INTRODUCTION

Cellulose is an essential constituent of the primary cell wall of green plants, many forms of algae and the oomycetes (Klemm et al. 2005). It is one of the most abundant natural biopolymer on the Earth and most dominating renewable agricultural waste with great potential for bioconversion to value-added bioproducts (Sadhu and Maiti 2013). Cellulases are the enzymes that hydrolyze β-1,4 linkages of cellulose chains and help in cellulose degradation. Cellulose degrading microorganisms produce all three types of cellulase components that include endo-1,4-β-D-glucanase (EC 3.2.1.4), exo-1,4-β-D-glucanase (EC3.2.1.91) and β-glucosidase (EC 3.2.1.21); and produced by bacteria, fungi, protozoans, plants, and animals (Zhang and Zhang 2013). These enzyme components either work separately in an enzyme catalyzed reaction or act synergistically in the form of a complex enzyme for complete cellulose hydrolysis. Cellulases produced by microorganisms have attracted worldwide attention because of their diverse applications in various industries including food and beverage industry. The major industrial applications of cellulases are as an additive in detergents, in food industry, textile industry, pulp and paper industry as well as in agriculture industry for controlling plant pathogens. The details of cellulase applications in various industries are presented in table 1 (Behera et al. 2017; Jayasekara and Ratnayake 2019). In food industry, cellulase is widely used in the coffee processing and wine making due to its cellulolytic activity (Anoop Kumar et al. 2018; Jayasekara and Ratnayake 2019). Cellulase degrades the skin of the grape and along with it removes tannins and the unpleasant aroma (Claus and Mojsov, 2018). Cellulase also used in reducing food spoilage and extraction of fruit juices along with other enzymes by working synergistically (Kuhad et al. 2011). Now cellulases account for a significant share of the world’s industrial enzyme market and expected to further increase due to application in pretreated cellulase hydrolysis and formation of bioethanol and other bio-based products at commercial level (Zhang and Zhang 2013; Sadhu and Maiti 2013). It is estimated that the global demand for enzymes will increase up to 4.6 percent through 2020 to $7.2 billion. Food and beverages will remain the largest market for enzymes by value, with gains driven by increasing consumption of products containing enzymes. United Nation Department of Economic and Social Affairs (UNDESA) estimates that food demand is expected to increase by 70% by 2050 due to increasing world population to 9.1 billion (http://www.un.org/waterforlifedecade/food_security.shtml). Food enzymes can resolve the shortage of quality food supply globally through increased food production and improvement in the quality such as flavor, texture and nutritional value (Niedelmann 1984; Raveendran et al. 2018).

**Table 1** Function and use of cellulases in various industry (modified from Behera et al. 2017).

| Industry       | Function                                      | Application/Use                                      | References                        |
|----------------|-----------------------------------------------|-----------------------------------------------------|-----------------------------------|
| Food industry  | Degradation of cell wall constituents; reducing viscosity of fruit juice, texture conservation | Fruits juice extraction, food colouring agent, Sensory properties modification of fruits, vegetables and oil, decreasing food spoilage | (Bhat 2000)                      |
| Beer and wine  | Hydrolysis of plant cell wall polysaccharides, Modification of aromatic residues | Improvement in skin maceration and colour extraction of grapes, quality, stability and clarification and aroma of wines | (Galante et al. 1998)             |
| Animal feed    | Pretreatment of agricultural silage and grain feed for partial hydrolysis of lignocellulosic materials | Improvement in the nutritional quality of animal feed; increasing weight of broiler chickens; decreasing pathogenic bacteria accumulation in large intestine | (Cowan 1996; Godfrey and West 1996) |
| Textile and laundry | Break off the small fibre ends on the cotton fabric, thereby loosening the dye after washing, prevention or permanent | Bio-stoning of denim fabrics; bio-polishing of non-denim fabrics, defibrillation of lyocell containing fabrics and bio finishing, production of high quality and environmentally | (Kirk et al. 2002) |
It is well documented that most of the commercial enzymes, including cellulases, used in various industries are derived from mesophilic or thermophilic microorganisms. However, in food and beverages industries application of cold-active enzymes are always preferred due to the following economic and environmental benefits: (1) high specific activity at low temperatures, (2) reduction in energy consumption, (3) preservation of compounds that are volatile and sensitive at high temperatures, (4) reduction in energy consumption, (5) easy inactivation of enzyme by denaturing of enzyme interaction with cellulose, and (6) minimizes undesirable chemical reactions, and (7) decrease the cost of required heat treatments. Due to the enormous applications of cold active cellulase enzyme in different industries, it is required to understand its structural and functional characteristics along with mechanism of cellulose hydrolysis.

### METHODOLOGY

**Reclamion of cellulase sequences and multiple sequence alignment**

In this study, sequences of cellulase enzyme were extracted from three different habitats such as psychrophilic, mesophilic and thermophilic. Based on potential industrial importance. Total 38 full-length cellulase sequences including 25 from bacteria, 11 from fungi and 2 from actinomycetes were selected from NCB (www.ncbi.nlm.nih.gov) in FASTA format. In silico analysis and multiple sequence alignment for all three categories of cellulases were performed by the modified method of Ramya and Pulicherla (2014).

**Phylogenetic investigation and dendrogram construction**

Sequences of cellulase from bacteria, fungi and actinomycetes were aligned by using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). The approaches given by Ramya and Pulicherla (2014) was used for phylogenetic trees construction.

**Crystal structures of cellulases**

In the present work, most prominent source of microbial cellulase from three different habitats viz. thermophilic, mesophilic and psychrophilic were selected. The crystal structure of cellulase from Rhodothermus marinus (thermophile), Bacillus sp. (mesophile) and Pseudoalteromonas haloplanktis (Psychrophile) were extracted from Protein Data Bank (PDB) (Table 2). The preference was given to the crystal structures that were obtained by X-ray diffraction method.

| Organism | PDB ID | Total residue (Amino acids) | UniProt Accession ID |
|----------|--------|-----------------------------|----------------------|
| Xanthomonas citri | 5HOS | 342 | Q8PRD3 |
| Xanthomonas citri | 4W7U | 332 | Q8PRD3 |
| Xanthomonas citri | 4W7V | 332 | Q8PRD3 |
| Xanthomonas citri | 4W7W | 332 | Q8PRD3 |
| Xanthomonas citri with one mutation | 5HPC | 377 | Q8PRD3 |
| Xanthomonas citri with triple mutation | 5HNN | 1011 | Q8PRD3 |
| Caldicellulosiruptor saccharolyticus | 5ECU | 555 | A4XHB2 |
| Bacillus sp. | 5E09 | 537 | D4P8C6 |
| Bacillus sp. | 5E0C | 537 | D4P8C6 |
| uncultured bacterium | 3WX5 | 488 | W8PWF3 |
| uncultured bacterium | 4HTY | 359 | I6PLH5 |
| uncultured bacterium | 4HU0 | 359 | I6PLH5 |
| uncultured bacterium | 3JI1 | 535 | A1E9A6 |
| uncultured bacterium | 3FW6 | 534 | A1E9A6 |
| Rhodothermus marinus | 3B7M | 864 | O33897 |
| Rhodothermus marinus | 2BW8 | 454 | O33897 |
| Rhodothermus marinus | 2BWA | 454 | O33897 |
| Rhodothermus marinus | 2BWC | 454 | O33897 |
| Rhodothermus marinus | 1H0B | 512 | O33897 |
| Pseudoalteromonas haloplanktis | 1TVN | 586 | O86699 |
| Pseudoalteromonas haloplanktis | 1TVP | 586 | O86699 |
Analysis of active site

Active site residues of cellulase enzymes was retrieved from PDB database and by docking studied as describe by the method of Laurie and Jackson (2005).

Molecular docking studies

Ligand preparation

The structures of cellulose unit (2D and 3D) was extracted from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) (Fig. 1). The structure was converted to .pdb format and optimized by means of ligand preparation using default settings in Molegro Virtual Docker (MVD-2010.4.2.0). In the structure, hydrogens were added through PyMol software and ligand was prepared for further docking studies (Ramya and Pulicherla 2014).

Preparation of receptors (proteins) and docking studies

For docking studies, X-ray crystal structure of thermophilic (3B7M), mesophilic (5E09) and psychrophilic (1TVN) cellulases were retrieved from PDB and molecular structure was optimized through MVD and HYPERCHEM software (Adam 2008). The docking was performed by MVD software and best-docked ligand was determined based on MoleDockScore (Ramya and Pulicherla 2014).

Parameters for docking search algorithms

Parameters for algorithm and scoring functions for docking was used as per the method described by Srikanth et al. (2017).

RESULTS

Reclamation of cellulase sequences and multiple sequence alignment

On the basis documentation and as a maximum enzyme producer, total 38 cellulase sequences from different organisms including bacteria, fungi and actinomycetes were extracted from NCBI. The selection was also based on different habitats of organisms which includes thermophiles, mesophiles and psychrophiles and their potential applications at industrial level as mentioned in table 1 (Behera et al. 2017). The details of organisms, their accession numbers and number of amino acids found in enzymes are presented in Table 3. The result of multiple sequence alignment (Clustal Omega) shows major amino acids that are involved in active site (Fig. 2).

| Sl. No. | Microorganism                  | Accession number | Length (Amino acids) |
|--------|--------------------------------|------------------|----------------------|
| 1      | Acidothermus cellulolyticus    | ABK51910.1       | 649                  |
| 2      | Anoxybacillus flavithermus     | GAC90965.1       | 355                  |
| 3      | Anoxybacillus sp.              | EPZ39606.1       | 361                  |
| 4      | Bacillus coagulans             | AEH52529.1       | 352                  |
| 5      | Bacillus sp.                   | BAB19360.1       | 824                  |
| 6      | Bacillus sp. BG-CS10           | ADD62401.1       | 569                  |
| 7      | Bacillus sp. HY2-3             | AAV34758.1       | 499                  |
| 8      | Bacillus sp. NBL420            | AAK73277.1       | 440                  |
| 9      | Bacillus sp. WRD-2             | AAX54913.1       | 499                  |
| 10     | Cellulomonas fimii              | AEE44521.1       | 567                  |
| 11     | Clostridium acetobutylicum      | KHD37670.1       | 878                  |
| 12     | Clostridium autoethanogenenum   | ALU36597.1       | 319                  |
| 13     | Clostridium cellulosovorans    | ADL00682.1       | 404                  |
| 14     | Eubacterium cellulosolvens     | EIM77521.1       | 775                  |
| 15     | Eubacterium sp.                | OLA09410.1       | 342                  |
| 16     | Fibrobacter succinogenes       | ABU45500.1       | 910                  |
| 17     | Methanosarcina thermophila     | BAW29155.1       | 357                  |
| 18     | Microbispora rosea             | SIR96585.1       | 510                  |
| 19     | Microbispora sp.               | ETK32165.1       | 536                  |
| 20     | Pseudalteromonas haloplanktis  | AD47126.1        | 493                  |
| 21     | Pseudomonas sp.                | AEM45646.1       | 467                  |
| 22     | Pseudomonas stutzeri           | AFN77157.1       | 348                  |
| 23     | Rhodothermus marinus           | AAB65594.1       | 260                  |
| 24     | Ruminococcus albus             | ADU21423.1       | 644                  |
| 25     | Ruminococcus flavefaciens      | AAB19708.1       | 455                  |
|        | **BACTERIA**                   |                  |                      |
|        | **Fungi**                      |                  |                      |
|        | **ACTINOMYCETES**              |                  |                      |

Figure 1 Structure of cellulose unit, 2D (a) and 3D (b)
The selected protein sequences obtained from bacteria, fungi and actinomycetes were aligned with the Clustal Omega program. The dendrogram was constructed through MEGA software. The result shows that all the organisms appeared in three different clusters. In addition, the bacteria displayed different clusters. The organisms showed a different group (Fig. 3).

**Phylogenetic tree and dendrogram**

The selected protein sequences obtained from bacteria, fungi and actinomycetes were aligned with the Clustal Omega program. The dendrogram was constructed by using most acceptable and commonly used maximum likelihood method through MEGA software. The result shows that all the organisms appeared in three different clusters. In addition, the bacteria displayed different clusters. The organisms showed a different group (Fig. 3).

![Figure 3 Phylogenetic tree of cellulase sequences](image-url)
Validation of active sites

Protein sequences related to thermophile (3B7M), mesophile (5E09) and psychrophile (1TVN) were extracted from PDB (Fig. 4, 5 and 6). All the three modeled cellulase enzymes viz. thermophilic from *Rhodothermus marinus*, mesophilic from *Bacillus* sp. and psychrophilic from *Pseudoalteromonas haloplanktis* were also confirmed for their active site residues by using blind docking method. Active site residues of thermophilic, mesophilic and psychrophilic cellulases are presented in Table 4 and also represented in Figure 7, 8 and 9, respectively.

**Table 4** Amino acid residues for active site of cellulase enzyme from thermophilic, mesophilic and psychrophilic microorganisms.

| Source                  | Active site residues                                      |
|-------------------------|----------------------------------------------------------|
| Thermophilic Rhodothermus marinus | HIS75, LEU76, LYS77, LEU120, CYS122, ASP239, ILE241, ASP260, LYS329 |
| Mesophilic Bacillus sp.   | ASN84, CY99, THR104, GLN148, GLN155, ASN157, ASN174, PHE175, ASN200 |
| Psychrophilic Pseudoalteromonas haloplanktis | TYR27, ASP30, THR31, GLN32, ARG48, PRO235, ALA236, GLY237, ASP238, GLY239, THR240 |
Molecular docking

The enzyme cellulase (receptor) from thermophilic, mesophilic and psychrophilic bacteria were used with the substrate cellulose (ligand) for further docking studies. The proteins were docked with cellulose unit and their binding energy were calculated as presented in Table 5 (Figure 7, 8 and 9). The binding energy of the substrate cellulose for thermophilic, mesophilic and psychrophilic cellulase enzymes were -93.29, -75.54 and -126.60 Kcal/mole, respectively. The results concluded that based on different binding affinities of these enzymes, psychrophilic cellulase has shown to be most promising enzyme for substrate binding.

CONCLUSION

Nowadays cold-active enzymes from cold adapted microorganisms referred as an important and valued component in different food and beverages industry. Due to their exclusive low temperatures activity; along with retention of volatile compounds, prevention of contamination and energy saving; make them very attractive for food scientist globally. The present study comprises in silico characterization of cellulases obtained from thermophilic, mesophilic and psychrophilic bacteria namely Rhodothermus marinus, Bacillus sp. and Pseudoalteromonas haloplanktis, respectively; and docking studies of enzyme with cellulose substrate was performed. This study may be considered as initial stage for additional in vitro research and in industrial applications. The reported cold-active cellulase is more effective than mesophilic and thermophilic cellulases. Additional investigation needed to explore cold-active cellulases for commercial application especially in food and beverages industries.

Conflict of interest: Nothing to declare.

REFERENCES

Adam, J., Klčíž, Z., Prokop, M., Wimmerová, M., & Koča, J. (2008). In silico mutagenesis and docking studies of Pseudomonas aeruginosa PA-IL lectin — numerous industrial applications especially in food and beverage industries where it is widely used in coffee processing, wine making, degrading skin of the grape, reducing food spoilage and extraction of fruit juices (Behera et al. 2017). The enzymes used at industrial level are mostly isolated from mesophiles or thermophiles. Out of mesophilic and thermophilic enzymes, the second one is preferred due to its thermal stability at high temperature. However, maintaining low temperature (<15°C) play crucial role during in many food processing that maintain quality and proportion of final products (Molina et al. 2007; Ramya and Pulicherla 2014). Cold active enzymes play a significant role in many food industries as the process require mild condition to maintain the taste of products and to avoid spoilage of food materials (Hamid and Mohiddin 2018; Feller 2013; Gerdal et al. 2000; Margesin and Schinner 1994; Russell 1998). In biofuel production, cold-active cellulase can produce ethanol from cellulose at low temperature resulting in saving production costs (Li et al. 2019). Cold-active enzymes has more flexibility in comparison to mesophilic and thermophilic enzymes (Adapa et al. 2014; Methé et al. 2005; Somero 2004). Along with cold-active enzymes, its producing microorganisms also possess significant characteristics such as modifications in the primary sequences of the proteins and greater number of flexible regions in order to tolerate the lower temperatures, in comparison to mesophiles and thermophiles.

In this study, cellulase enzymes from thermophilic, mesophilic and psychrophilic bacteria were selected by using bioinformatics tools for the study of similarity at the sequence level between these enzymes. The results of docking studies concluded that cold-active cellulase have strong affinity with the substrate cellulose and are energetically favorable in comparison to thermophilic and mesophilic cellulases. These binding is due to hydrogen bond formation among cellulose and amino acids of cellulase active site that are sufficient for strong bonding affinity (Patil et al. 2010). The cold-active cellulase possess reasonable low binding energy in comparison to its meso and thermo counterparts that clearly indicates higher efficiency of psychrophilic enzymes (Table 5). This finding would be encouraging for further research with respect to cold-active enzymes utilization in food and beverage processing among others. It is important to maintain low temperature in food processing rather than processing at high temperatures because low temperature treatments help in retaining nutritional value and taste and also avoid food spoilage which is very common problems in food processing industries (Nakagawa et al. 2004). The present in silico investigation about industrial enzymes concludes that cold-active enzymes, such as psychrophilic cellulase, have more efficient and beneficial than its counterparts mesophilic and thermophilic cellulases. Further research with different cold-active enzymes will be useful to evaluate and explore their potential at industrial level and their economic feasibility compared to thermophilic and normal mesophilic enzymes.

Table 5 Docking results of cellulase binding with cellulose ligand

| Type               | Protein          | Ligand          | Binding Energy | Affinity | Ranked score | Torsion |
|--------------------|------------------|-----------------|----------------|----------|--------------|---------|
| Thermophilic       | Cellulase        | Cellulose       | -93.29         | -20.54   | -33.85       | 5       |
| (Rhodothermus marinus) |                |                 |                |          |              |         |
| Mesophilic         | Cellulase        | Cellulose       | -75.54         | -19.00   | -56.63       | 5       |
| (Bacillus sp.)     |                |                 |                |          |              |         |
| Psychrophilic      | Cellulase        | Cellulose       | -126.60        | -24.10   | -44.00       | 5       |
| (Pseudoalteromonas haloplanktis) |          |                 |                |          |              |         |

Predicting binding modes and energies. Journal of Chemical Information and Modeling, 48(11), 2234–2242. https://doi.org/10.1021/ci8002107

Adapa, V., Ramya, L. N., Pulicherla, K. K., & Rao, K. R. S. S. (2014). Cold active pectinases: advancing the food industry to the next generation. Applied Biochemistry and Biotechnology, 172(5), 2324–2337. https://doi.org/10.1007/s12010-013-0685-1

Anoop Kumar, V., Suresh Chandra Karup, R., Snishalom, C., & Nagendra Prabhu, G. (2018). Role of cellulases in food, feed, and beverage industries. Green Bio-Processes, 323–343. https://doi.org/10.1007/978-981-13-32635-0_17

Bayer, E. A., Morag, E., Wilchek, M., Lamed, R., Yaron, S., & Shoham, Y. (1995). Cellulosomes domains for novel biotechnological application. Carbohydrate Bioengineering, Proceedings of an International Conference, 251–259. https://doi.org/10.1016/j.0921-0432(96)08108-5

Beguin, P., & Aubert, J.P. (1994). The biological degradation of cellulose. FEMS Microbiology Reviews, 13(1), 25–58. https://doi.org/10.1111/j.1574-6976.1994.tb00035.x

Behera, B. C., Sethi, B. K., Mishra, S. R., Dutta, S. K., & Thatoi, H. N. (2017). Microbial cellulases – Diversity and biotechnology with reference to mangrove environment: A review. Journal of Genetic Engineering and Biotechnology, 15(1), 197–210. https://doi.org/10.1016/j.jgeb.2016.12.001
Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology. Biotechnology Advances, 18(5), 355–383. https://doi.org/10.1016/S0734-970X(00)00041-0

Claus, H., & Mosjov, K. (2018). Enzymes for wine fermentation: Current and perspectives. Fermentation, 4(3), 52. https://doi.org/10.3390/fermentation4030052

Cowan, W. D. (1996). Animal feed. In: Industrial enzymology. T. Godfrey and S. West, Eds. Macmillan Press, London, UK, 2nd edition, pp. 360–371.

Feller, G. (2013). Psychrophilic enzymes: from folding to function and biotechnological applications. Biochemistry, 52(9), 13913–13918. https://doi.org/10.1016/j.biochem.2013.05.12840

Galante, Y. M., De Conti, A. & Monteverdi, R. (1998). Application of trichoderma enzymes in food and feed industries. In: Harman, G. F., Kubicek, C. P. (eds). Trichoderma & Gliocladium – Enzymes, biological control and commercial applications. Vol. 2. London: Taylor & Francis, pp. 327-342.

Galante, Y., Attaleb, M., Benhamia, J-P., Claverie, P., Cottin, T., … Feller, G. (2000). Cold-adapted enzymes: from fundamentals to biotechnology. Trends in Biotechnology, 18(3), 103–107. https://doi.org/10.1016/S0167-7799(99)01413-4

Godfrey, T. & West, S. (1996). Textiles. In: Industrial Enzymology, Macmillan Press, London, UK, 2nd edition, pp. 360–371.

Gupta, R., Mehta, G., Deswal, D., Sharma, S., Jain, K. K., Kuhad, R. C., & Singh, A. (2013). Cellulases and their biotechnological applications. Biotechnology for Environmental Management and Resource Recovery, 89–106. https://doi.org/10.1016/j.biortech.2011.01.022

Hamid, B., & Mohamid, F. A. (2018). Cold-active enzymes in food processing. Enzyme Technology, 383–400. https://doi.org/10.1016/j.enzyme.2018.01.013

Kirk, O., Borchert, T.V., & Fugbangan, C.C. (2002). Industrial enzyme applications. Current Opinion in Biotechnology, 13(4), 345–351. https://doi.org/10.1016/S0958-1669(02)00328-2

Klemm, D., Heublein, B., Fink, H.-P., & Bohn, A. (2005). Cellulose: fascinating biopolymer and sustainable raw material. Angewandte Chemie International Edition, 44(22), 3358–3393. https://doi.org/10.1002/anie.200406987

Kuhad, R. C., & Ramakrishna, A. (2010). Bioethanol production from Lantana camara (red sage): Pretreatment, saccharification and fermentation. Bioresearch Technology, 101(21), 8348–8354. https://doi.org/10.1016/j.biortech.2010.06.043

Kuhad, R. C., Gupta, R., & Khasa, Y. P. (2010b). Bioethanol production from lignocellulosic biomass: An overview. In: Lal B. Ed., Wealth from Waste, Teri Press, New Delhi, pp. 53-106.

Kuhad, R. C., Mehta, G., Gupta, R., & Sharma, K. K. (2010a). Fed batch enzymatic saccharification of newspaper cellulose improves the sugar content in the hydrolysates and eventually the ethanol fermentation by Saccharomyces cerevisiae. Bioresourrce and Biopowerie, 34(8), 1189–1194. https://doi.org/10.1016/j.biombioe.2010.03.009

Laurie, A. T. R., & Jackson, R. M. (2005). Q-SiteFinder: an energy-based method for the prediction of protein-ligand binding sites. Bioinformatics, 21(9), 1908–1916. https://doi.org/10.1093/bioinformatics/bti315

Li, Y., Wang, Z., Zhou, Y., Zhu, G., & Lin, L. (2019). Enzymatic identification and functional studies of a novel cold-active cellulase (McCel5) from Microbacterium kitamiensea. Applied Microbiology and Biotechnology, 103(3), 739–747. https://doi.org/10.1007/s00253-018-8766-0

Margesin, R., & Schinner, F. (1994). Properties of cold-adapted microorganisms and their potential role in biotechnology. Biotechnology, 33(1), 1–14. https://doi.org/10.1007/bf01022877

Methé, B. A., Nelson, K. E., Deming, J. W., Momen, K., Mehta, G., & Momen, K. A. (2010). Optimized hydrophobic interactions and hydrogen bonding at the target-ligand interface leads the pathways of drug-designing. PLoS ONE, 5(8), e12029. https://doi.org/10.1371/journal.pone.0012029

Neidleman, S. L. (1984). Applications of biocatalysis to biotechnology. Biotechnology and Genetic Engineering Reviews, 1(1), 1–38. https://doi.org/10.1007/978-1-472-765X-804-100153-0