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Bioprospecting of Thermostable Cellulolytic Enzymes through Modeling and Virtual Screening Method

R. Navanietha Krishnaraj[1,2], Dipayan Samanta[1], Anand Kumar[1] and Rajesh Sani[2*]

[1]Department of Biotechnology, National Institute of Technology Durgapur, Mahatma Gandhi Avenue, Durgapur 713209, INDIA

[2]Department of Chemical and Biological Engineering, South Dakota School of Mines and Technology, Rapid City 57701, USA

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Abstract

Cellulolytic enzymes are promising candidates for the use of cellulose in any bioprocess operations and for the disposal of the cellulosic wastes in an environmentally benign manner. Cellulases from thermophiles have the advantage of hydrolyzing cellulose at wider range of operating conditions unlike the normal enzymes. Herein we report the modeled structures of cellulolytic enzymes (endoglucanase, cellobiohydrolase and β-glucosidase) from a thermophilic bacterium, *Clostridium thermocellum* and their validation using Root Mean Square Deviation (RMSD) and Ramachandran plot analyses. Further, the molecular interactions of the modeled enzyme with cellulose were analyzed using molecular docking technique. The results of molecular docking showed that the endoglucanase, cellobiohydrolase and β-glucosidase had the binding affinities of -10.7, -9.0 and -10.8 kcal/mol, respectively. A correlation between the binding affinity of the endoglucanase with cellulose and the enzyme activity was also demonstrated. The results showed that the binding affinities of cellulases with cellulose could be used as a tool to assess the hydrolytic activity of cellulases. The results obtained could be used in virtual screening of cellulolytic enzymes based on the molecular interactions with the substrate, and aid in developing systems biology models of thermophiles for industrial biotechnology applications.

Keywords: Cellulose, Thermozymes, Molecular docking, Binding affinity, Molecular interaction

Introduction

Cellulose is the major component of plant biomass, and strongly meshed by covalent and non-covalent interactions [1]. The cellulosic wastes are produced in very large amounts from domestic, industrial and agricultural sources [2, 3]. As per the report of the U.S. Department of Energy (2011), total primary residues from agricultural source alone were projected to be 320 million dry tons by 2030 [4]. Microorganisms are potential candidates for degradation of the cellulosic wastes. The microorganisms are capable of producing different cellulolytic enzymes such as endoglucanase, β-glucosidase and cellobiohydrolase [5]. Several bacteria such as *Geobacillus* sp., *Bacillus licheniformis*, *Cellulomonas cellulans*, *Clostridium thermocellum* and *Staphylococcus aureus* have been reported for the production of cellulolytic enzymes [6-8].

These mesophilic organisms demand the need for ambient conditions of temperature for growth and catalytic activity. The enzymes from the mesophilic organisms are fragile and have a very narrow range of operating conditions such as temperature and pH. They are prone to denaturation even with small increments/decrements in temperature. The use of thermophiles could confer thermostability to the enzyme besides helping in accelerating the catalytic rates [9, 10]. Because of the stability of the cellulolytic thermozymes and advantage that they can be synthesized in the laboratory at large scale, the cellulases from thermophiles are realized to be the promising options for industrial applications. Attempts have also been made to engineer organisms/enzymes to confer thermostability and enhanced catalytic rates [11, 12].

Clostridium thermocellum is one such thermophilic organism which possesses cellulolytic activity and is shown to have applications in consolidated bioprocessing (CBP) applications [13]. Reports are available on the production of ethanol, biohydrogen and formate using *Clostridium thermocellum* with cellulose as a substrate [14-16]. Hirano et al. (2016) made a detailed investigation on the enzymatic diversity of the *Clostridium thermocellum* cellulosome and showed that the hydrolysis of different cellulosic feedstocks by the cellulosome requires different enzymatic composition of cellulosome as well as different structures of cellulosome [17].

Herein we model the structures of three different thermozymes of *Clostridium thermocellum* namely endoglucanase, cellobiohydrolase and β-glucosidase which play a key role in the hydrolysis of cellulose. The modeled structures of these three key enzymes can help to predict the hydrolytic activity based on the molecular interactions with cellulose. The computational strategies developed in

*Corresponding author: Email: Rajesh.Sani@sdsmt.edu

current study can be used for screening the thermozymes for cellulolytic activity based on the molecular interactions between the structure of the enzyme and the structure of cellulose. Screening the ideal enzyme with high catalytic activity and stability to slightly broader range of operating conditions is a key step for developing a bioprocess for real time application. *In silico* techniques can help to screen the enzymes based on the virtual screening approach in a simple manner and in a shorter time. The experimental techniques are laborious, time consuming and demand sophisticated facilities. The binding energy between the enzyme and the substrate provides the clues for the substrate specificity and catalytic rates of the enzymes. These modeled structures can also be helpful for the molecular dynamics investigations to study the thermostability of the enzymes as well as the enzyme-substrate interactions at different physical conditions such as temperature. The structures of these three key enzymes will help in developing a systems biology model for understanding the cellulolytic activity/mechanisms of *Clostridium thermocellum*.

Materials and Methods

Homology modeling

The amino acid sequences of thermozymes endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* ATCC 27405 were retrieved from Swissprot protein sequence database using their accession numbers P0C2S2, Q9L3J2 and P26208, respectively [18]. The sequences of thermozymes endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* had 742, 660 and 442 amino acids, respectively. The sequences of these thermozymes served as the target sequences for the experiment. The respective template protein structure for each of these three target sequences were identified based on the maximum similarity with the cellulolytic enzyme sequences (query sequences) and used as a template for modeling the structures of the three target enzymes. The protein sequence that is homologous to the query sequence was identified from the Brookhaven Protein Data Bank (PDB) using PSI-BLAST [19, 20].

The best template for the sequences of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* were identified based on the e-value and sequence identity. The Clustal Omega was used to align the template protein sequence and the target sequences [21]. Homology modeling of the sequences of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* were carried out with their respective templates using Modeller 9.17. The modeled structures were ranked based on the internal scoring function. The modeled structure of target sequence with the least internal scores was identified and utilized for model validation.

Validation of the modeled structure

The overall stereochemical quality of the modeled structures of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* were analyzed using Ramachandran plot analysis and RMSD analysis. The RAMPAGE server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) was used to analyze the Ramachandran plot [22, 23]. The root mean square deviation (RMSD) was calculated between the structure of the template and the newly modeled thermozyme to compare their structures. The RMSD of the modeled thermozyme with its template was obtained by superimposing the modeled thermozyme with its respective template structure using PYMOL viewer.

Docking experiment

Molecular docking investigations were carried out to study the molecular interactions of the modeled structure of the thermozyme with its substrate (cellulose) and to find the binding energy between them. The structure of cellulose was modeled (with 5, 7, and 9 glucose subunits) using Chem sketch and chosen as the ligand for the computational investigation. The structure of cellulose (with 9 glucose subunits) and modeled structures of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* were processed by adding hydrogen atom with the Molecular Graphics Laboratory tools (http://mgltools.scripps.edu/). The energy of the cellulose ligand was minimized by computing gasteiger charges before storing in the pdbqt format for the docking studies with the Autodock vina 4.2 (http://vina.scripps.edu/). The entire ligand binding region of the enzyme was covered within the GRID. The dimensions of the Grid were kept as 40 along all the three directions. Docking studies were performed with the Autodock Vina algorithm with AMBER force field and Monte Carlo simulated annealing [24]. The structures of the thermozymes were kept as rigid and the cellulose structure was kept flexible throughout the docking process.

Results and Discussion

The sequences of endoglucanase from *Clostridium cellulolyticum* (PDB Id: 1GA2), beta-glycosidase from *Pyrococcus horikoshii* (PDB Id: 1VFF) and a catalytic domain of *Cidothermus cellulolyticus* endocellulase (PDB Id: 1ECE) had the maximum similarity of 45, 36 and 35.569%, respectively with the sequences of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum*, respectively. The crystal structure of endoglucanase from *Clostridium cellulolyticum*, beta-glycosidase from *Pyrococcus horikoshii* and a catalytic domain of endocellulase of *Cidothermus cellulolyticus* were chosen as template to model the structures of the enzymes endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum*, respectively. The modeled structures of the enzymes endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* is shown in the Fig. 1.

Homology modeling of thermozymes with the Modeller 9.17 yielded 5 models for each of these three thermozymes of *Clostridium thermocellum*. Among the five different models generated to each of these target sequence, the model with the least Discrete Optimized Protein Energy (DOPE) score was chosen as the best model of the thermozyme. The DOPE scores of all the models generated for the three different sequences of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* are shown in Table 1.

The best model for sequences of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* was identified based on the minimal DOPE score. The chosen model for each of these thermozymes was subjected to validation of its stereochemical characteristics. The structures with the minimal DOPE score had the lowest potential energy and hence they had the stable structure. The percentages of phi and psi angles that occur in the allowed and disallowed regions for the chosen structures of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* were calculated from the Ramachandran plots analysis. The Ramachandran plots for structures of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* are shown in Fig. 2 along with their Ramachandran plot characteristics.

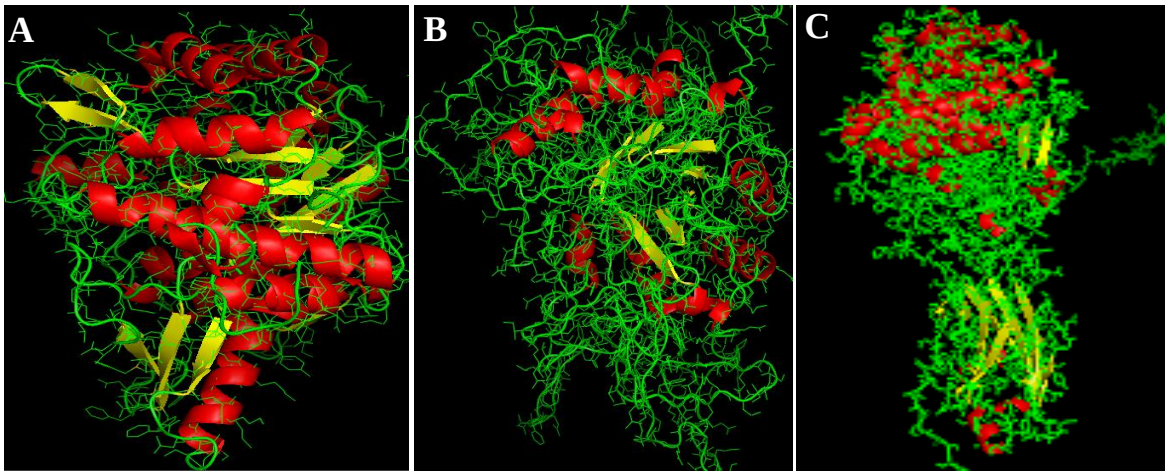

Fig. 1: Modeled structures of **(A)** endoglucanase, **(B)** cellobiohydrolase and **(C)** β-glucosidase of *Clostridium thermocellum* ATCC 27405.

Table 1: Dope score of produced models of enzymes of *Clostridium thermocellum*.

Sl. No.	Enzymes	Dope Scores				
		Structure 1	Structure 2	Structure 3	Structure 4	Structure 5
1	Endoglucanase	-75758.0783	-75950.09375	-75806.03406	-75735.28125	-75689.46875
2	Cellobiohydrolase	-54825.86719	-54428.46875	-55106.41016	-56659.23828	-56393.57422
3	β-glucosidase	-52574.55078	-52413.66797	-52179.46484	-52034.78516	-51749.16797

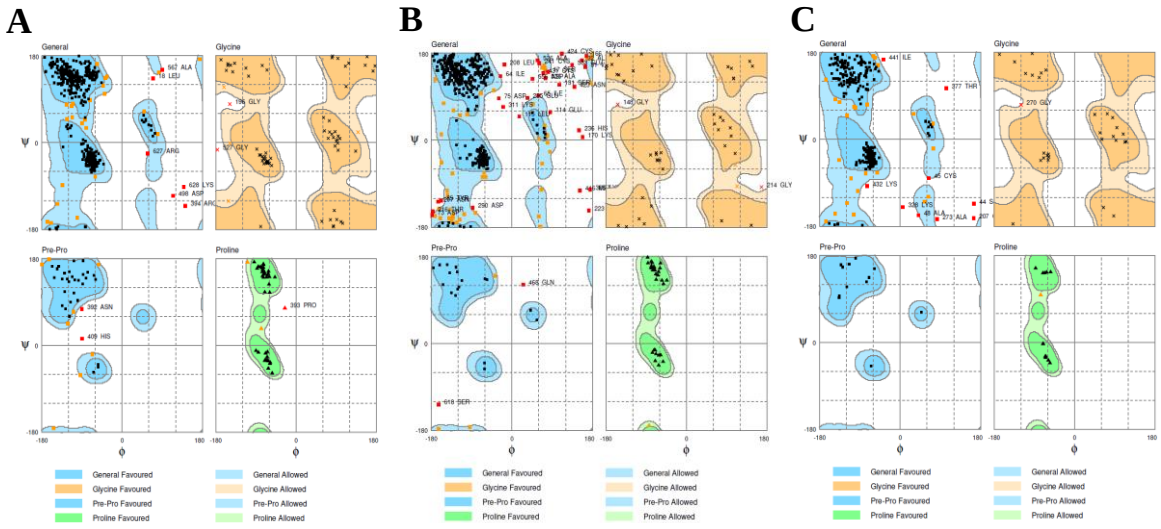

Fig. 2: Ramachandran Plot of the modeled structures of **(A)** endoglucanase, **(B)** cellobiohydrolase and **(C)** β-glucosidase.

The modeled structures of endoglucanase and β-glucosidase had 93.4% and 92% of the residues of the modeled structure in the favorable region. The modeled structure of cellobiohydrolase also had 86.5% of its residues in the favorable region (Table 2). This indicates the good stereochemical characteristics of the modeled structures of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum*.

Further the chosen models of endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* were validated with the RMSD between the modeled thermozymes and their template structures. The RMSD values between the target and template sequences were calculated by superimposing the modeled structure with its respective template structure using the software PYMOL. The RMSD values of endoglucanase, cellobiohydrolase and β-glucosidase with its template structures were found to be 0.280 Å, 0.770 Å and 0.210 Å, respectively. All the three models had very small RMSD values indicating their good stereochemical characteristics. The RMSD values indicate the inter-distance between the structures of target structure and the template structure after the structural alignment.

Fig. 3 depicts the modeled structure of cellulose. Finally, the molecular interactions of the modeled structures of endoglucanase, cellobiohydrolase and β-glucosidase with the modeled structure of cellulose (ligand) were analysed by molecular docking technique. The docking studies were carried out using Autodock vina and their binding energies were calculated. The thermozymes endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum* had the affinity of -10.7 (kcal/mol), -9.0 (kcal/mol) and -10.8 (kcal/mol), respectively. The molecular interactions of modeled endoglucanase, cellobiohydrolase and β-glucosidase with its substrate are shown in the Fig. 4.

Further investigations were carried out to analyze the effect of number of subunits of glucose in cellulose on the binding affinity with endoglucanase, cellobiohydrolase and β-glucosidase of *Clostridium thermocellum*. Table 3 depicts the binding affinity between modeled structure of cellulose (5, 7 and 9 glucose subunits) and thermozymes of *Clostridium thermocellum*. The results show that there is a linear increase in binding affinity between the enzyme and the substrate with increase in number of subunits of glucose. The increase in binding affinities of thermozymes with size (number of glucose subunits) is due to the increase in molecular weight as well as the contact area of modeled cellulose. The increase in size of the cellulose also increases the hydrophobicity which will result in effective binding with the thermozymes and increasing binding affinity of the enzyme cellulase with cellulose.

Table 2: Ramachandran Statistics of the modeled structures of enzymes of *Clostridium thermocellum*.

Sl. No.	ENZYMES	RAMACHANDRAN STATISTICS		
		Residues in favorable region (%)	***Residues in the allowed region (%)***	***Outlier Region (%)***
1	Endoglucanase	93.4	5.1	1.5
2	Cellobiohydrolase	86.5	8.2	5.3
3	β-glucosidase	92.0	5.7	2.3

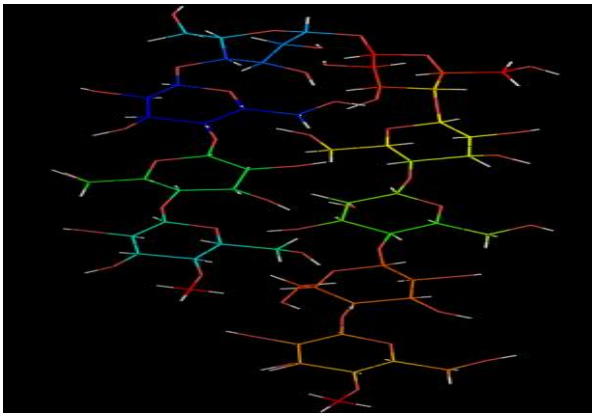

Fig. 3: Modeled structure of cellulose with 5 glucose subunits.

Table 3: Binding affinity between modeled structure of cellulose (5, 7 and 9 glucose subunits) and thermozymes of *Clostridium thermocellum*.

Number of glucose subunits in cellulose	Endoglucanase (kcal/mol)	Cellobiohydrolase (kcal/mol)	β-glucosidase (kcal/mol)
9 glucose subunits	-10.7	-9.0	-10.8
7 glucose subunits	-8.7	-7.5	-7.8
5 glucose subunits	-8.2	-6.8	-7.0

Molecular investigations were also carried out to analyze the correlation between the binding affinity of thermozymes with the modeled structure of cellulose. Molecular docking experiments were carried out with the endoglucanases structure of *Acidothermus cellulotyticus*, *Clostridium cellulolyticum*, *Thermoascus aurantiacus* and *Aspergillus niger* with the structure of cellulose (9 glucose subunits). The endoglucanases of *Acidothermus cellulotyticus*, *Clostridium cellulolyticum*, *Thermoascus aurantiacus* and *Aspergillus niger* had binding affinity of -6.1, -7.2, -7.6 and -9.1 kcal/mol, respectively. The molecular interactions of endoglucanases of *Thermoascus aurantiacus* and *Aspergillus niger* with cellulose is shown in the Fig. 5. The results of binding affinity of cellulose with endoglucanase of different organisms are compared. These binding affinities were correlated with the enzyme activities of the endoglucanase of *Thermoascus aurantiacus*, *Aspergillus niger* and *Clostridium thermocellum*. The enzyme activities of *Thermoascus aurantiacus*, *Aspergillus niger* and *Clostridium thermocellum* of the endoglucanase had the values of 45, 35.7 and 30 IU/mg, respectively (taken from the literature) [25-27]. The binding affinity of cellulose with the thermozymes (calculated using molecular docking) was used to assess the hydrolytic activity of cellulose and for screening the enzyme. Among the three different endoglucanases analyzed, the endoglucanase from *Clostridium thermocellum* had the most minimum binding affinity (-10.1 kcal/mol) and the maximum enzyme activity. The lower binding energy indicates that minimum energy is required to convert the reactant to product which leads to enhanced kinetic rates of the enzyme. Similarly, endoglucanase from *Thermoascus aurantiacus* had the highest binding affinity (-6.1 kcal/mol) and the minimum enzyme activity.

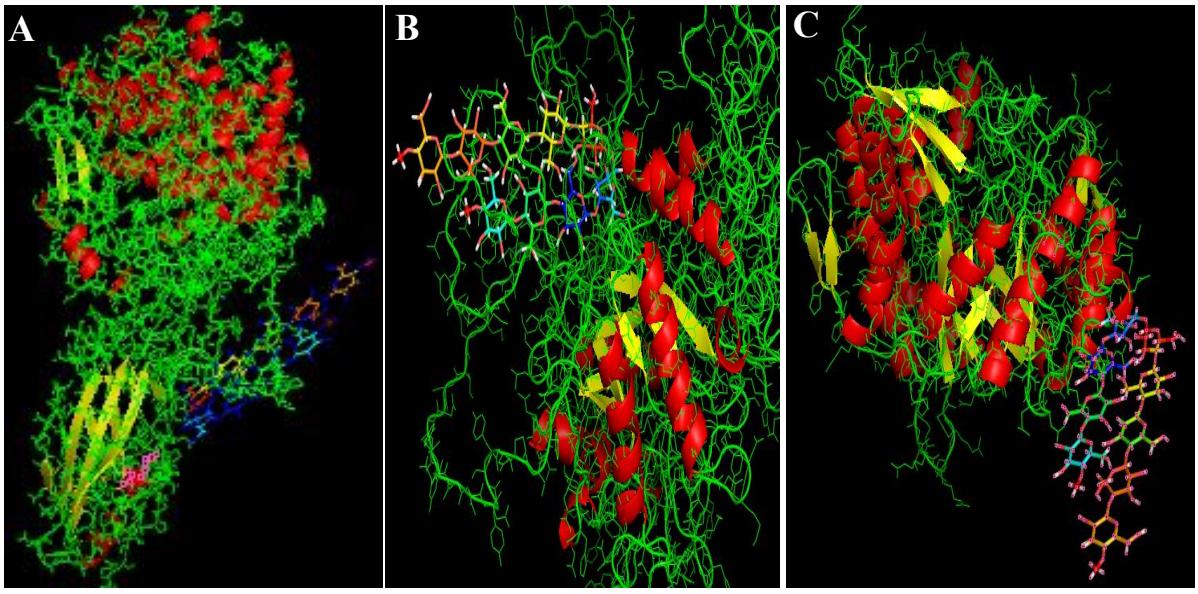

Fig. 4: Molecular Interaction of **(A)** endoglucanase, **(B)** cellobiohydrolase and **(C)** β-glucosidase of *Clostridium thermocellum* with cellulose.

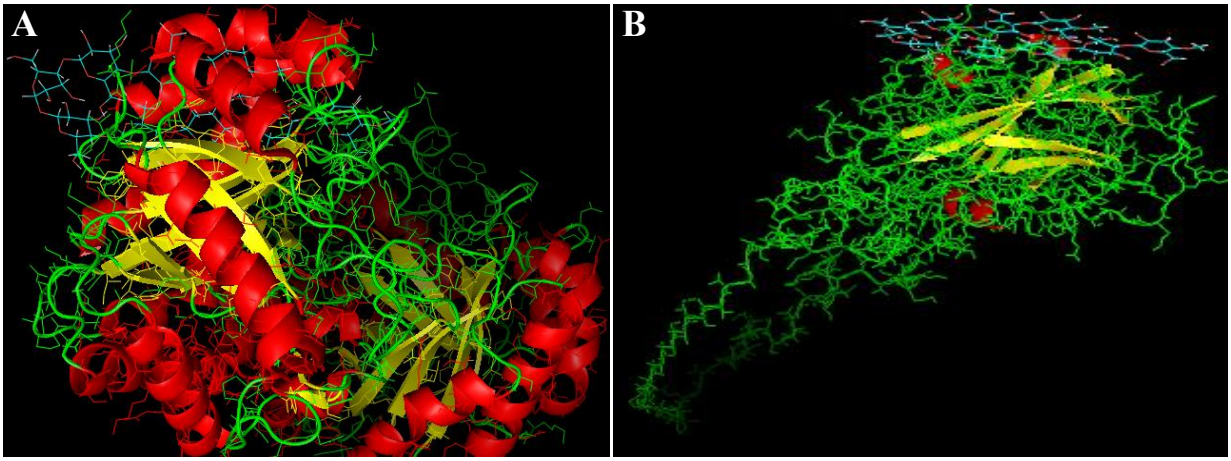

Fig. 5: Molecular interactions of endoglucanases of **(A)** *Thermoascus aurantiacus* and **(B)** *Aspergillus niger* with the modeled structure of cellulose.

These structures will help in virtual screening of cellulolytic enzymes based on the molecular interactions with the substrate. These binding energy values can be used as the standard for predicting the catalytic rates of these enzymes with different lignocellulosic substrates such as corn stover, sugarcane bagasse, switchgrass, wheat straw, etc. Previously, we have reported computational investigations for the screening of the pesticides, drugs, toxicity of nanomaterials and enzymes for bioenergy applications [28-34]. This strategy will also serve as a tool for screening the other cellulolytic enzymes.

Conclusion

Clostridium thermocellum is one of the model thermophilic organism for cellulolytic activity. A new strategy for assessing the cellulolytic activity of the enzyme was developed based on its binding affinity. The endoglucanase from *Clostridium thermocellum* had the minimum binding affinity (-10.1 kcal/mol) among others indicating that minimum energy is required to convert the reactant to product. The correlation between binding affinity and enzyme activity was demonstrated. The lower binding energy leads to enhanced kinetic rates of the enzyme. This study provides insights for developing a systems biology model for elucidating the cellulolytic activity of *Clostridium thermocellum*. This study will also pave way for further developing techniques to screen the cellulolytic enzymes based on the enzyme activity, sensitivity, selectivity and stability of the enzymes.

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Conflict of Interest

Authors declare no conflict of interest.

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