant PHS protein Lane 3 Total cellular extracts after induction.

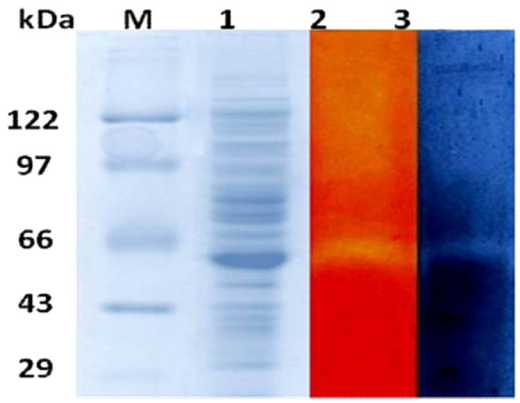

Fig. 5. Zymogram analysis and native polyacrylamide gel electrophoresis (Native – PAGE) analysis of purified recombinant PHS protein Lane M Molecualr Weight Marker Lane 1 Total cellular extracts after induction Lane 2 Zymogram analysis of Purified PHS protein Lane 3 Purified PHS protein on Native PAGE gel.

amino acid residues along with two catalytic residues are conserved in GH5 family cellulases [34,35] (Fig. 3).

Three models of PHS were generated using Swiss model server based on homology modeling. Models generated were validated using Procheck program of NIH SAVES server. Comparative analysis of values used for the analysis of various predicted models indicated Model 2 as the best model. According to the Ramachandran plot, 85.4% of the amino acid residues were scattered within the most favoured regions, 12.3% in the allowed regions and only 1.1% residues were in disallowed regions for Model 2 (Table 1). Three dimensional structure was visualized and quality image was generated using PyMOL software. A superimposition of PHS model with the 3D structure of endoglucanase 5A having 67.4% sequence identity revealed a relative mean standard difference of 0.64 A (less than 1).

3.4. Cloning, overexpression and purification of the PHS endoglucanase

The full-length 1665 bp PHS gene was amplified from recombinant plasmid pUC-PHS using specific primers based on ORF sequence of endoglucanase. Restriction site prediction in the predicted ORF was done using NEB cutter tool and no restriction sites were found for *Bam*H1 and *Not*1 restriction enzymes. Primers were synthesised using primer 3 tool and various parameters were checked using oligo sofware tool (www.oligo.net)

Cell forward primer 5' GGGGGATCCATGGCTGCGGAAAAAGT

ATTTTCGAAAAAT3′(Tm 60.5)

Cell Reverse primer 5' AAAGCGGCCATGTCGCGATTCCAG

TTTGTTCTGCTC3′(Tm 62.8)

The full length endoglucanase gene was cloned into pET-28a (+) expression vector. PHS was over expressed as soluble protein by transferring the recombinant plasmid pET- PHS into *E. coli* BL21 (DE3) after induction with 1 mM IPTG and incubation for 12 h at 30 °C. His 6- tagged PHS was purified from cell lysate using Ni^{2+}-NTA affinity chromatography leading to 7.82 –fold purification and 90% activity yield (Table 2). Purified protein appeared as a single band of 61 kDa on SDS-PAGE, which was in accordance with PHS theoretical molecular mass (Fig. 4) The size of PHS has been found comparable to endoglucanases derived from *bacillus* or *bacillus* related species ranging from 52 kDa to 62 kDa where as in case

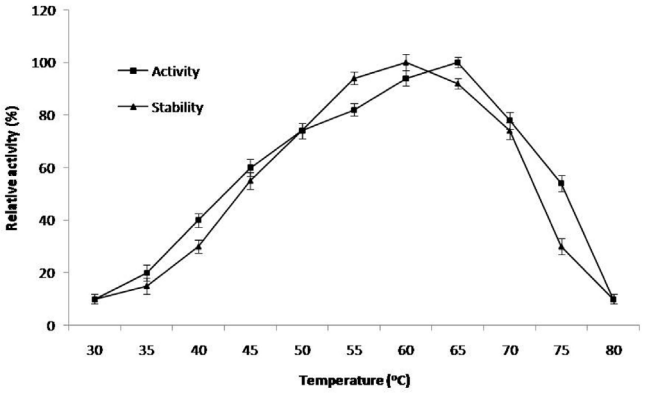

Fig. 6. Effect of temperature on the activity and stability of recombinant PHS endoglucanase. The enzyme was incubated at various temperatures (30 °C–80 °C) with 1% CMC as the substrate. The PHS thermostability was determined by incubating the reaction mixture at various temperatures for 30 min (pH 8).

of metagenomic derived endoglucanases size varies from 42 kDa to 72 kDa. Zymogram analysis of PHS produced a clear band connoting the cellulose hydrolysis at a position corresponding to the molecular mass of protein on SDS- PAGE (Fig. 5)

3.5. Biochemical characterization of PHS endoglucanase

The temperature optima and temperature stability of recombinant purified PHS were measured at different temperatures ranging from 30 °C–80 °C. The temperature optima of the recombinant purified PHS was 65 °C and it was stable in the temperature range of 45 °C–70 °C. The enzyme also showed high thermal stability, retaining 70% of its activity between 50 °C and 70 °C with maximum stability at 60 °C complying with the enzyme stability requirements during various industrial processes (Fig. 6)

Thermostable endoglucanases with optimum temperature ranging from 55° to 80 °C are available from various sources. However most of the endoglucanases reported from metagenomic libraries have temperature optima of 45 °C–60 °C. PHS displayed around 5 °C higher optimal temperature than several metagenomic derived thermostable endoglucanases, including Umcel5G [36], Umcel 9B [37], celM2 [38], C671 [39] and endoglucanases from bacterial sources such as *Bacillus subtilus* [40], *Bacillus licheniformis* [41,42].

The optimum pH was evaluated (pH 4.0–10.0) for recombinant PHS activity and pH stability. PHS displayed the optimal activity at pH 8.0 with 68% and 65% of relative activities at pH 6.5 and pH

Table 1
Model validation of recombinant PHS cellulase using NIH SAVES server.

Regions	Model 1	Model 2	Model 3
Residues in most favoured regions	81.7%	85.4%	83.5%
Residues in additional allowed regions	17.1%	12.3%	13.8%
Residues in generously allowed regions	1.2%	1.1%	2.3%
Residues in disallowed regions	0.0%	1.1%	0.4%
Number of non-glycine and non-proline residues	100	100	100
Number of end-residues (excl, Gly and Pro)	2	2	2
Number of glycine residues	28	28	28
Number of proline residues	9	9	9

Table 2
Purification of recombinant PHS cellulase.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Fold purification	Activity yield (%)
Cell lysate	75.36	2104.08	27.92	1.00	100
Ni-NTA chromatography	7.9	1725.35	218.4	7.92	82

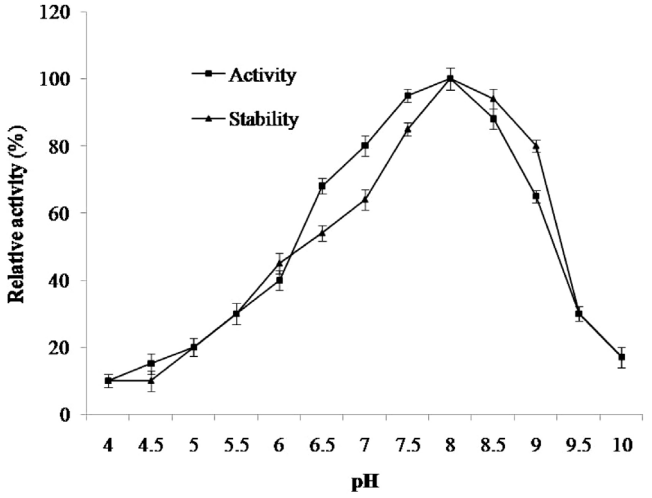

Fig. 7. Effect of pH on the activity and stability of recombinant PHS cellulase. The recombinant PHS endoglucanase was incubated along with reaction mixture in buffers of different pH (pH 4.0–10.0) with 1% CMC as the substrate The PHS stability was determined by incubationg the reaction mixture at various pH for 30 min.

9.0, respectively.It also displayed optimum stability at pH 8.0 with 54% and 84% relative stability at pH 6.5 and pH 9.0, respectively (Fig. 7). Most of the thermostable endoglucanases isolated from various metagenomes and *Bacillus* sp. have lower thermostability than recombinant PHS isolated in the present study. Additionally, most of the reported thermostable endoglucanases have acidic or neutral optimum pH for their activity [42–49] where as recombinant PHS is a thermostable endoglucanase with optimum activity at alkaline pH. Therefore, the recombinant PHS having activity and stability at elevated temperatures and wide pH range finds its prospective in various industrial applications such as laundry detergents, paper recycling and pretreatment processs during bioethanol production [50].

GH5 family cellulases have the capacity to hydrolyse cellulosic as well non cellulosic substrates due to presence of *endo*-β-1,4-glucanase, β-glucosidase, glucan β-1,3-glucosidase, licheninase, *exo*-β-1,4-glucanase, xylanase [51]. Activity towards crystalline forms of cellulose such as avicel for GH 5 cellulases is due to presence of carbohydrate binding module (CBM) [52,53]. Majority of GH 5endoglucanases hydrolyse a variety of cellulosic substrates such as carboxymethyl cellulose (CMC), barley β-glucan, Avicel [45,54–56] where as some are resistant to avicel hydrolysis [39,57]. Substrate specificity of the recombinant PHS was determined using 1% of selected substrate under optimal conditions. The enzyme

Table 3
Substrate specificity of recombinant PHS cellulase towards different substrates.

Substrate	Specific activity
Carboxy methyl cellulose	218.4 U mg^{-1}
Barley β-glucan	89.2 U mg^{-1}
Avicel	0.04 U mg^{-1}
Birchwood xylan	N.D
Oat spelt xylan	N.D
Laminarin	N.D
Cellobiose	N.D

N.D Not detectable.

activity was high for CMC (218.4 U/mg) as compared with Barley β-glucan (89.2 U/mg) and Avicel (0.8 U/mg). No endoglucanase activity was detected on xylan polysaccharides (birchwood xylan and oat spelt xylan) and other polysaccharides such as laminarin, cellobiose. The K_m and V_{max} of PHS for CMC were 3.85 mg/ml and 370.37 μmol/min/1 mg, respectively.

PHS showed high activities for β-1,4-linked glucans, such as CMC and β- glucan from barley, with limited acivity against as avicel. PHS displayed no cellulase activity for β-1,3-linked glucan i.e laminarin, xylan polysaccharides (β −1,4-linked xylose), and non cellulosic substrates (Table 3). Analysis of the utilization of substrates indicated that PHS can hydrolyze β-(1,4)-linked polysaccharides efficiently where as limited activity towards avicel is due to presence of CBM in PHS endoglucanase.

PHS displayed V_{max} (370.37) higher than mostly reported thermostable endoglucanases from metagenomes, including dairy cow rumen metagenome (178.57 μmol/min/mg), mangrove soil metagenome (16.3 μmol/min/mg), *Bursaphelenchus xylophilus* metagenome (333.33 μmol/min/mg), *Bacillus* sp. (25 μmol/min/mg) except for soil metagenome (1941 μmol/min/mg) [(Fig. 8). PHS showed K_m (3.85 mg/ml) lesser than most of the metagenomic derived thermostable endoglucanases [11,46,50,55,58] (Fig. 8). Low K_m value and high V_{max} value of PHS makes it all together unique from already known thermo-alkalistable endoglucanses.

Metal ions such as Co^{2+}, Ca^{2+} and EDTA has no effect on PHS activity where as Fe^{3+} increased the endoglucanase activity by 5%. Cu^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+} reduce the enzyme activity to 92%, 89%, 86%, 58% respectively (Table 4). Low inhibitory effect of Mn^{2+} on endoglucanase activity is in contrast to with previous reports of *Bacillus amyloliquefaciens* DL-3 [59] and *Bacillus flexus* [60]. It has been reported that the inhibition of cellulase activity by Hg^{+} ion might be related to its binding with thiol groups, tryptophan residue, or the carboxyl group of amino acid residues in the enzyme. The inhibition of PHS by Co^{2+}, and Cu^{2+} ions could be due to competition between the exogenous cations and the

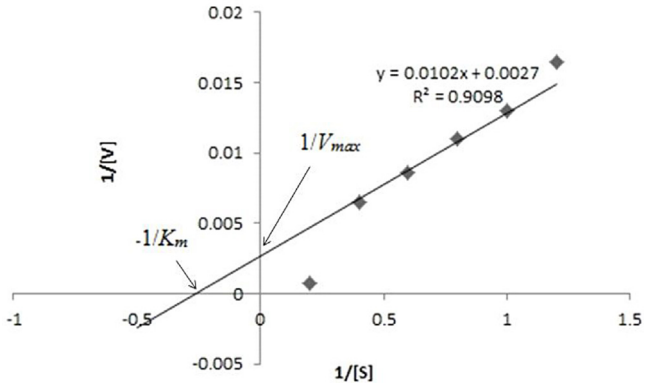

Fig. 8. Kinetic characterisation of PHS cellulase using lineweaver-Burk double reciprocal plot (K_m and V_{max} values calculated were 3.85 mg/ml and 370.37 μmol/min/mg).

Table 4
Effects of various metals ions on the stability of recombinant PHS cellulase.

Metal Ion	Concentration	Relative activity
None	–	100 ± 0.37
Cu^{2+} ($CuSO_4$)	10 mM	88 ± 1.87
Co^{2+} ($CoCl_2$)	10 mM	85 ± 1.34
Mn^{2+} ($MnCl_2$)	10 mM	60 ± 1.246
Fe^{3+} ($FeCl_3$)	10 mM	34 ± 1.13
EDTA	10 mM	34 ± 0.98

Table 5
Effects of various detergents on the stability of recombinant PHS cellulase.

Detergent	Concentration	Relative activity
None	–	100 ± 0.34
Tween 20	0.5%	115 ± 1.28
Tween 40	0.5%	108 ± 1.17
Tween 80	0.5%	105 ± 0.84
Triton X 100	0.5%	95 ± 1.24
PEG	0.5%	108 ± 0.21
SDS	0.5%	5 ± 0.84
CTAB	0.5%	30 ± 0.34

protein-associated cations, resulting in decreased metallo-enzyme activity. Metal ions such as Fe^{2+}, Cu^{2+} and Zn^{2+} has inhibitory effect on the activity of majority of metegenomic derived GH5 family hydrolases [36,37,57,61]. For GH5 endoglucanases, Cu^{2+} and Zn^{2+} exert inhibitory effect [56,62] whereas Fe^{2+} can lead to enhancement as well reduction in enzyme activity [43,32,62,63]. Mn^{2+} has inhibitory, stimulatory and no effect on GH5 endoglucanases [46,61,63]. Addition of Mn^{2+} imparts molecular rigidity leading to decrease in enzyme activity as reported in the literature.

Non-ionic detergents such as Tween 20, Tween 40, Tween 80, Triton X- 100 and PEG increased the activity by 22%, 30%, 42%, 56% and 18% respectively, whereas cationic detergent CTAB and SDS decreased the enzyme activity to 30% and 5% respectively (Table 5) Non ionic detergents like Tween 20, Triton- 100 can increase, decrease or no effect on enzyme activity GH5 family hydrolases [11,36,39,43,62,64,65]. Tween-20 and Triton-100 increases enzyme activity by reducing thermal/mechanical stress [50]. In the present study, Cations (Cu^{2+}, Zn^{2+}, Mn^{2+}) has inhibitory effect where as Fe^{3+} and non ionic detergents like Tween 20, Triton X-100 increased the PHS activity but detergents such as SDS, CTAB reduced the PHS activity drastically. No effect of EDTA, Ca^{2+} ion on PHS activity clearly depicted that PHS is not a metallo enzyme. In industry, various amounts of non-ionic detergents as well as ions are present during enzymatic hydrolysis step of the process. No inhibition by non-ionic detergents as well ions make PHS process suitable for various biotechnology industry.

PHS endoglucanase is thermostable as well as alkalistable with activity over broader range of pH and over elevated temperatures. Additionally, PHS endoglucanase is resistant in the presence of several divalent cations, non-ionic detergents, and chelating agents that are commonly used in various industrial processes. The distinctive attributes of the recombinant PHS endoglucanase makes it a potential candidate for various process suitable applications that could be exploited for the production of products of commercial importance such as biofuel production, detergents and paper recycling.

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