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Cloning, expression of β-1,3-1,4 glucanase from *Bacillus subtilis* SU40 and the effect of calcium ion on the stability of recombinant enzyme: *in vitro* and *in silico* analysis

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Abstract:
A new glucanolytic bacterial strain, SU40 was isolated, and identified as *Bacillus subtilis* on the basis of 16S rRNA sequence homology and phylogenetic tree analysis. The gene encoding β-1,3-1,4-glucanase was delineated, cloned into pET 28a+ vector and heterologously overexpressed in *Escherichia coli* BL21(DE3). The purified recombinant enzyme was about 24 kDa. The enzyme exhibited maximum activity (36.84 U/ml) at 60°C, pH 8.0 and maintained 54% activity at 80°C after incubation for 60 min. The enzyme showed activity against β-glucan, lichenan, and xylan. Amino acid sequence shared a conserved motif EIDIEF. The predicted three-dimensional homology model of the enzyme showed the presence of catalytic residues Glu105, Glu109 and Asp107, single disulphide bridge between Cys32 and Cys61 and three calcium binding site residues Pro9, Gly45 and Asp207. Presence of calcium ion improves the thermal stability of SU40 β-1,3-1,4-glucanase. Molecular dynamics simulation studies revealed that the absence of calcium ion fluctuate the active site residues which are responsible for thermostability. The high catalytic activity and its stability to temperature, pH and metal ions indicated that the enzyme β-1,3-1,4-glucanase by *B. subtilis* SU40 is a good candidate for biotechnological applications.

Background:
The β-glucan, a homo-polysaccharide of glucose molecule linked by the glycoside bond forms the major constituents of the cell wall of higher plant family *Poaceae*. This group of carbohydrates is made up of approximately 1,200 glucose units, linked through β-1,3-1,4 glycosidic bonds and β-1,3 linkage account for 25-30%. Enzymatic degradation of β-1,3-1,4-glucans is activated by three different endogenous glycosyl hydrolases: 1,4-β-D-glucan 4-glucanohydrolase (EC 3. 2. 1. 4), 1,3- β-D-glucan 3-glucanohydrolase (EC 3. 2. 1. 39) and 1,3-1,4-β-D 4-glucanohydrolase (EC 3. 2. 1. 73). Among endo-glycosidases, the 1,3-1,4- β-D-glucan 4-glucanohydrolase (β-1,3-1,4-glucanase or lichenase) is reported to be most active. The β-1,4-glucosidic bonds are present conterminous to the β-1,3 linkages of lichenan or barley β-glucan and yield the final products such as cellobiosyltriose and cellotriosyltetrose **[1].** The β-1,3-1,4-glucanase has attracted commercial interest due to its importance in beer fermentation and poultry feed formulations. The β-glucans cause several industrial processing problems during malting. They reduced the barley seed extract, the amount of wort separation, formation of brumous condition and viscous precipitates in beer. Now a days, malt enzymes are replaced by β-1,3-1,4-glucanase to reduce these problems **[2].** In order to increase the feed conversion ratio (FCR) of non-ruminal animals β-1,3-1,4-glucanase is added as a supplement. In poultry industries, the water-soluble non-starch

polysaccharide acts as an anti-nutritional agent. Poultry feed mixed with β-1,3-1,4-glucanase and xylanase enzymes enhances the weight gain, feed intake and the digestibility of nitrogen (+5.6%) and lipids (+6.2%) and also reduces the sticky droppings which is a major sanitary problems.

The β-1,3-1,4-glucanase genes have been reported from prokaryotes and eukaryotes. While microbial enzymes are classified as members of glycosyl hydrolase family 16 plant enzymes are classified as glycosyl family 17 **[3].** Genes encoding β-1,3-1,4-glucanase have been delineated and characterized from the members of *Bacillaceae* family and non-*Bacillus* bacteria such as *Clostridium thermocellum*, *Streptococcus bovis*, *Fibrobacter succinogenes* and *Paenibacillus* sp.

The β-1,3-1,4-glucanase enzymes produced by *Bacillus* and non-*Bacillus* sp. have shown high degree of sequence homology in their catalytic domains and proteins shared a conserved amino acid sequence "EIDIEF" **[4].** Presence of two glutamic acid residues in the motif plays a key role in hydrolytic activity. Bacterial β-1,3-1,4-glucanases contains calcium ion binding sites in its structure **[5].** Glycoside hydrolases such as β-1,3-1,4-glucanases, hemicellulases are sensitively depends upon the calcium ions for their stability. The removal of calcium ions generally destabilize the thermostability of enzymes. Even though the *in vitro* effects of calcium ions have been studied, *in silico* analyses have not been studied. Present investigation reports the isolation, cloning, expression, purification, characterization and molecular dynamics simulation of a novel recombinant enzyme from *B. subtilis* SU40.

Methodology:

Isolation and identification of bacteria

Soil sample was collected from agricultural field located at Puducherry. Serially diluted soil suspension (10^{-10} - 100 µl) were spread-plated onto barley glucan congo red agar medium and incubated at 30°C for 2 days. Single colonies that produce clear zones were picked up and used for further study. Universal primers, fD1 (5'-GAG TTT GAT CCT GGC TCA-3') and rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') were used to amplify 16S rRNA as described **[6]** and sequenced. The nucleotide sequence has been deposited at Genbank with an accession number HQ834723.

Delineation, cloning and heterologous expression of SU40-glu gene

Gene-specific primers, BSF (5'-ACA ACG GAATTC ATG CAA ACA GGT GGA TCG TTT TTT G-3') and BSR (5'-ATA GTT TA GCGGCCGC TTA TTT TTT TGT ATA GCG CAC CCA G-3') were used **[7]** to amplify the β-1,3-1,4 glucanase gene. The SU40-glu gene was sequenced and deposited in Genbank with an accession number HQ834722. The SU40-glu gene was cloned into pET 28 a+ vector and overexpressed in *Escherichia coli* BL21(DE3) using 1 mM IPTG.

Purification of recombinant β-1,3-1,4 glucanase

Recombinant protein was purified as described earlier **[8].** Purification was achieved by HIS-Select nickel affinity gel (Sigma). Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the eluted fractions.

Characterization of recombinant β-1,3-1,4 glucanase enzyme

Optimum temperature was identified by performing standard assays at various temperatures ranged from 30 to 90°C and the residual activity was measured using dinitrosalicylic method. In order to determine the effects of metal ions, the enzyme solution was incubated in the assay buffer containing 10 mM of Ca^{2+}, Mn^{2+}, Na^{+}, K^{+}, Mg^{2+} and Zn^{2+} metal ions and the enzyme activity was measured. The effect of calcium ion concentration on the recombinant glucanase was studied. Various concentration of Ca^{2+} (5, 10, 15, 20, 25 mM) to the enzyme and enzyme activity was measured as described earlier. Effect on the thermostability was determined by incubating the enzyme with optimum concentration of Ca^{2+} ions at various temperatures ranged from 30 to 90°C.

Molecular modelling

The DNA nucleotide sequence was translated into protein sequence and the deduced amino acid sequence of β-1,3-1,4 glucanase was analyzed with the Expert Protein Analysis System (http://expasy.org/). Translated protein sequence was used as an input for protein Basic Local Search Alignment Tool (BLAST) to identify the best protein template for homology model building. The tertiary structure was modeled using Modeler9v9 and 1GBG as a template. Best model was selected based on the Discrete Optimized Protein Energy (DOPE) score and further verified using SAVeS server **[9]** for Procheck, ERRAT and Verify3D. Verified model was further scrutinized for the protein stability with and without Ca ion using Molecular Dynamics Study.

Molecular Dynamics Simulations

A 5000 ps molecular dynamics (MD) simulation of the β-1,3-1,4 glucanase was carried out with GROMACS4.5. package **[10]** using the GROMOS9643a1 force field. The initial conformation was taken from the one with binding energy closest to experimental binding energy and binding constant. The topology parameters of β-1,3-1,4 glucanase were created by using Gromacs program. Then the protein was immersed in a cubic box (7.335×6.135×8.119 nm) of extended simple point charge (SPC) water molecules. The solvated system was neutralized by adding chlorine ions in the simulation box. To release conflicting contacts, energy minimization was performed using the steepest descent method of 1000 steps, followed by the conjugate gradient method for 1000 steps. The MD simulation studies consist of equilibration and production phases. Finally, the full system was subjected to 5000 ps MD at 300 K temperature and 1 bar pressure **[11, 12].**

Results & Discussion:

Isolation and identification of bacteria

Based on the 16S rRNA gene sequence homology and molecular phylogenetic tree analysis, the new glucanolytic bacterial strain SU40 was identified as *Bacillus subtilis*.

Delineation, Characterization of SU40-glu gene

Using gene-specific primers the putative SU40-glu gene (650 bp) was delineated and sequenced. BLAST search and phylogenetic tree analysis of amino acid sequence showed 97% homology with the β-1,3-1,4 glucanase enzyme (AAO18342) of *B. licheniformis*. The ORF containing 650 bp encodes 214 amino acids with a theoretical molecular mass and pI of 24.2 kDa, 5.78

respectively. The conserved motif "EIDIEF" of family GH-16 was found.

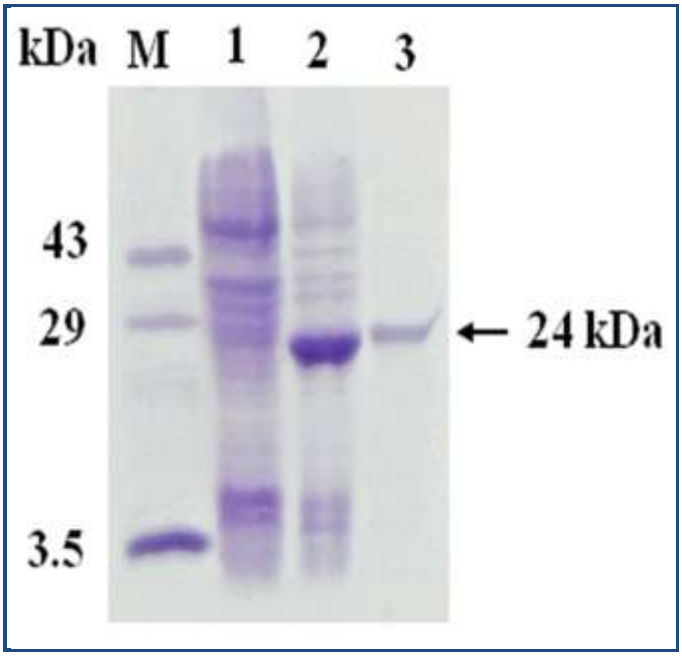

Figure 1: SDS-PAGE of overexpressed and purified SU40-β-1,3-1,4 glucanase. Lane 1, molecular weight marker (Bangalore Genei, India); Lane 2, the un-induced sonicated cell supernant of Escherichia coli pET28a+ - SU40 as control; lane 3, sonicated cell supernatant after IPTG induction; Lane 4, purified β-1,3-1,4 glucanase

Purification of recombinant β-1,3-1,4 glucanase

Purified enzyme was confirmed by SDS-PAGE **(Figure 1).** The molecular weight was determined as 24 kDa which is similar with the theoretically calculated molecular weight. The yield of purified enzyme was 44.88% with 37.85 U/mg of specific activity.

Characterization of recombinant β-1,3-1,4 glucanase enzyme

Various substrates such as β-glucan, lichenan, laminarin, carboxymethylcellulose, PNPG and xylan were used to determine the substrate specificity. Maximum activity was observed against 0.5% β-glucan (34.07 U/ml) followed by lichenan (26.48 U/ml). The recombinant enzyme also showed activity against 0.5% xylan (8.14 U/ml). Hence, SU40-glu acts as a bi-functional enzyme with single catalytic domain. The optimum temperature was found to be 60°C. Around 54% of the total activity was remained after incubating the enzyme upto 85°C for 60 min at pH 8. In the presence of Ca^{2+} ions the enzyme activity was slightly increased. Maximum enzyme activity (142%) of was observed in the presence of 15 mM Ca^{2+} ions and 25 mM concentration decreased the activity to 86%. Thermostability of the enzyme was increased upto 65% at 85°C in the presence of 15 mM Ca^{2+}. Calcium ion has a general stabilizing effect on *Bacillus* β-1,3-1,4 glucanases. Thermostability was highly affected by the removal of the bound calcium ion. In bacterial β-1,3-1,4 glucanases, N-terminal loop contributes to the higher thermostability when complexed with calcium ion. Calcium ion also plays the role in stabilizing the native protein **[5].** Calcium is bound to the backbone carbonyl oxygens of Pro9, Gly45, Asp207 and carboxylate oxygen of Asp207 and two water molecules **[13].** The enzyme SU40-glu also showed Pro9, Gly45 and Asp207, which may acts as a binding domain for calcium ion.

Metal ions such as Mn^{2+}, Na^{+}, K^{+}, Mg^{2+} and Zn^{2+} showed little or no effect on the enzyme activity. However, the mercury ion totally inhibited the activity because Hg^{2+} ion may reacts with the amino acid cysteine in their side group –SH moiety. The SU40-glu enzyme showed the presence of four histidine residues (His130, His145, His166 and His205) and the mercury ion that may be involved in binding of imidazole ring of histidine. This altered cysteine and histidine residues on the protein chain, affect the protein folding in its tertiary structure and the shape of the active site and thus, inhibits the activity of the enzyme **[14].**

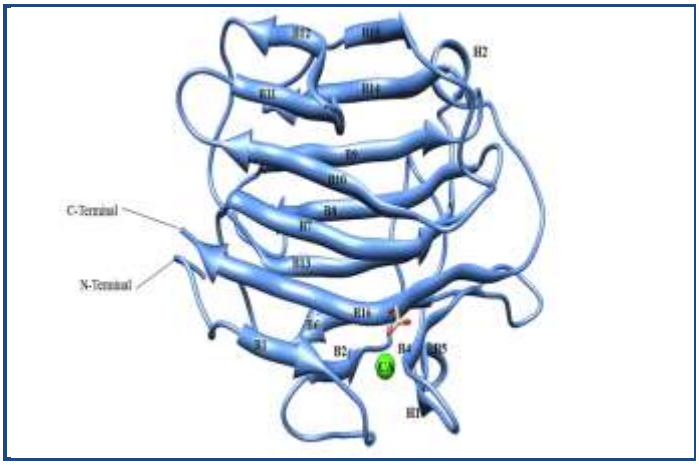

Figure 2: Molecular three-dimensional model of SU40- β-1,3-1,4 glucanase with CA ion. The predicted structure of β-1,3-1,4 glucanase exhibited the occurrence of two α-helices, 16 β-strands and 19 loop turns.

Molecular modelling

The template protein (1GBG) and SU40-glu showed 90% sequence identity and the homology model of SU40-glu were generated using Modeler9v9 with and without Ca ion **(Figure 2).** The predicted structure of β-1,3-1,4 glucanase showed one disulphide bridge between Cys32 and Cys61 residues. The active site residues were identified as Trp103, Asp104, Ile106, Ile108 and Glu109 in the β-strand. Binding sites in SU40-glu were predicted as Phe33, Val91, Ser93, Phe95, Trp106, Glu108, Asp110, Glu112, Tyr126, Asn185 and Trp187 by CASTp server (http://sts-fw.bioengr.uic.edu/castp/calculation.php). Earlier study indicated that Asp107, Glu105 (as nucleophile) and Glu109 (as acid catalyst) are essential for enzyme activity **[6].** The PROCHECK analysis confirmed the presence of 95.7% of amino acids in the favored region, 3.8% of amino acids in the additional allowed region and 0.5% of amino acicds in the generously allowed region with a Verify3D of 99.08% and ERRAT value of 80.838. The three-dimensional model of SU40-glu revealed the presence of all these amino acids in the same reported positions which may be responsible for the enzyme activity.

Molecular Dynamics Simulations

The MD simulation studies of SU40 β-1,3-1,4 glucanases with and without Ca ions have been performed for 5ns. The protein shows its stability throughout the simulations period. But in the

absence of Ca, protein energy slightly moves to high with compare to Ca bound protein complex **(Figure 3A).** Root Mean Square Deviation (RMSD) clearly state that there is no dramatic change in the protein backbone deviation. Marginal fluctuations difference was noticed during the trajectory period **(Figure 3B),** while RMSF graph revealed that in the lack of Ca ion some of the Ca bound residues and active site residues [DMFNCTWRAN (31-40), T (45 CA bound residue), SYNKFD (58-63), W(106), D(193) were fluctuated abnormally **(Figure 3C).** Radius of gyration **(Figure 3D)** also suggests that the Ca bound structure of SU40 Beta 1, 3-1, 4 glucanase is more stable which supports our experimental results.

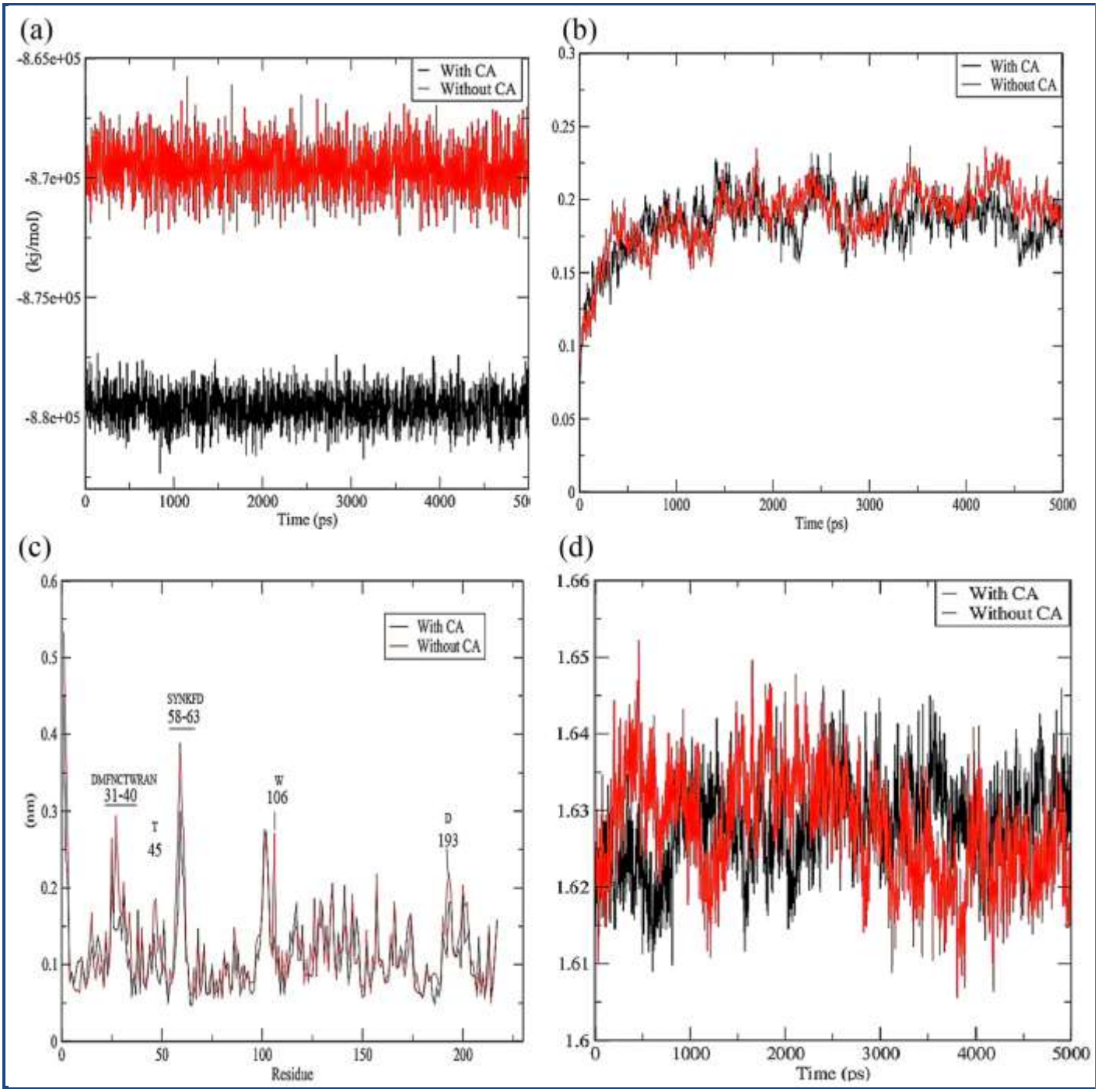

Figure 3: Evaluation of the trajectory 5,000 ps for the calculation of **a)** Potential energy; **b)** Root Mean Square Deviation; **c)** RMSF and **d)** Radius of gyration. The backbone RMSF of Ca^{2+} free SU40- β-1,3-1,4 glucanase (red) showed fluctuation in active site residues. (Black colour – with Ca^{2+} and Red colour – without Ca^{2+}).

Conclusion:

The gene encoding β-1,3-1,4-glucanase was delineated from *Bacillus subtilis* SU40, cloned into pET 28a+ vector and overexpressed in *Escherichia coli* BL21(DE3). The recombinant enzyme (24 kDa) was purified to homogeneity. Purified enzyme exhibited a high catalytic activity and stability to temperature, pH and metal ions. Experimental analysis suggested that the presence of Ca ions improved the thermostability of SU40 β-1,3-

1,4 glucanase. Molecular dynamics simulation studies also revealed that the absence of calcium ion fluctuate the active site residues which are responsible for thermostability.

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