

Production, characterization, *in silico* and crystal structure analyses of recombinant endo- β -1,3-glucanase of family 81 glycoside hydrolase (GH81) from *Clostridium thermocellum* ATCC 27405 and synthesis of laminarioligosaccharides for prebiotic applications

PhD Thesis

by

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September 2019

**DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
GUWAHATI – 781039, ASSAM, INDIA**



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A Thesis

Submitted in partial fulfillment of the requirements for the Degree of

Doctor of Philosophy

by

Krishan Kumar

Under supervision of

Professor Arun Goyal



September 2019

**DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING
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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOSCIENCES & BIOENGINEERING

STATEMENT

I do hereby declare that the content embodied in this thesis entitled as **“Production, characterization, *in silico* and crystal structure analyses of recombinant endo β -1,3-glucanase of family 81 glycoside hydrolase (GH81) from *Clostridium thermocellum* ATCC 27405 and synthesis of laminarioligosaccharides for prebiotic applications”** is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India under the guidance of Professor Arun Goyal.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

It is certified that the work described in this thesis entitled “Production, characterization, *in silico* and crystal structure analyses of recombinant endo β -1,3-glucanase of family 81 glycoside hydrolase (GH81) from *Clostridium thermocellum* ATCC 27405 and synthesis of laminari-oligosaccharides for prebiotic applications” by Krishan Kumar (Roll No. 146106035) for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision at the Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India and this work has not been submitted elsewhere for a degree.

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ACKNOWLEDGEMENTS

It is a pleasant task for me to express my gratitude to all those who contributed in many ways and had profound impact on this study. It would not have been possible without the support and encouragement of many people including my supervisor, doctoral committee members, my family, my friends and colleagues.

First, I would like to express my sincere gratitude to my family for the blessings and strength bestowed upon me to complete this work.

I wish to thank and acknowledge my thesis supervisor, Professor Arun Goyal, Department of Biosciences and Bioengineering, IIT Guwahati for his guidance, support, encouragement and providing me with the necessary instructions and research facilities. I thank him for reviewing my thesis progress and making corrections numerous times.

I would also like to express my sincere gratitude to all my doctoral committee members Dr. Ranjan Tamuli, Dr. Vimal Katiyar and Dr. Sachin Kumar for their valuable suggestions and constructive criticism that has led to the successful completion of my thesis.

I am thankful to Department of Biosciences & Bioengineering and Central Instrumentation Facility (CIF), IITG for providing me instruments for my research work.

I would also like to thank the present and previous heads of the Department of Biosciences & Bioengineering, IIT Guwahati, Prof. Latha Rangan, Prof. K. Pakshirajan, and Prof. Venkata. V. Dasu for providing me with the necessary facilities.

I would also like to thank Prof. Carlos M.G.A. Fontes and Virgínia R. Pires from CIISA – Faculdade de Medicina Veterinária, Universidade de Lisboa and Márcia Correia and Ana Luísa Carvalho from UCIBIO-REQUIMTE,

Departamento de Química, Faculdade de Ciências e Tecnologia-Universidade NOVA de Lisboa for crystallization and solving the structure of CtLam81A

I am also thankful to my seniors Dr. Arun Dhillon, Dr. Aruna Rani and Dr. Ruvoo Baruah for their help and suggestions. I am immensely thankful to my research group members Vikky, Sumitha, Kedar, Shweta, Priyanka, Abhijeet, Kaustubh, Dishant, Parmeshwar, Jebin. I am grateful to all the people with whom I have worked in the lab at the Department of Biosciences and Bioengineering for their cooperation and support.

I would like to thank my friends Abdul, Ajit, Papu, Shatrudhan, Tarik, Sahil and Gautam for their support.

I wish to acknowledge the support received from other teaching and non-teaching staff of the Department of Biotechnology, IIT Guwahati.

I wish to acknowledge M.H.R.D, Govt. of India for providing financial assistance and also Department of Biotechnology, Govt. of India, New Delhi for providing me facility for CD analysis and protein melting study.

My PhD endeavor would not have been successful without the love, trust, support and blessings of my parents. I owe my achievements to my family and friends.

Krishan Kumar

September, 2019

Synopsis

Introduction

β -1,3-glucans are major carbohydrates found in bacteria (*Alcaligenes faecalis*, *A. radiobacter*, *A. rhizogenes*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*), yeast (*Candida albicans*, *Saccharomyces cerevisiae*), fungi (*Lentinus edode*, *Sclerotium rolfii*, *schizophyllum commune*) and algae (*Laminaria digitata*) (McIntosh *et al.*, 2005; Volman *et al.*, 2008). β -1,3-glucan is part of the callose-containing substructures of plant cell walls, being required in pollen development and cell plate formation (McCormick *et al.*, 1993; Chen and Kim *et al.*, 2009). In bacteria, β -1,3-glucan is found as a linear chain of β -1,3-linked glucose (McIntosh *et al.*, 2005). In fungi and algae, the β -1,3-glucan main chain may contain branches of glucose with β -1,6-glycosidic linkage (Volman *et al.*, 2008), while in yeast the long linear chain of β -1,3-glucan is branched with short chains of β -1,3-glucan and β -1,6-glycosidic linkages (Volman *et al.*, 2008). Thus, a variety of β -1,3-glucans exist in nature displaying mainly a structural role but assuming a storage function in algae.

β -1,3-glucanases catalyse the hydrolysis of the β -1,3-glycosidic linkages in β -glucan chains. In the last years, a wide variety of biotechnological applications was explored for β -1,3-glucanases. In agriculture, β -1,3-glucanase is used in combination with chitinase to control fungal diseases (Joosten and Dewit, 1989; Castresana *et al.*, 1990). β -1,3-glucanases are also major players in biomass conversion for bioethanol

production from algae biomass (John *et al.*, 2011). In addition, the combination of β -1,3-glucanases with papain was shown to increase the production of yeast extract (Ryan *et al.*, 1985). Furthermore, the partial hydrolysis of β -1,3-glucan produces oligosaccharides that can be used as prebiotics (Salyers *et al.*, 1977), as immune-activation precursors (Akramiene *et al.* 2007) or anticancer agents (Miyanishi *et al.*, 2003, Jamois *et al.*, 2005). Optimization of the above-mentioned processes and development of novel applications for β -1,3-glucanases is hampered by our current lack of knowledge on the mechanism of action of these enzymes.

β -1,3-glucanases can display an endo mechanism of attack, referred as endo- β -1,3-glucanases (EC 3.2.1.39) or an exo-mode of action, named as exo- β -1,3-glucanases (EC 3.2.1.58). Endo- β -1,3-glucanases hydrolyze β -1,3-glucan randomly and release a mixture of oligosaccharides while exo- β -1,3-glucanases hydrolyze the terminal β -1,3-linkage and release predominantly glucose monomers or dimers (Mouyana *et al.*, 2013). According to the classification of Carbohydrate-Active enZymes (CAZy; <http://www.cazy.org/>, Cantarel, 2009), endo- β -1,3-glucanases are found in six glycoside hydrolase (GH) families: 16, 17, 55, 64, 81 and 128 (Sakamoto *et al.*, 2011). In contrast, exo- β -1,3-glucanases are classified in GH families 3, 5, 17 and 55. Thus, GH17 and GH55 families contain β -1,3-glucan specific enzymes that act following an exo- or endo-mechanism. In contrast, families such as GH81 only contain endo- β -1,3-glucanases. Presently (September 2017), family GH81 contains 645 members widely distributed in plants, yeast, fungi, bacteria, archaea and viruses (<http://www.cazy.org/GH81.html>). GH81 enzymes were shown to display a diversity of physiological roles. Thus, β -1,3-endoglucanase (Eng1p) from *Saccharomyces pombe* plays a role in cell cycle and helps in the dissolution of septum while β -1,3-

endoglucanase Eng2P is involved in actin assembly (Martin-Cuadrado, 2003). In addition, CAENG1 from *Candida albicans* was found to be involved in cell wall separation (Esteban *et al.*, 2005), while the β -1,3-endoglucanase from *Pneumocystis* regulates cell wall β -glucan processing during *Pneumocystis* life cycle (Villegas *et al.* 2010). In *S. pombe*, Eng2 is required for the hydrolysis of the ascus wall that resulted in the release of ascospores (Del Dedo *et al.*, 2009). In plants, β -1,3-endoglucanase provides part of the enzymatic defense mechanism against pathogenic fungi (Umemoto *et al.*, 1997, Fliegmann *et al.*, 2005).

Some of the members of the family 81 GH have been biochemically characterized such as Eng2 from fungus *Saccharomyces cerevisiae* (Martín-Cuadrado *et al.*, 2008), TfLam81 from bacterium *Thermobifida fusca* (McGrath *et al.*, 2006) and β -glucan-binding protein (GBP) from plant Glycine max (Fliegmann *et al.*, 2005). According to the CAZy database, within family 81 GHs, the structural characterization has been reported for β -1,3-glucanase from *B. halodurans* C-125 in complex with various β -1,3-glucooligosaccharides (Pluvinage *et al.*, 2017) and endo- β -1,3-endoglucanase (Lam81A) from *Rhizomucor miehei* CAU432 (Zhou *et al.*, 2013).

The mechanism used by highly diverse multi-enzyme complexes, such as the cellulosome, to degrade β -1,3-glucans remains poorly understood. GH81 from *Clostridium thermocellum* is a typical β -1,3-glucanase, here termed CtLam81A, that belongs to the high molecular mass multi-enzyme cellulosome complex produced by this thermophilic bacterium (Wei *et al.*, 2014). Therefore, the enzymes produced by this bacterium may also be thermostable, which are on current demand for industrial applications (Gomes *et al.*, 2016). Moreover, most of the family 81 GH characterized

so far, are of fungal origin as per CAZy database, of which while only two enzymes from bacteria are characterized.

Present investigation is carried out on **biochemical and structural characterization of recombinant endo β -1,3-glucanase of family 81 glycoside hydrolase (GH81) from *Clostridium thermocellum* ATCC 27405 and synthesis of laminari-oligosaccharides for prebiotic applications**. The thesis work comprises 5 chapters.

Chapter 1 is the general introduction that starts with the structure of different glucan polysaccharides. The basic structural monomeric units and applications of polysaccharides such as β -1,4-glucan (cellulose), β -1,3-glucan (curdlan, paramylon, pachyman, callose, laminarin and chrysolaminarin), β -1,3;1,6-glucan (lentinan, grifolan and botryosphaeran) and β -1,3;1,4-glucan (cereal glucan and lichenan) are described. This chapter also includes a brief description about the different carbohydrate active enzymes with special emphasis on glycoside hydrolases. The description on β -1,3-glucanase enzymes has been included followed by the work that has been done on the family 81 GH. This chapter also enlists the applications of β -1,3-glucanase enzymes such as antifungal activity, yeast extract production, prebiotic properties and anticancer activity. The chapter mentions why microorganism *Clostridium thermocellum* was selected for this study. This chapter defines the significance of investigation undertaken and then lists the specific objectives.

Chapter 2 describes the cloning, expression and purification of family 81 glycoside hydrolase (CtLam81A) from *Clostridium thermocellum* ATCC 27405. The sequence analysis of β -1,3-glucanase (locus tag *Cthe_0660*) from *Clostridium thermocellum* showed the presence of an N-terminal family 81 GH catalytic domain (CtLam81A) and a type I dockerin domain at the C-terminal connected via linker (Fig. 1). The dockerin domain binds to cohesin domain, located on the scaffoldin protein in cellulosome complex (Ding et al, 2001). The 2151 bp of DNA sequence encoding the CtLam81A was amplified by PCR from genomic DNA of *Clostridium thermocellum*. The amplified fragment of CtLam81A was digested with *NheI* and *XhoI* restriction enzymes and cloned into pET-28a(+) expression vector. The recombinant plasmid was transformed into *E. coli* BL-21 competent cells and hyper-expressed by using IPTG. The purified protein showed the molecular size of 82.4 kDa. This protein was further used for biochemical and structural characterization.

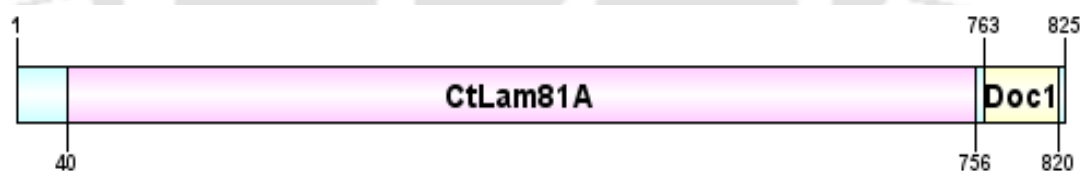


Fig. 1 Molecular architecture of β -1,3-glucanase (*Cthe_0660*) from *Clostridium thermocellum*.

Chapter 3 describes the biochemical properties of recombinant CtLam81A from *Clostridium thermocellum*. CtLam81A showed maximum activity against laminarin (100 U/mg), followed by curdlan (65 U/mg), at pH 7.0 and 75°C. CtLam81A displayed K_m , 2.1 ± 0.12 mg/ml and V_{max} , 109 ± 1.8 U/mg, against laminarin under optimized conditions. CtLam81A displayed stability in a wide range of pHs (6.5-9.0). It retained 100% of its activity at 70°C and 93% of its activity at 75°C after 1h incubation indicating the thermophilic nature of the enzyme. The thermal tolerance displayed by

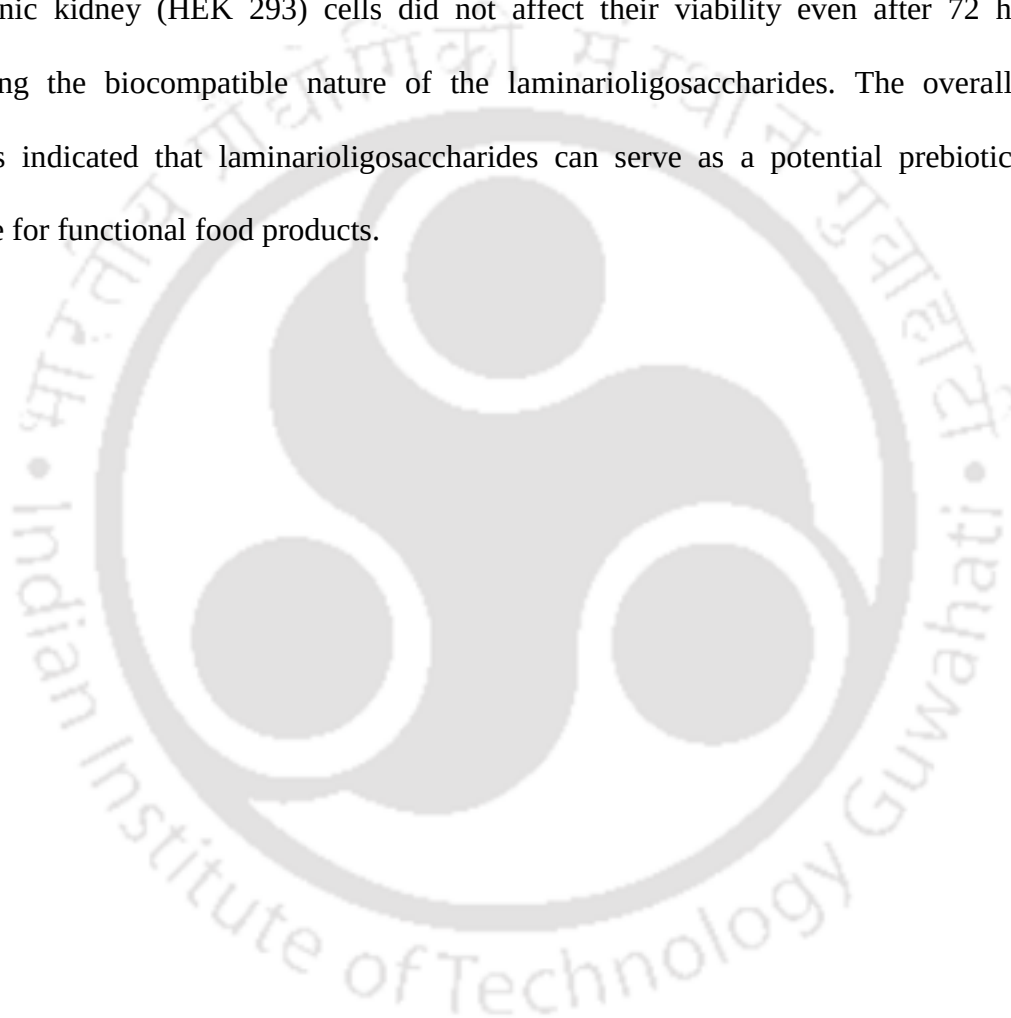
CtLam81A makes it suitable for industrial application, as several industrial processes require thermostable enzymes for easy mixing, better substrate solubility, high mass transfer rate and reduced risk of contamination. *CtLam81A* activity was significantly enhanced by Ca^{2+} or Mg^{2+} ions. Melting curve analysis of *CtLam81A* showed an increase in melting temperature from 91°C to 96°C by Ca^{2+} or Mg^{2+} ions and decreased to 82°C by EDTA. The shifting of melting temperature by 5°C confirmed its involvement in catalysis and also in protein stability. TLC and MALDI-TOF analysis of β -1,3-glucan hydrolysed products released initially, showed β -1,3-glucan-oligosaccharides degree of polymerization (DP) from DP2 to DP7, confirming an endo-mode of action. The capacity of *CtLam81A* to produce β -1,3-glucan oligosaccharides of different DP may be applied to the production of prebiotics, immune-activation precursors or anticancer agents.

Chapter 4 elaborates the Structural characterization of endo β -1,3-glucanase of family 81 glycoside hydrolase (*CtLam81A*) from *Clostridium thermocellum* by *in silico* homology modelling and X ray crystallography. The amino acid sequence of *CtLam81A* was analysed for secondary structure elements by PsiPred tool and CD. The secondary structure analysis of *CtLam81A* by Circular Dichroism showed the presence of α -helices (23.6%) and β -strands (23.6%). The secondary structure prediction analysis by PsiPred corroborated the CD results. The 3D modeled structure of *CtLam81* generated by Swiss-Model server and energy minimized by Swiss-PDB viewer displayed an N-terminal β -sandwich domain, a $(\alpha/\alpha)_6$ domain and a short β -sandwich domain at C-terminal. The quality assessment of modelled structure by Ramachandran plot displayed 94% residues in favoured region, 4.6% in allowed region and 1.4% in outlier region. The superposition of *CtLam81* modeled structure with its closest

homologue Bh0236 (PDB ID: 5t49) from *Bacillus halodurans* indicated that Glu515 acts as a catalytic base, while Asp438 acts as a catalytic acid which hydrolyze β -1,3-glucan by inverting mechanism. Multiple sequence alignment showed that these catalytic residues are conserved within the family 81 glycoside hydrolase. The docking study of CtLam81A with ligands showed the lowest binding energy against laminari pentaose, because active site pocket of CtLam81A can occupy 5 glucose residues of β -1,3-glucan and the aromatic amino acid residues Tyr363, Trp364, Tyr430, Trp588, Trp589 and Trp637, Trp 589 and Trp 588 create a binding pocket for holding the ligand at the catalytic site. The catalytically inactive mutant CtLam81A-E515A generated by site-directed mutagenesis was co-crystallized and tetragonal crystals diffracting up to 1.4 Å resolution were obtained. CtLam81A-E515A contained 15 α -helices and 38 β -strands forming a four-domain structure viz. a β -sandwich domain I at N-terminal, an α/β -domain II, an $(\alpha/\alpha)_6$ barrel domain III, and a small 5-stranded β -sandwich domain IV.

Chapter 5 reports the hydrolysis of Curdlan, a β -1,3-glucan by β -1,3-endoglucanase (CtLam81A) to produce laminarioligosaccharides. TLC and MALDI-TOF analysis of hydrolysed curdlan showed the presence of laminarioligosaccharides of degree of polymerization, DP2-DP7. This mixture of laminarioligosaccharides was explored for *in vitro* prebiotic activities. laminarioligosaccharides showed increase in the growth of probiotic bacteria such as *Lactobacillus acidophilus* and *Lactobacillus plantarum* DM5 while it did not promote the growth of non-probiotic bacteria (*Escherichia coli* and *Enterobacter aerogenes*). Laminarioligosaccharides showed higher resistance or low digestibility against artificial gastric juice, intestinal fluid and α -amylase than inulin

indicating their bioavailability to the probiotic bacteria present in the gastrointestinal tract of human. The probiotic bacteria consumed laminaribiose and laminaritriose more readily than higher laminarioligosaccharides as carbon source for their growth. The *in vitro* cytotoxicity assay of laminarioligosaccharides (1 mg/mL) on human embryonic kidney (HEK 293) cells did not affect their viability even after 72 h indicating the biocompatible nature of the laminarioligosaccharides. The overall findings indicated that laminarioligosaccharides can serve as a potential prebiotic additive for functional food products.



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Chapter 1

General Introduction

1. β -Glucan

β -Glucans are polysaccharides of D-glucose units linked by β -glycosidic bonds. β -glucans are found in bacteria, yeast, fungi, algae and plants (Zhu *et al.*, 2016). The linkage in β -glucan can vary with respect to the source. β -Glucan from bacteria contains β -1,3-linked linear glucan while in yeast the main chain of β -1,3-glucan contains short chain branches of β -1,3-glucan linked by β -1,6-linkage (Zhu *et al.*, 2016). Cereals contain β -1,3;1,4-linked glucan while cellulose polymers of plant contain β -1,4-linkage between glucose unit (Zhu *et al.*, 2016). β -Glucan displays broad range of biological activities. Fungal β -glucan showed immunomodulation effect and anti-cancerous activity (Du *et al.*, 2015). β -Glucan from cereals help to lower cholesterol level (Zhu *et al.*, 2015). The supplementation of β -1,3-glucan (curdlan) stimulated the growth of *Bifidobacterium* sp. with production of short chain fatty acids and lactate in rat (Shimizu *et al.*, 2001). The feeding of laminarin (β -1,3-glucan from algae) to weaning pig reduced the population of the *E. coli* in faecal samples and enhanced the weight (O'Doherty *et al.*, 2010). Lentinan (β -glucan from fungus *Lentinus edodes*) showed immunomodulating activities by stimulating the mouse macrophages for releasing the tumor necrosis factor- α (TNF- α) and nitric oxide (NO) (Minato *et al.*, 1999).

1.1 Types of β -glucan and its sources

1.1.1 β -1,4-Glucan

β -1,4-Glucan is a long linear polysaccharide of β -1, 4-linked D-glucose units (Fig. 1.1). β -1,4-glucan from plants known as Cellulose is the most abundant organic polymer on earth and major structural component of the plant cell wall (Ummartyotin *et al.*, 2015). The percentage of cellulose is about 40% of the total plant carbon (Demain *et al.*, 2005). Its occurrence is widespread, from higher plants to primitive organism such as sea-weeds, flagellates. β -1,4-Glucan is also produced from bacteria known as microbial cellulose. It is an exopolysaccharide produced by various species of bacteria, such as those of the genera *Gluconacetobacter*, *Agrobacterium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, and *Salmonella* (Chawala *et al.*, 2009). Bacterial cellulose is preferred over the plant cellulose as it can be obtained in higher purity and exhibits a higher degree of polymerization and crystallinity index. It also has higher tensile strength and water holding capacity than that of plant cellulose, making it more suitable raw material for producing high fidelity acoustic speakers, high quality paper and dessert foods (Chawla *et al.*, 2009). Cellulose can be hydrolysed chemically or enzymatically to produce monomeric sugar which can be utilized by yeast to produce ethanol (Demirbaş, 2005). Cellulose is the main ingredient of textiles made from cotton, linen, and other plant fibers (Stanković *et al.*, 2008).

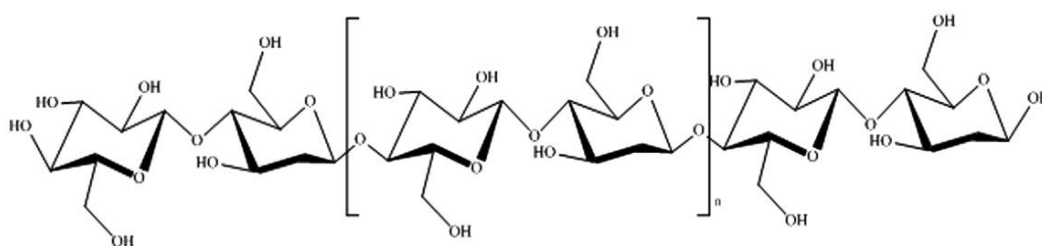


Fig. 1.1 Chemical structure of cellulose (Ummartyotin *et al.*, 2015).

1.1.2 β -1,3-Glucan

β -1,3-Glucan is a long linear polysaccharide of β -1,3-linked D-glucose units. Depending upon the source of isolation β -1,3-glucan named as curdlan, paramylon, pachyman, callose, laminarin and lentinan.

1.1.2.1 Curdlan

It is an extracellular polysaccharide of β -1,3-linked glucose (Fig. 1.2) produced by bacteria (*Alcaligenes faecalis*, *A. rhizogenes*, *A. radiobacter*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia* etc.) (Xu & Zhang, 2016). Curdlan is used as a gelling agent for desserts and starch jelly and as thickener or food stabilizer for low calorie foods (Spicer *et al.*, 1999). The supplementation of curdlan stimulated the growth of *Bifidobacterium* sp. with production of short chain fatty acids and lactate in rat (Kuda *et al.*, 2005).

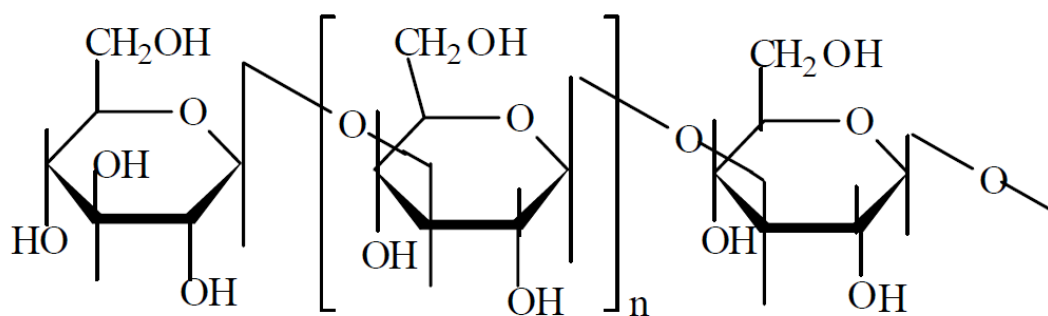


Fig. 1.2 Chemical structure of curdlan (Cremer *et al.*, 2010).

1.1.2.2 Paramylon

It is a linear β -1,3-glucan that represents the reserve of photosynthetic protista, *Euglena gracilis* (Barras & Stone, 1968). Paramylon recovered the hepatic injury in rats induced by carbon tetrachloride (Sugiyama *et al.*, 2009). The sulfated derivatives of paramylon showed anti-HIV activity (Koizumi *et al.*, 1993).

1.1.2.3 Pachyman

It is a linear β -1,3-linked polysaccharide of glucose produced by a sclerotium of fungus, *Poria cocos* (Chihara *et al.*, 1970). Pachyman displayed several properties such as diuretic (Narui *et al.*, 1980), mitogenic, complement activating (Yamada *et al.*, 1992), anti-inflammatory (Schinella *et al.*, 2002) and immunomodulation properties (Shuxiu *et al.*, 1995). The derivatives of pachyman have also been used in pharmaceutical and biomedical applications like carboxymethylated derivative of pachyman (CMP) which has been used as delivery vehicles for anti-tumor drugs (Hu *et al.*, 2008).

1.1.2.4 Callose

Callose is a linear β -1,3-glucan found in the cell wall of plants (Currier *et al.*, 1957). Callose has been known to take part in many biological processes in plants. Callose is temporarily deposited at cell plates during cytokinesis and also involved in pollen development (Shi *et al.*, 2016).

1.1.2.5 Laminarin

Laminarin is a storage polysaccharide in brown algae. The main chain of laminarin is made of β -1,3-linked glucose units with occasionally β -1,6-linked glucose (Synytsya *et al.*, 2014). The degree of polymerization of water-soluble laminarin is of about 20–25 glucose units (Read *et al.* 1996). The feeding of laminarin (β -1,3-glucan) to weaning pig reduced the population of the *E. coli* in faecal samples and enhanced the weight (O'Doherty *et al.* 2010). The ingestion of laminarin by rat modulates the intestinal metabolism by affecting the mucus composition, intestinal pH and short chain fatty acid concentration (Deville *et al.*, 2007).

1.1.2.6 Chrysolaminarin

It is a polymer of β -1,3-linked glucose unit branched with β -1,6-linked glucose in a ratio of 11:1 (Beattie *et al.*, 1961). Chrysolaminarin is a storage polysaccharide typically found in phytoplankton such as Bacillariophyta. Chrysolaminarin is stored inside the cells of these organisms dissolved in water and encapsulated in vacuoles whose refractive index increases with chrysolaminarin content (Beattie *et al.*, 1961). Chrysolaminarin exhibited both antioxidant and immunomodulatory activities (Xia *et al.*, 2014).

1.1.3 β -1,3:1,6-Glucan

β -1,3:1,6-Glucan contains the main chain of β -1,3-glucan branched with glucose by β -1,6-linkage. The proportion of β -1,6-linkage to β -1,3-linkage varies with respect the source of isolation. Depending upon the source of isolation and linkage β -1,3;1,6-glucan named as lentinan, grifolan and botryosphaeran.

1.1.3.1 Lentinan

Lentinan is a polysaccharide produced from the fruiting bodies of fungus, *Lentinus edodes*. The repeating unit in the lentinan consists of five β -1,3-linked glucose residues and two β -1,6-linked branched glucose (Fig. 1.3) (Zhang *et al.*, 2008). Lentinan showed immunomodulating activities by stimulating the mouse macrophages for releasing the tumor necrosis factor- α (TNF- α) and nitric oxide (NO) (Minato *et al.*, 1999). Lentinan suppressed the hepatic CYP1As expression through the production of tumor necrosis factor- α from macrophages accompanied by an increase in the DNA binding activity of nuclear factor- κ B (NF- κ B) (Hashimoto *et al.*, 2002).

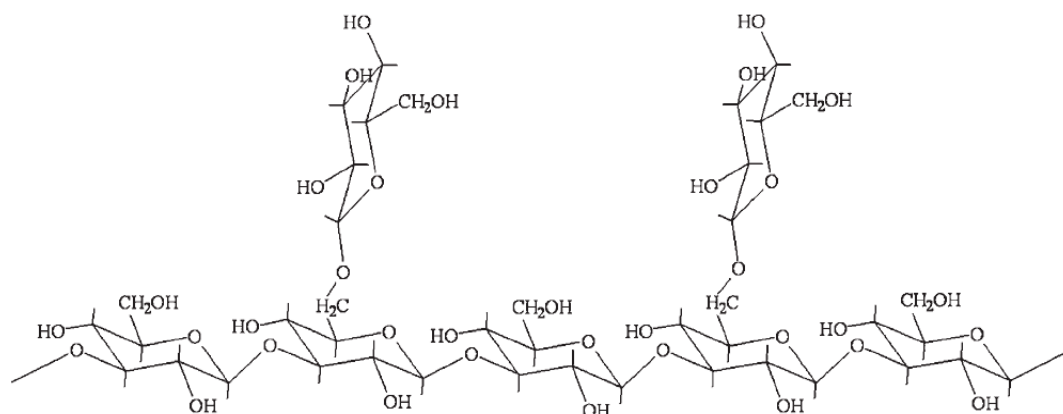


Fig. 1.3 Repeating unit of lentinan (Zhang *et al.*, 2008).

1.1.3.2 Grifolan

Grifolan is a protein-bound polysaccharide or proteoglycan found in the cell wall of fungus, *Grifola frondosa*. Grifolan is a polymer of β -1,3-glucan containing β -1,6-linkage with glucose for every third glucopyranose residue (He *et al.*, 2018) (Fig. 1.4). A large number of pharmacological analyses and clinical experiments confirmed that grifolan showed anticancerous and antiviral (hepatitis B and human immunodeficiency virus) properties and improve the immune system, cholesterol levels, blood fat, regulate blood sugar, and blood pressure without any side effects (He *et al.*, 2018). Grifolan showed a protective effect on alcoholic oxidative injury in mice and also showed antidiabetic activity (He *et al.*, 2018). It exhibited anti-hypertensive activity and showed the protective effect on the heart and kidney disorders caused by high blood pressure (Zhang *et al.*, 2013). It also improved cell-mediated and humoral immune functions and non-specific immune function, and protect the mice from radiation injuries (Yan *et al.*, 2010).

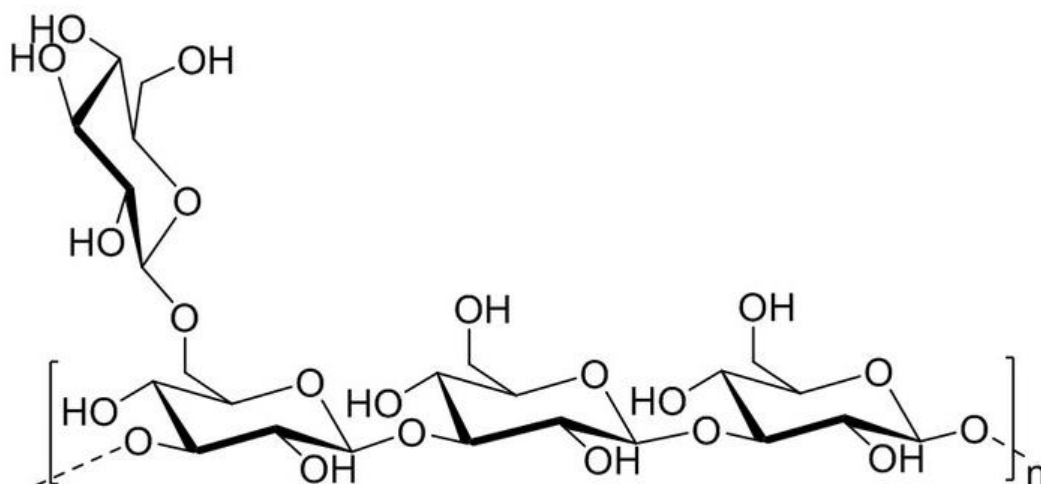


Fig. 1.4 Chemical structure of Grifolan (He *et al.*, 2018).

1.1.3.3 Botryosphaeran

Botryosphaeran is an exopolysaccharide produced by endophytic fungus *Botryosphaeria rhodina*. Botryosphaeran is composed of main chain β -1,3-glucan branched with either glucose or gentiobiose linked by β -1,6-bonds at every fifth glucose unit along the backbone (Fig. 1.5) (Barbosa *et al.*, 2003). Botryosphaeran showed strong anticlastogenic activity in mice (Miranda *et al.*, 2008) and promoted hypoglycaemia and hypocholesterolaemia in diabetic and hyper lipidaemic conditioned rats, respectively (Miranda *et al.*, 2011). The sulfonylation derivative of botryosphaeran exhibited anticoagulant and antithrombotic activities (Mendes *et al.*, 2009). Botryosphaeran also possesses free-radical scavenging and antioxidant activity in vitro (Giese *et al.*, 2015). Botryosphaeran and its sulfonated derivatives also showed antiviral activity against herpes simplex and dengue fever (Sacchelli *et al.*, 2019).

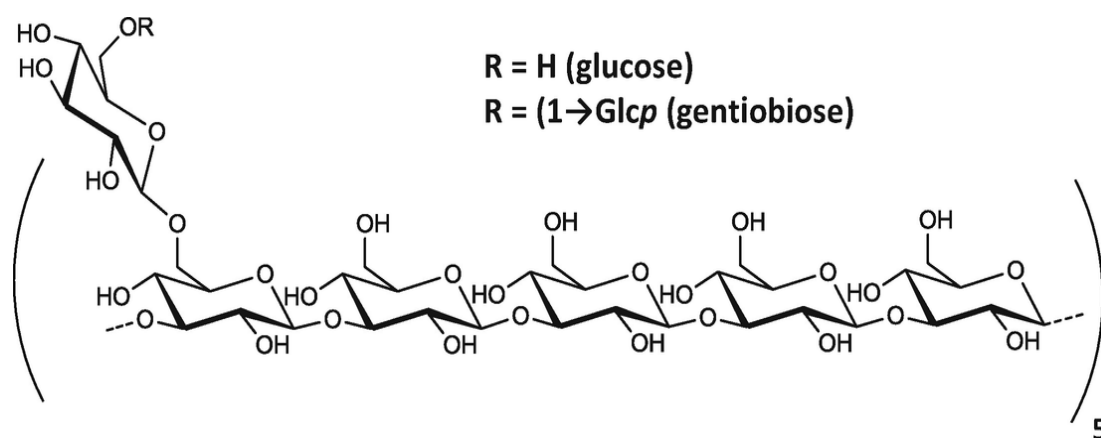


Fig. 1.5 Chemical structure of botryosphaeran (Dekker *et al.*, 2019).

1.1.4 β -1,3:1,4-Glucan

β -1,3:1,4-Glucan contains the main chain of β -1,3-glucan branched with glucose by β -1,6-linkage. The proportion of β -1,3-linkage to β -1,4-linkage varies with respect the source of isolation.

1.1.4.1 Cereal Glucan

The glucan from cereals such as barley and oat contain β -glucans as part of their endosperm cell walls. The structure of β -glucan in cereals is a linear chain of glucose unit linked by β -1,3 and β -1,4 glycosidic bonds (Fig. 1.6). In cereal β -glucans, β -1,3-linked cellotetraosyl units are produced in abundance and together these two features account for more than 90% of the polysaccharide (Tosh *et al.*, 2004). β -Glucans from cereals reduced the risk of cardiovascular diseases by lowering the level of cholesterol (LDL), without effecting the level of high density lipoprotein (HDL) (Nakashima *et al.*, 2018). These glucan are fermented by the microorganisms residing in the colon and converted to short chain fatty acids (SCFA), propionic acid and butyric acid. SCFA produced in the colon have beneficial effect to host like immunomodulation, cancer

apoptosis and prevention of obesity (Nakashima *et al.*, 2018). Cereals glucan showed the antioxidant activity by scavenging of ROS (Nakashima *et al.*, 2018).

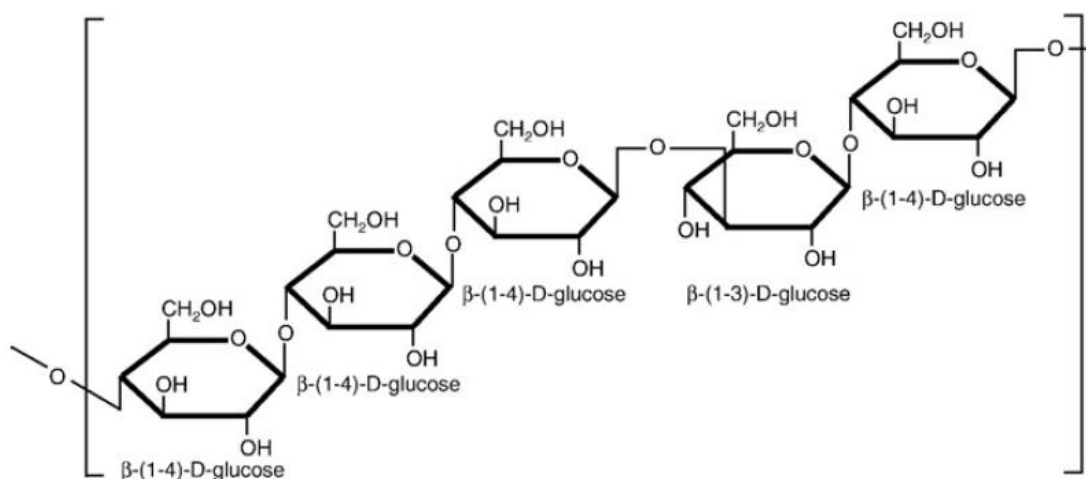


Fig. 1.6 Repeating unit of cereal glucan (Volman *et al.*, 2007).

1.1.4.2 Lichenan

Lichenan is present in a wide range of habitats throughout the world and dominates terrestrial ecosystems accounting for 20,000 species globally among them 2040 species found in India (Upreti *et al.*, 2005). The resultant structure of lichenan contains 82% of β -1,3-linked cellotriosyl and cellotetraosyl units (Tosh *et al.*, 2004). It exhibited antiviral activity in *Nicotiana tabacum* against tobacco mosaic virus. Lichenan can also be converted in to bioethanol using lichenase and yeast (Menon *et al.*, 2011). Lichenan from *Cetraria islandica* at a concentration of 100 $\mu\text{g/mL}$ induced terminal cellular differentiation of primary human keratinocytes (Zacharski *et al.*, 2018)

1.2 Carbohydrate-active enzymes

Carbohydrates are dynamic molecules that are constantly synthesized and broken down. These carbohydrates are synthesized or breakdown by varieties of enzymes. The glycosyltransferases (GTs) enzymes are involved in the synthesis or formation of the glycosidic bond of carbohydrates. The other carbohydrate related enzymes like polysaccharide lyases (PLs), glycoside hydrolases (GHs) and carbohydrate esterase (CEs) are involved in the breakdown of polysaccharides. In summary, the carbohydrate-active enzymes are grouped into 347 families based on amino acid sequence similarity and are listed in the continually updated carbohydrate-active enzyme (CAZy) database (www.cazy.org). As per carbohydrate-active enzymes database about 165 families belong to GHs, 107 families from glycosyltransferases (GTs), 37 from Polysaccharide Lyases, and 16 families belong to Carbohydrate esterases. The information available at CAZy database provide a wealth of gene sequences (many yet to be characterized) to study the structure and function of carbohydrate-active enzymes.

1.2.1 Glycosyltransferases

Glycosyltransferases (GTs) are enzymes that catalyse the transfer of any sugar moiety from activated donor molecules to a specific acceptor molecule, forming glycosidic bonds (Sinnot, 1990). These enzymes utilize 'activated' sugar phosphates as glycosyl donors and catalyze glycosyl group transfer to a nucleophilic group, usually an alcohol (Campbell *et al.*, 1997). As of now, nearly 107 families of GTs have been recognized and are listed in the CAZy database (<http://www.cazy.org/GlycosylTransferases.html>).

1.2.2 Polysaccharide lyases

Polysaccharide lyases (PLs) are group of enzymes that break the uronic acid containing polysaccharide chains following a β -elimination mechanism to liberate an unsaturated hexenuronic acid residue and a new reducing end (<http://www.cazy.org/Polysaccharide-Lyases.html>). The CAZy database contains 37 families of these enzymes classified, based on amino acid sequence similarities, which also reflect their structural features (Lombard *et al.*, 2010).

1.2.3 Carbohydrate esterase

The carbohydrate esterase (CEs) catalyzes the de-O or de-N-deacylation of substituted saccharides. Two types of substrates have been considered for carbohydrate esterases: i) those in which the sugar plays the role of the "acid", such as pectin methyl esters for 4-O-methyl-glucuronoyl methylesterase from *Schizophyllum commune* (Li *et al.*, 2007) and ii) those in which the sugar behaves as the alcohol, such as acetylated xylan for acetyl xylan esterase (family 1 and 2 CEs) from *Clostridium thermocellum* ATCC 27405 (Montanier *et al.*, 2009).

1.2.4 Glycoside hydrolase

The glycoside hydrolases are a group of enzymes that exists in most living organism (Cantarel *et al.*, 2009). Glycoside hydrolases (GHs) are enzymes that catalyze the hydrolysis of the glycosidic linkage of glycosides, leading to the formation of a sugar hemiacetal or hemiketal and the corresponding free aglycone (Henrissat, 1991). Glycoside hydrolases are also referred to as glycosidases, and sometimes also as glycosyl hydrolases. Glycoside hydrolases can catalyze the hydrolysis of O-, N- and S-linked glycosides. They hydrolyze the glycosidic linkages between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety

(<http://www.cazy.org/Glycoside-Hydrolases.html>). The carbohydrate-active enzymes database (CAZy) provides a continuous updated list of the glycoside hydrolase families. They are classified into different families based on homology of their primary sequence (Henrissat, 1991). As of now, the GHs are grouped into 165 families based on amino acid sequence similarity. GHs are also grouped into 14 clans based on the fold of proteins as it was found to be better conserved than their amino acid sequence (Cantarel *et al.*, 2009). Even the closely related catalytic GHs are very often found to differ in substrate specificity (<http://www.cazy.org/>), while enzymes with very different enzyme activities are found among glycoside hydrolase homologs (Coutinho *et al.*, 2003).

GHs are important plant cell wall degrading enzymes and thus cell wall degradation by microbial enzymes is pivotal to many biological and industrial processes (Gilbert *et al.*, 2010). The polysaccharides of plant cell walls are relatively recalcitrant to enzymatic degradation and due to this the microbes, with time, have evolved and developed a complex enzymatic system in order to counter this problem. For example, *Clostridium thermocellum* and *Clostridium cellulolyticum* secrete a mega-dalton multi-modular enzyme complex called the “cellulosome” (Bayer *et al.*, 2007; Fontes and Gilbert, 2010). The cellulosome is a macromolecular complex, whose components interact in a synergistic manner to catalyze the efficient degradation of cellulose. The cellulosome complex is composed of numerous kinds of cellulases and related enzyme subunits, which are assembled into the complex by virtue of a unique type of scaffolding subunit (Bayer *et al.*, 1998; Bayer *et al.*, 2004).

1.2.4.1 Mechanism of action of Glycoside hydrolase

Glycoside hydrolases usually hydrolyze glycosidic bonds by adding water across the glycosidic bond. Asp or Glu are the most commonly found catalytically active residues, although a few exceptions do exist (Davies *et al.*, 2002; Rajan *et al.*, 2004). There are two fundamental mechanisms of action by which these enzymes hydrolyze the substrates (www.cazy.org). These include i) Retaining mechanism and ii) Inverting mechanism and are explained in the sub sections 1.2.4.1.1 and 1.2.4.1.2.

1.2.4.1.1 Retaining mechanism

Glycoside hydrolase involves in retaining mechanism generally have a nucleophile and catalytic acid/base residues approximately, 6Å apart (Zechel and Withers, 2000). This reaction proceeds *via* a double displacement mechanism (Fig 1.7A). In the first step, the nucleophilic attack occurs at the anomeric centre with immediate protonation of the glycosidic oxygen by the general acid (Fig. 1.8A). This occurs through a transition state that has oxocarbenium ion character and results in the formation of a glycosyl-enzyme intermediate and results in retention of stereochemistry at the anomeric carbon as shown in Fig. 1.8A (Zechel and Withers, 2000). Hydrolysis of the intermediate occurs and simultaneously, the general base deprotonates an incoming water molecule, which further attacks the anomeric centre causing hydrolysis of the glycosyl-enzyme intermediate (Fig. 1.8A).

1.2.4.1.2 Inverting mechanism

Inverting glycoside hydrolases require a larger distance between general base and general acid residues in order to accommodate the direct attack of a water molecule and the substrate (Zechel and Withers, 2000). This reaction proceeds *via* a single displacement mechanism (Fig. 1.8B). Water is deprotonated by the general base and

attacks the anomeric centre while the general acid concomitantly protonates the leaving group (Fig. 1.8B). The inverting mechanisms proceed via an oxocarbenium ion-like transition state and results in overall inversion of the stereochemistry at the anomeric carbon (Zechel and Withers, 2000).

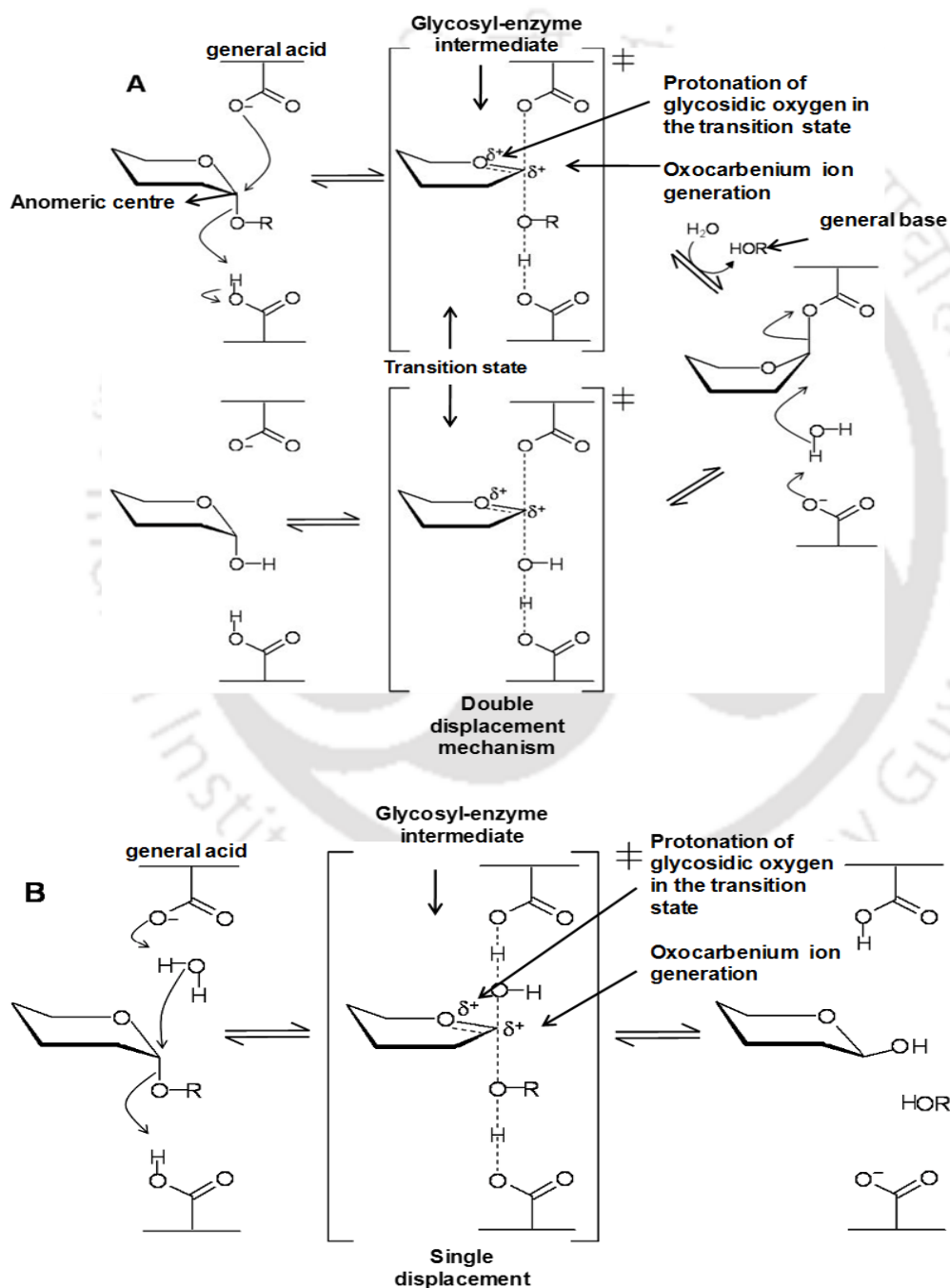


Fig. 1.7 Mechanisms of action of hydrolysis by glycoside hydrolases. A) Retaining mechanism, B) Inverting mechanism (Zechel and Withers, 2000).

1.2.4.2 Cellulosome

Cellulosome is a multienzyme complex produced by many cellulolytic microorganisms. Cellulosome is efficiently degrade plant cell wall polysaccharides, notably cellulose, the most abundant organic polymer on Earth. Each of the cellulosomal subunits consists of a multiple set of modules, two classes of which (dockerin domains on the enzymes and cohesin domains on scaffoldin) govern the incorporation of the enzymatic subunits into the cellulosome complex (Bayer *et al.*, 1998). Within a cellulosome, multiple degradative enzymes work synergistically to attack heterogeneous, insoluble cellulosic substrates (Fontes *et al.*, 2010). This is accomplished by the interaction of two complementary classes of module, located on the two separate types of interacting subunits, i.e., a cohesin module on the scaffoldin and a dockerin module on each enzymatic subunit (Fig. 1.7). The high-affinity cohesin-dockerin interaction defines the cellulosome structure. Attachment of the cellulosome to its substrate is mediated by a scaffoldin-borne cellulose-binding module (CBM) that comprises part of the scaffoldin subunit. The realization about the discovery of the multienzyme complex (cellulosome) occurred gradually with discoveries of different catalytic and binding domains, dockerins and scaffoldins, first in clostridial species and then subsequently in other bacteria (Doi *et al.*, 2004).

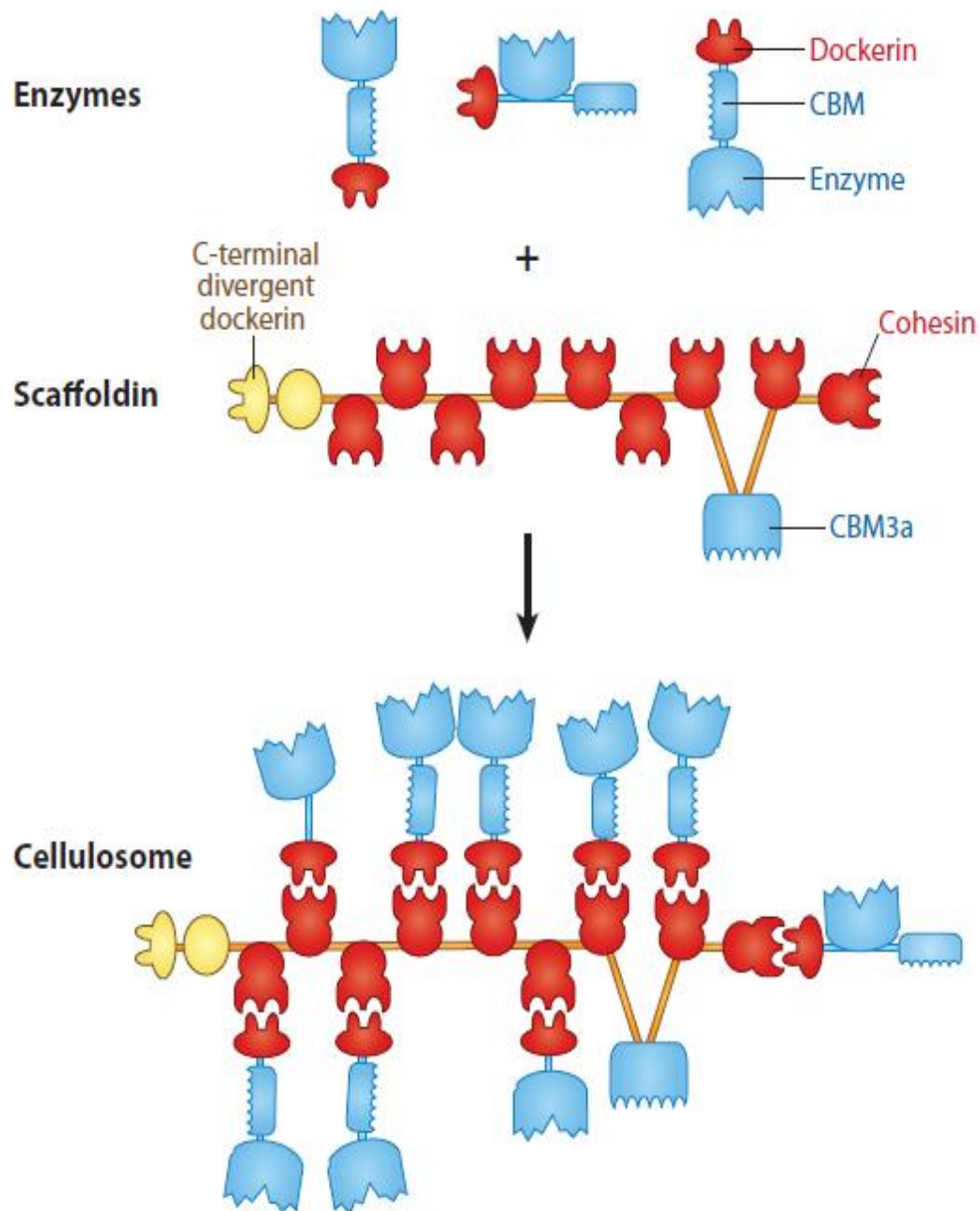


Fig.1.8 structure of cellulosome (Fontes *et al.*, 2010)

1.3 β -1,3-Glucanases

β -1,3-Glucanases are the enzymes that catalyze the hydrolysis of β -1,3-glucan chain by acting on the O-glycosidic linkage in the glucan chain. β -1,3-Glucanases are widely distributed among plant, fungus, yeast, algae and bacteria and some of them have been characterized. Their physiological roles vary with respect to their origin of the source. In plants, these enzymes provide defense against pathogenic fungi (Umemoto *et al.*, 1997, Fliegmann *et al.*, 2005). In fungi it helps in the cell wall separation and ascus wall hydrolysis that result into the release of ascospores (Del *et al.*, 2009). They are also used for commercial purposes like yeast extract production and conversion of algal biomass to fermentable sugars (Ryan *et al.*, 1985).

The products released by the β -1,3-glucanase depend on their mode of action either endo- β -1,3-glucanase (EC 3.2.1.39) or exo- β -1,3-glucanase (EC 3.2.1.58). Endo- β -1,3-Glucanase hydrolyze the β -1, 3-glucan randomly and release the mixture of short oligosaccharides while the exo- β -1, 3-glucanases hydrolyze the terminal β -1, 3-D linkage and release glucose monomers as the product (Fig. 1.9) (Mouyana *et al.*, 2013). Endo- β -1,3-glucanase are gradually gaining popularity because of their application in yeast extract production (Ryan *et al.*, 1985), antifungal activity (Hong *et al.*, 2003), conversion of algal biomass to fermentable sugar. The oligosaccharides can be produced by partial hydrolysis of β -1,3-glucan that can be used as prebiotics (Salysers *et al.*, 1977) and for other clinical purposes. According to the classification of Carbohydrate-Active enZymes (CAZy; <http://www.cazy.org/>, Cantarel, 2009), endo- β -1,3-glucanases are grouped into six glycoside hydrolase (GH) families: 16, 17, 55, 64, 81 and 128 (Sakamoto *et al.*, 2011). In contrast, exo- β -1,3-glucanases are classified in GH families 3, 5, 17 and 55. Thus, GH17 and GH55 families contain β -1,3-glucan

specific enzymes that act following an exo- or endo-mechanism. In contrast, families such as GH81 contain endo- β -1,3-glucanases. Most of the family 81 GHs proteins characterized so far belong only to either fungi or plants and less work has been done on GH81 from bacteria.

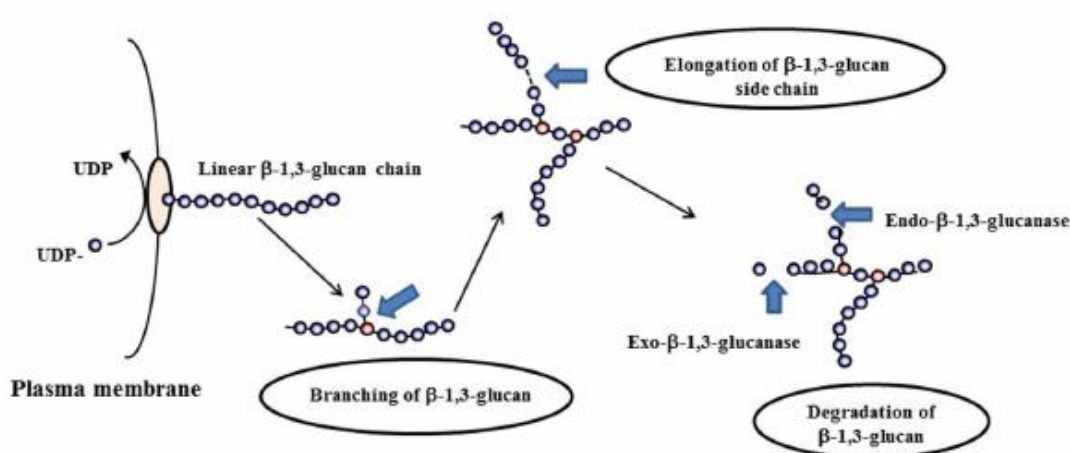


Fig. 1.9 Endo and exo activities of β -1,3-glucanase enzymes (Mouyna *et al.*, 2013).

1.3.1 Family 81 glycoside hydrolase

The family 81 GH shares a common region of approximate 650 amino acids. Some of the proteins of this family from bacteria, fungi and plants have been characterized. All of the characterized proteins show endo- β -1,3-glucanase activity. The laminarin degradation studies of three enzymes *Saccharomyces cerevisiae* ScEng2, *Schizosaccharomyces pombe* SpEng1 and SpEng2 revealed that the active site of enzymes recognizes the 5 glucose units (Martin-Cuadardo *et al.*, 2008). Studies of Lam81A of *Thermobifida fucsa*, β -glucan-binding protein (GBP) of Soybean revealed that family GH81 shows inverting mode of the catalytic mechanism (Fliegmannel *et al.*, 2005, McGrath & Wilson, 2006). Physiological studies of *Saccharomyces pombe* showed that family GH81 protein β -1,3-endoglucanase (Eng1p) together with chitinase

play role in cell cycle and helps in the dissolution of septum while β -1,3-endoglucanase Eng2P involved in actin assembly (Martin-Cuadrado, 2003). Family 81 GH enzymes Eng1P from *Saccharomyces cerevisiae* and CAENG1 from *Candida albicans* were found to be involved in cell wall separation (Baladron et al, 2002, Esteban *et al.*, 2005). Endo- β -1,3 Glucanase gene PC Eng2 in *Pneumocystis* was found to regulate the cyst cell wall β -glucan in the *Pneumocystis* life cycle (Villegas *et al.*, 2010). In *S. pombe* Eng2 with Agn2 (endo- α -1, 3-glucanase of family GH71) hydrolysed the ascus wall that resulted in the release of ascospores (Del *et al.*, 2009).

In plants, GH81 β -glucanases are involved in the defence mechanism, for example β -glucan elicitor binding protein (GEBP) was released by the soybean root cells on exposure to phytopathogenic fungus *Phytophthora megasperma* (Umemoto *et al.*, 1997). GEBP bound to β -glucan elicitor molecule (GE) that was released from the cell wall of the infecting fungus and triggers the defence against the fungus (Umemoto *et al.*, 1997). This glucan elicitor binding protein also showed endo- β -1,3-glucanase activity and acts by an inverting glycoside hydrolase mechanism (Fligemann *et al.*, 2005). In bacteria Lam81, isolated from *Thermofibidia fucsa* was used for the biomass conversion (McGrath & Wilson 2006)

So far crystal structure of only one member of family GH81 from *Rhizomucor miehei* has been solved (Zhou *et al.*, 2013). Most of the family 81 GH proteins characterized so far belongs to either fungi or plants. Very less work has been done on bacterial GH81 and only one structural has been solved in family 81 GH form *B. halodurans* in bacteria. Secondary structure prediction of family GH81 endo- β -1,3-glucanases showed that they contain two independent domains. N-terminal domain was found to be composed of β -strands and C-terminal domain was composed of α -helix

(Martin-Cuadrado, 2008). One study of *S. cerevisiae* ScEng2 (endo- β -1, 3-glucanase) showed that α -helix rich domain is responsible for the catalytic activity and it was hypothesized that another domain may be required for substrate binding or stabilizing the carbohydrate enzyme interaction (Martin-Cuadrado, 2008). They also showed that aspartate and glutamate amino acids at position D518, D588, E609 and E613 are necessary for catalytic activity. The study on *Thermobifida fusca* family GH81 protein Lam81 showed that the catalytic residues of the enzymes are E499 and E503 and with the help of azide rescue method, it was found that the residue E499 is a catalytic base (McGrath *et al.*, 2009). In another study family GH81 protein RmLam81A from *Rhizomucor miehei* was crystallized and the structure was solved (Zohu *et al.*, 2013). The structure of RmLam81A protein consists of three domains, N-terminal β -sandwich domain A, a C-terminal $(\alpha/\alpha)_6$ domain C and an additional domain B between them. By analyzing the structure, it was proposed that E553 is a proton donor and E557 is the catalytic base. But this result of acid/base catalyst is contradictory to the previous study done by McGrath *et al* 2009 on *Thermobifidia fusca* Lam81.

1.4 Applications of β -1, 3-endoglucanase

1.4.1. Antifungal activity

Chitin and β -1,3 glucan are the major constituents of fungal cell wall. β -1,3-Endoglucanases can catalyze the hydrolysis of β -1,3-glucan of cell wall of fungi, resulting in lysis of the fungus cell wall. It was shown that endoglucanase (*Tm* β G) of *Thermotoga maritima* MSB8 inhibits the growth of the fungus *Candida albicans* indicating its antifungal activity (Woo *et al.*, 2014). In another study, endo- β -1,3 glucanase from *Paenibacillus* species showed antifungal activity against *Pythiumaphanidermatum* and *Rhizoctonicsolani* AG-4 (Hong *et al.*, 2003)

1.4.2. Yeast extract production

β -1,3-Endoglucanase from *Basidiomycetes* QM806 on addition along with Papain resulted in augmentation of the effect of papain in yeast autolysis that increased the yield of the extract (Ryan *et al*, 1985).

1.4.3 Prebiotic properties

The supplementation of β -1,3-glucan (curdlan and laminarin) stimulated the growth of *Bifidobacterium* sp. with production of short chain fatty acids and lactate in rat (Shimizu *et al.*, 2001; Kuda *et al.*, 2005). The feeding of laminarin (β -1,3-glucan) to weaning pig reduced the population of the *E. coli* in faecal samples and enhanced the weight (O'Doherty *et al.*, 2010).

1.4.4 Clinical applications

Partial hydrolysis of the β -1,3-glucan gives rise to a mixture of oligosaccharides which can be used in many clinical conditions. In one study conditioned medium was prepared by incubating β -1,3-glucan oligomers ($DP \geq 4$) with monocytes and its inhibitory activity against human leukemic U93 cells was checked (Miyanishi *et al.*, 2003). It was found that oligomers stimulated the monocytes to produce the cytotoxic cytokines that inhibited the proliferation of human leukemic U93 cells (Miyanishi *et al.*, 2003). In another study it was found that β -1,3-glucan oligomers with higher content of β -1,6-linkage stimulated the monocytes to produce cytokines for inhibiting the human leukemic U93 cells (Pang *et al.*, 2005). Laminaritetraose and laminaripentaose stimulated granulocytes macrophages that resulted in phagocytosis of necrotic tissue (Jamois *et al*, 2005).

1.5 Industrial production of endo- β -1,3-glucanase

The use of endo- β -1,3-glucanase in various application has increased the demand for its commercial production. Several companies have started producing endo- β -1,3-glucanases belonging to family GH16, GH17 and GH81. Megazyme u.c., Ireland, Merck INC., USA, Prozomix Limited, USA, NZYTech Ltd, Portugal, Prokazyme Ltd, Iceland, MyBioSource Inc., USA and Bio-Cat Inc., USA are the companies known for industrial production of endo- β -1,3-glucanase enzymes.

1.6 The microorganism

Clostridium thermocellum is an anaerobic, thermophilic, cellulolytic and ethanologenic, Gram-positive bacterium capable of directly converting cellulose biomass into ethanol (Tachaapaikoon *et al.*, 2012). The general cellular structure of *C. thermocellum* is similar to most rod-shaped bacteria as shown in Scanning Electron Microscopic images in Fig. 1.10 (Lamed *et al.*, 1987). *C. thermocellum* contained the cellulosome complex known to degrade recalcitrant polysaccharides (Fontes *et al.*, 2010). *C. thermocellum* is a thermophilic bacterium, therefore enzymes will also be thermostable (Zamost *et al.*, 1991; Haki *et al.*, 2003).

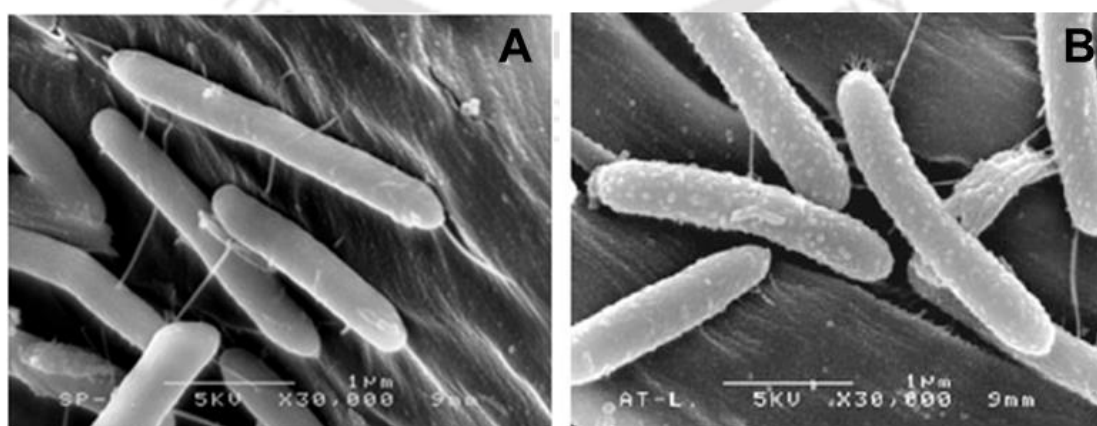


Fig. 1.10 Scanning electron micrographs (SEM) of (A) *C. thermocellum* S14 and (B) *C. thermocellum* ATCC 27405 (Tachaapaikoon *et al.*, 2012).

1.7 Significance of present study

1.7.1 Why study family 81 GHs from *Clostridium thermocellum*

The reasons for undertaking the study on family 81 glycoside hydrolase (GH81) from *Clostridium thermocellum* are summarized below:

1. The β -1,3-endoglucanase of family 81 GH from *Clostridium thermocellum* is a part of cellulosome. The cellulosomal enzyme complex is 50 times more efficient than *Trichoderma reesei* system in degrading crystalline substrates as reported by Fontes and Gilbert (2010).
2. This shall be the first study on about family 81 glycoside hydrolase (GH81) from *Clostridium thermocellum*.
3. Functional characterization of family 81 glycoside hydrolase (GH81) from *Clostridium thermocellum* will be important as very less work has been done on family 81 GH from bacteria.
4. Structural characterization of family 81 glycoside hydrolase (GH81) from *Clostridium thermocellum* is important for identifying the catalytic amino acid residues and mechanism of catalysis.
5. Laminarioligosaccharides (LOS) generated by hydrolysis of curdlan by endo- β -1,3-glucanase (GH81) can be explored for prebiotic properties.

In the present study, the cellulosomal enzyme, β -1,3-glucanase of family 81 glycoside hydrolase (locus tag *Cthe_0660*) from *Clostridium thermocellum* ATCC27405 having gene accession number ABN51895.1 and uniprot ID A3DD6 was identified. The catalytic module of family 81 glycoside hydrolase here termed as *CtLam81A* will be cloned in pET28a(+) and expressed in *E. coli* BL21 cells. *CtLam81A* will be biochemically characterized to see the effect of temperature and pH on activity

and stability. Effect of metal ion on activity and thermostability of CtLam81A will also be examined. The *in silico* and crystal structures of CtLam81A will be also solved to identify the catalytic amino residues that are taking part in the hydrolysis of β -1,3-glucan. Docking analysis of ligand on the surface of modeled CtLam81A structure will be done to identify the amino acid residues involved in binding the substrates in the catalytic site. The laminarioligosaccharides will be generated by hydrolysis of linear β -1,3-glucan (curdlan) by CtLam81A and its prebiotic properties will be examined. The following specific objectives are defined for the proposed thesis work:

1.7.2 Specific Objectives

1. Cloning, expression and purification of family 81 glycoside hydrolase (CtLam81A) from *Clostridium thermocellum* ATCC 27405.
2. Biochemical and functional characterization of CtLam81A from *Clostridium thermocellum* ATCC 27405.
3. Structural characterization of endo- β -1,3-glucanase of family 81 glycoside hydrolase (CtLam81A) from *Clostridium thermocellum* by *in silico* homology modeling and X-ray crystallography.
4. *In vitro* prebiotic and biocompatibility properties of Laminarioligosaccharides.

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Chapter 2

Cloning, expression and purification of family 81 glycoside hydrolase (CtLam81A) from *Clostridium thermocellum* ATCC 27405

2.1 Introduction

β -1,3-Glucans are major carbohydrates found in bacteria (such as *Alcaligenes faecalis*, *A. radiobacter*, *A. rhizogenes*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*), fungi (such as *Candida albicans*, *Saccharomyces cerevisiae*, *Lentinus edode*, *Sclerotium rolfii*, *schizophyllum commune*) and algae (such as *Laminaria digitata*) (McIntosh *et al.*, 2005; Volman *et al.*, 2008). β -1,3-glucan is a part of the callose-containing substructures of plant cell walls, required in pollen development and cell plate formation (McCormick *et al.*, 1993; Chen and Kim *et al.*, 2009). In bacteria, β -1,3-glucan is found as a linear chain of β -1,3-linked glucose (McIntosh *et al.*, 2005), whereas, in fungi and algae, the β -1,3-glucan main chain contain branches of glucose linked by β -1,6-glycosidic bond (Volman *et al.*, 2008). However, in yeast the long linear main chain of β -1,3-glucan contains branches of short chain of β -1,3-glucan linked to the main chain by β -1,6-glycosidic linkages (Volman *et al.*, 2008). Thus, a variety of β -1,3-glucans exists in nature, displaying a structural role in general but assuming a storage function in algae.

β -1,3-Glucanases can display either an endo- or an exo- mode of action. Endo- β -1,3-glucanase (EC 3.2.1.39) hydrolyzes β -1,3-glucan randomly within the chain and releases a mixture of oligosaccharides. While, exo- β -1,3-glucanase (EC 3.2.1.58) hydrolyzes the terminal β -1,3-linkage and releases predominantly the glucose monomer or dimers (laminaribiose) (Mouyana *et al.*, 2013). According to the classification of Carbohydrate-Active enZymes (CAZy; <http://www.cazy.org/>, Cantarel *et al.*, 2009), endo- β -1,3-glucanases are found in six glycoside hydrolase (GH) families: 16, 17, 55, 64, 81 and 128 (Sakamoto *et al.*, 2011). Exo- β -1,3-glucanases are classified in GH families 3, 5, 17 and 55. Thus, GH17 and GH55 families contain β -1,3-glucan specific enzymes that act following an exo- or endo-mechanism. In contrast, families such as GH81 only contain endo- β -1,3-glucanases. Presently (May 2019), family GH81 contains 729 members widely distributed in plants, fungi, bacteria, archaea and viruses (<http://www.cazy.org/GH81.html>). GH81 enzymes have been shown to display diverse physiological roles. β -1,3-Endoglucanase (Eng1p) from *Saccharomyces pombe* plays a critical role in cell cycle by helping in the dissolution of the septum while another β -1,3-endoglucanase (Eng2P) is involved in actin assembly (Martin-Cuadrado *et al.*, 2003). In addition, β -1,3-endoglucanase (CAENG1) from *Candida albicans* was found to be involved in cell wall separation (Esteban *et al.*, 2005) and β -1,3-endoglucanase from *Pneumocystis* regulates cell wall β -glucan processing during its life cycle (Villegas *et al.* 2010). In *S. pombe*, β -1,3-endoglucanase (Eng2) is required for the hydrolysis of the ascus wall that resulted in the release of ascospores (Del Dedo *et al.*, 2009). In plants, β -1,3-endoglucanase provides part of the enzymatic defense mechanism against pathogenic fungi (Fliegmann *et al.*, 2005). Some of the members of the family 81 GH have been biochemically characterized such as Eng2 from fungus

Saccharomyces cerevisiae (Martín-Cuadrado *et al.*, 2008), Tflam81 from bacterium *Thermobifida fusca* (McGrath *et al.*, 2006) and β -glucan-binding protein (GBP) from plant *Glycine max* (Fliegmann *et al.*, 2005). According to the CAZy database, within family 81 GHs, the structural characterization has been reported for β -1,3-glucanase from *B. halodurans* C-125 in complex with various β -1,3-glucooligosaccharides (Pluvinage *et al.*, 2017) and endo- β -1,3-endoglucanase (Lam81A) from *Rhizomucor miehei* CAU432 (Zhou *et al.*, 2013).

The mechanism used by highly diverse multi-enzyme complexes, such as the cellulosome, to degrade β -1,3-glucans remains poorly understood. One such cellulosomal β -1,3-glucanase (locus tag *Cthe_0660*) belongs to family 81 GH was found in thermophilic bacterium *Clostridium thermocellum* (Wei *et al.*, 2014). Therefore, the enzymes produced by this bacterium may also be thermostable, which are on current demand for industrial applications (Gomes *et al.*, 2016). Moreover, most of the family 81 GH characterized so far, are of fungal origin as per CAZy database, of which only two enzymes from bacteria are characterized. To gain a deeper insight about the biochemical properties of GH81 enzymes, the gene encoding family 81 GH catalytic module (*CtLam81A*) from *Clostridium thermocellum* was amplified, cloned and the corresponding recombinant enzyme expressed and purified.

2.2 Materials and Methods

2.2.1 Bacterial strain, plasmid, chemicals and kits

Escherichia coli strains DH5 α (Novagen) and BL-21 (Novagen) were used as hosts for cloning and expression, respectively. The expression vector pET-28a(+) was used for both cloning and expression of the gene encoding CtLam81A. Taq DNA polymerase for PCR amplification and restriction enzymes *Nhe*I and *Xho*I were acquired from New England Biolabs UK. The oligonucleotide primers of CtLam81A were synthesized by GCC Biotech (India) Pvt. Ltd. Trizma base (Tris free base). Ethidium bromide, Bradford reagent, DNase-RNase free water (pH 8.0), antibiotics kanamycin and components of polyacrylamide gel electrophoresis were obtained from Sigma-Aldrich Co. LLC, USA. Coomassie Brilliant Blue R250 for protein staining, Luria Bertani medium, disodium ethylenediamine tetra acetate salts (EDTA), glucose, sodium hydroxide and sodium dodecyl sulphate (SDS) was acquired from Himedia, whereas, methanol was supplied by Merck, India. The miniprep plasmid isolation kit and gel extraction kit was purchased from Sigma-Aldrich Co. LLC, USA. The DNA ladder was purchased from New England Biolabs UK, while protein ladder was purchased from GeneI, India. T4 DNA ligase was purchased from Takara Bio Inc.

2.2.2 Sequence analysis of β -1,3-glucanase from *Clostridium thermocellum*

The amino acid sequence of family 81 glycoside hydrolase β -1,3-glucanase (locus tag *Cthe_0660*) from *Clostridium thermocellum* ATCC27405 having gene accession number ABN51895.1 and uniprot ID A3DD6 was retrieved from CAZy database (<http://www.cazy.org/>). The boundaries of the family 81 GH catalytic domain (CtLam81A) in the amino acid sequence of Cthe_0660 protein was identified by using the conserved domain database (<http://www.ncbi.nlm.nih.gov/cdd/>).

2.2.3 PCR amplification of CtLam81A

The genomic DNA of *Clostridium thermocellum* ATCC 27405 was obtained from DSMZ, Germany. The 2151 bp gene of β -1,3-glucanase encoding CtLam81A protein (Gen Bank Accession No: ABN51895.1) was amplified by using the forward and reverse primers (Table 2.21). The forward primer contains the *NheI* restriction site while reverse primer contains an *XhoI* restriction site. The details of PCR reaction mixture (60 μ l) and PCR cycles for amplification are shown in Tables 2.2.2 and 2.2.3, respectively. The PCR amplification was performed using a thermal cycler (Applied Biosystems, GeneAmp® PCR System 9700).

Table 2.2.1 Oligonucleotide primer sequences used for cloning CtLam81A from *Clostridium thermocellum*.

Construct	Primer sequence
CtLam81A	Forward: 5'-CTCGCTAGCCAGTATTACCGTGAG-3' (T _m : 59°C)
	Reverse: 5'-CACCTCGAGTTAACTGCCGGTTGAAACTGC-3' (T _m : 64°C)

Table 2.2.2 PCR mixture for amplification of gene encoding CtLam81A from *Clostridium thermocellum*.

PCR components	Volume (μ l)	Final concentration
10x reaction buffer	6.0	1x
dNTP mix (100 mM)	0.12	0.2 mM
Forward primer (15 μ M)	1.8	0.45 μ M
Reverse primer (15 μ M)	1.8	0.45 μ M
Sigma water, pH 8.0	48.58	--
Genomic DNA (20.0 μ g/ml)	0.5	10.0 ng
Taq DNA polymerase (1 U/ μ l)	1.2	0.02 U/ μ l
Total	60.0	--

Table 2.2.3 PCR cycles for amplification of *CtLam81A* from *Clostridium thermocellum*.

Steps	Time
I. Denaturation at 95°C	4 min
II. 30 cycles of	
i) Denaturation at 95°C	30 s
ii) Annealing at 50°C	45 s
iii) Extension at 68°C	2:30 min
III. Final extension at 68°C	10 min

2.2.4 Agarose gel electrophoresis of PCR amplified DNA

The PCR amplified DNA was analysed by agarose gel electrophoresis. 0.8% agarose gel was prepared by dissolving 400 mg agarose in 1x TAE buffer followed by heating in a microwave oven till the solution gets transparent. Then 5.0 µl of ethidium bromide (5.0 mg/ml) was added and mixed into the solution when its temperature was around 50°C. The solution was poured on to the casting tray and kept for 30 min. The PCR amplified DNA was mixed with the 5X DNA loading dye in 4:1 ratio and loaded on to the agarose gel. The PCR amplified DNA was run on agarose gel using constant electric field (50 Volts) for 1h in 1X TAE buffer. The bands were then visualized under UV illumination in a gel documentation system (Kodak, Gel Logic 1500).

2.2.4.1 Preparation of 10x TAE buffer and DNA loading dye

A stock solution of 10x TAE buffer was prepared by dissolving 400 mM Tris-acetate and 10 mM EDTA in water and the pH was adjusted at 8.0, (Sambrook and Russell 2001). The DNA loading dye was prepared by mixing the components mentioned below in Table 2.2.4. The final pH of the DNA loading dye was adjusted to pH 8.0.

Table 2.2.4 Composition of 5x DNA loading dye.

Components	Final concentration (5x)
Tris-HCl	50 mM
Glycerol	25% (w/v)
EDTA	5.0 mM
Bromophenol blue	0.2% (w/v)
Xylene cyanol	0.2% (w/v)

2.2.5 DNA extraction from agarose gel

The PCR amplified DNA was purified from agarose gel using a gel extraction kit (GenElute™ Gel Extraction Kit). The protocol for DNA purification from agarose gel was provided by the manufacturer as described in Section 2.2.5.1. The extracted DNA was eluted in 20 µl elution buffer provided by Gel Extraction Kit.

2.2.5.1 DNA gel extraction protocol

1. The PCR or plasmid DNA band was excised from gel using sharp sterile scalpel and transferred to an empty microcentrifuge tube.
2. Three volume of Gel Solubilization Solution was added to every 1 volume of gel (100 mg ~ 100 µl) in the microcentrifuge tube and incubated at 50°C for 10 min (or until the gel slice has completely dissolved).
3. 1 gel volume of 100% isopropanol was added to the microcentrifuge tube and mixed until homogenous.
4. GenElute Binding Column G (DNA binding column) was placed in 2 ml collection tube provided with the kit. 500 µl of the Column Preparation Solution was added to the binding column and centrifuged at 13,000g for 1 min.
5. The above solution containing PCR-amplified or plasmid DNA was added to DNA binding columns and centrifuged at 13,000g for 1 min at room temperature and the flow through was discarded.

6. 700 mL of Wash Solution was added to the binding column and Centrifuged at 13,000g for 1 minute. Centrifugation was repeated again for 1 minute without any additional wash solution to remove excess ethanol.
7. Now the column containing bound DNA was placed in a fresh 1.5 ml sterile microcentrifuge tube. 30 μ l of DNase free water (Sigma-Aldrich Co. LLC, USA) or elution buffer (10 mM Tris-Cl, pH 8.5) was added at the centre of the column. The column was incubated for 2 min at room temperature and centrifuged at 13,000g for 1 min.
9. The PCR amplified or plasmid DNA was eluted from QIAquick spin columns was collected in 1.5 ml sterile microcentrifuge tube. The DNA was stored at -20°C for further use.

2.2.6 Restriction enzyme digestion of the PCR amplified DNA

The purified PCR DNA (200 ng/ μ l) was digested with restriction enzymes (RE) *NheI* and *XhoI* as per the set up described in Table 2.2.5. The reaction mixtures were incubated in a water bath at 37°C for 2h. The digested PCR fragments were run on 0.8% agarose gel and the desired fragments were extracted using gel extraction kit as mentioned in Section 2.2.5.1 and eluted in 20 μ l of elution buffer.

Table 2.2.5 Restriction enzyme digestion set up of PCR amplified DNA.

RE digestion set up	1x (μ l)
10x reaction buffer	0.8
PCR DNAs (200 ng/ μ l)	2.0
DNase free water	3.2
<i>NheI</i> (10 U/ μ l)	1.0
<i>XhoI</i> (20 U/ μ l)	0.5
Total	8.0

2.2.7 Restriction digestion of pET-28a(+) expression vector for cloning of CtLam81A amplified PCR fragments

The pET-28a(+) vector (50 ng/μl) of 5369 bp was subjected to restriction digestion with *NheI* and *XhoI* to prepare it for ligation with PCR amplified CtLam81A shown in Table 2.2.6. The digestion mixture was incubated in a water bath at 37°C for 2h. The *NheI-XhoI* digested pET-28a(+) vector was run on 1% (w/v) agarose gel and purified as described in Section 2.2.5.1 and eluted in 20 μl of elution buffer.

Table 2.2.6 Restriction enzyme digestion set up of pET-28a(+).

RE digestion set up	1x (μl)
10x reaction buffer	2.4
vector pET-28a(+) (50 ng/μl)	20.0
DNAse free water	2.0
<i>NheI</i> (10 U/μl)	1.0
<i>XhoI</i> (20 U/μl)	0.5
Total	24.0

2.2.8 Ligation of *NheI-XhoI* digested PCR fragments into pET-28a(+) vector

The *NheI-XhoI* digested PCR fragments and pET-28a(+) vector were ligated using T4 DNA ligase enzyme. The insert: vector molar ratio was kept at 3:1 in the reaction mixture. The amounts of *NheI-XhoI* digested insert DNA of CtLam81A required for cloning was calculated using the following formula (Engler and Richardson, 1982). The ligation set up used for digested PCR fragment and pET-28a(+) vector is shown in Table 2.2.7. The ligation reaction was incubated at 16°C for 16h.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert :vector molar ratio} = \text{ng of insert}$$

$$\frac{60\text{ng} \times 2.1 \text{ kb}}{5.369\text{kb}} \times 3:1 = 70.4 \text{ ng of insert (CtLam81A)}$$

Table 2.2.7 Ligation set up of PCR inserts and pET-28a(+).

Ligation set up	1x (μl)
10x buffer	1.0
pET-28a(+) vector (30 ng/μl)	2.0
PCR DNA insert (23.5 ng/μl)	3.0
DNase free water	3.0
T4 DNA ligase (350 U/μl)	1.0
Total	10.0

2.2.9 Preparation of *E. coli* (DH5α) competent cells by calcium chloride method

Day 1

1. 50 μl of culture of *E. coli* (DH5α) from glycerol stocks were inoculated into 5.0 ml LB medium and grown at 37°C at 180 rpm for overnight.
2. 0.1 M CaCl₂, 0.1 M MgCl₂ and 0.1 M CaCl₂ solution containing 15% glycerol solution was prepared and filter-sterilized via 0.22 μm membrane filter in laminar air flow and stored at 4°C.

Day 2

3. 1.0 ml of grown culture from above was taken and inoculated into 100 ml LB medium in 250 ml conical flask. The flask was incubated at 37°C, 180 rpm till cell OD reached 0.4 at 600 nm.
4. When the absorbance (280 nm) reached at 0.4, the culture was chilled on ice for 15 min.
5. The culture was centrifuged at 2710g at 4°C for 10 min.
6. The supernatant was discarded and the cell pellet was gently resuspended in 5 ml of sterile, ice-chilled 0.1 M CaCl₂ solution for 10 min.
7. The resuspended cells were centrifuged again at 2710g at 4°C for 10 min.

8. The supernatant was carefully removed and the pellet was gently resuspended in 5.0 ml of sterile, ice chilled 0.1 M MgCl_2 solution.
9. The resuspended cells were centrifuged again at 2710g at 4°C for 10 min.
10. The supernatant was carefully removed and the pellet was gently resuspended in 1.0 ml of sterile, ice chilled 0.1 M CaCl_2 solution containing 15% glycerol. 100 μl of competent cells were aliquoted into sterile 1.5 ml microcentrifuge tube and kept at -80°C for further use.

2.2.10 Transformation of ligated DNA using *E. coli* (DH5 α) competent cells

The ligated DNAs (recombinant plasmid DNA) of CtLam81A were transformed using *E. coli* (DH5 α) competent cells. The steps followed in transformation protocol are as follows:

1. The microcentrifuge tube containing competent cell (100 μl) was taken out from -80°C and kept on ice for 5 min, then 10 μl of ligation mixture was added to it.
2. The tube was gently tapped 4-5 times and kept on ice for 30 min.
3. The cells were heat shocked at 42°C for 40s.
4. The cells were immediately transferred to ice for 5 min.
5. 1.0 ml of LB medium previously incubated at 37°C was added to the transformed cells.
6. The transformed cells were incubated at 37°C in a shaking incubator at 200 rpm for 1h.
7. The cells were harvested by centrifugation at 5000g at 25°C for 3 min.
8. 1.0 ml supernatant was discarded and the cell pellet was then resuspended in remaining 100 μl supernatant.

9. The cells were spread on LB agar plates supplemented with kanamycin at a final concentration 50 µg/ml.
10. The LB agar plates were incubated at 37°C for overnight.
11. The transformation efficiency was calculated using the following equation,

$$\text{Transformation efficiency} = \frac{\text{No. of colonies on LB agar plate} \times 1000}{\text{ng of insert DNA}} = \text{cfu}/\mu\text{g}$$

The colonies were picked at random from LB agar plates and inoculated in 5 ml LB medium supplemented with 50 µg/ml kanamycin and incubated at 37°C, 180 rpm for 12h for isolation of plasmid DNA to check for positive clones.

2.2.11 Isolation of recombinant plasmid DNA

The recombinant plasmid DNAs of *CtLam81A* was isolated from *E. coli* (DH5α) cells using non-ionic detergent method (Lezin et al., 2011) and eluted in 30 µl of sterile DNase free water. Plasmid DNA was also isolated using plasmid miniprep kit (Sigma-Aldrich Co. LLC, USA) for storage and transformation in expression host. The plasmid DNA was isolated by non-ionic detergent method and with plasmid miniprep kit as described in Sections 2.2.10.1 and 2.2.10.2, respectively.

2.2.11.1 Protocol for plasmid isolation by NID method

1. 2 ml of bacterial cultures were pelleted at 6000-7000 rpm for 1 min.
2. Resuspended the cell pellet in 150 µl extraction buffer (5%, w/v sucrose, 50 mM EDTA, 50 mM Tris pH 8, 0.75 M NH₄Cl, 0.5% Triton X-100, lysozyme 100 mg/ml and RNase A 25 mg/ml)
3. The bacterial suspension was incubated at 65°C for 5 min.

4. Suspensions were centrifuged at maximum rpm for 10 min The pellet was removed with a toothpick.
5. 100-120 μ l isopropanol was added, followed by mixing and centrifugation of the solution at 7000 rpm for 10 min at room temperature.
6. After discarding the supernatant, the DNA was centrifuged after adding 70% ethanol. Ethanol was removed and the DNA pellet was dissolved in 20-50 μ l TE buffer.

2.2.11.2 Plasmid isolation protocol (Sigma-Aldrich Co. LLC, USA)

1. 10 ml of an overnight grown recombinant *E. coli* culture was pelleted by centrifugation at $\geq 12,000$ g for 1 minute.
2. Bacterial pellet was resuspended with 200 μ l of the Resuspension Solution.
3. Resuspended cells were lysed by adding 200 μ l of the Lysis Solution and by inverting it 6-7 times.
4. The cell debris was precipitated by adding 350 μ l of the Neutralization/Binding Solution. The cell debris was precipitated by centrifuging at $\geq 12,000$ g or maximum speed for 10 minutes.
5. The column was prepared by passing 500 μ l of the Column Preparation Solution through GenElute Miniprep Binding Column at 12,000g for 1 minute.
6. The cleared lysate was transformed from step 3 to the column prepared in step 4 and centrifuge at 12,000 x g for 30 seconds to 1 minute.
7. 750 μ l of the diluted Wash Solution was added the column and centrifuged at 12,000g for 1 minute.
8. 50 μ l of Elution Solution was added to the column and centrifuged at 12,000g for 1 minute. The eluted DNA was stored at -20°C for further use.

2.2.12 Screening of recombinant plasmid DNA for identification of positive clones

The purified recombinant plasmid DNA was taken separately in a fresh sterile microcentrifuge tube for restriction enzyme digestion analysis. The recombinant DNA was digested with restriction enzymes, *NheI* and *XhoI*, to check for positive clones following the reaction set up as given in the Table 2.2.8. The reaction mixtures were incubated at 37°C in a water bath for 2h. The digested products were run on 0.8% agarose gel. The digested fragments viz. pET-28a(+) vector and the insert DNA of above mentioned recombinant derivatives were visualized under UV transilluminator. The digested fragments (insert and vector) were analyzed to check whether the size of insert DNA and vector pET-28a(+), were similar of their respective size. Based on this observation, the positive clones for the respective recombinant derivatives were identified. Glycerol stocks of *E. coli* (DH5α) cells harbouring the recombinant plasmids was prepared by keeping final concentration of glycerol to 20-25% (v/v). The glycerol stocks in cryo vials of 2 ml were stored at -80°C.

Table 2.2.8. Restriction enzyme digestion set up of recombinant DNA.

Digestion set up	1x (μl)
10x buffer	0.5
DNAse free water	1.4
Recombinant plasmid DNA (approx. 100 ng)	2.5
<i>NheI</i> (10 U/μl)	0.3
<i>XhoI</i> (20 U/μl)	0.2
Total	5.0

2.2.13 Transformation of recombinant plasmids in *E. coli* BL-21 (DE3) cells for protein expression

1 μl of the recombinant plasmid DNA isolated by Sigma miniprep method, was transformed in 100 μl of *E. coli* BL-21 (DE3) competent cells. The protocol for

transformation followed was same as described earlier. The cells harboring recombinant plasmid were spread on LB agar plates with 50 µg/ml kanamycin and grown at 37°C for 12h. The colonies were randomly picked from LB agar plates recombinant cells after 12h of and inoculated into 5.0 ml LB medium supplemented with 50 µg/ml kanamycin.

2.2.14 Hyper-expression of recombinant proteins

The *E. coli* BL-21 (DE3) cells harbouring recombinant plasmids of CtLam81A was analysed for protein expression. The colonies grown on LB agar plates supplemented with kanamycin (50 µg/ml) were picked and inoculated in 5 ml LB medium containing kanamycin (50 µg/ml) and incubated in a shaking incubator at 37°C and 180 rpm. The cells were allowed to grow up to mid-exponential phase till cell absorbance at 600 nm (A_{600}) reached ~0.6. The culture was then induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 1.0 mM final concentration for hyper-expression of recombinant proteins and further incubated at 24°C with shaking at 180 rpm for 16h. The 200 µl of uninduced and induced cells were centrifuged at 13,000g for 2 min and their supernatants were discarded. The cell pellet was resuspended in 40 µl distilled water and the protein expression was checked on SDS-PAGE by using 12% (w/v) gel.

2.2.15 Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) analysis of recombinant proteins

2.2.15.1 Preparation of SDS-PAGE gel

The components of a SDS-PAGE gel are acrylamide 30% (w/v), resolving gel (Tris-HCl, pH 8.8), a stacking gel (Tris-HCl, pH 6.8), SDS 10 % (w/v), APS 10% (w/v), TEMED, sample loading buffer (pH 6.8) and electrophoretic running or tank buffer (pH

8.3-8.5). The composition of each component of SDS-PAGE gels and buffers are described below in Sections 2.2.14.2 to 2.2.14.5.

2.2.15.2 Preparation of acrylamide 30% (w/v) solution

0.8 g of bis-acrylamide was weighed and transferred into an amber colour bottle and dissolved in 50 ml of ultra-pure deionized water collected at 18 MΩcm (Millipore, Milli-Q water purification system) using a magnetic stirrer (IKA, C-MAG HS7). After completely dissolving bis-acrylamide, 29.2 g of acrylamide was added to and stirred on a magnetic stirrer till the solution was clear. The final volume was adjusted to 100 ml with ultra-pure water by keeping the measuring cylinder (100 ml) wrapped in aluminium foil as acrylamide is light sensitive. The acrylamide solution was then filtered (Whatman No. 1) under dark condition and stored at 4°C.

2.2.15.3 Polymerization of SDS-PAGE gel

The resolving gel and stacking gels were prepared by following the protocols from Sambrook *et al.* (1989) using the composition as described in Tables 2.2.9 and 2.2.10. The resolving gel was prepared by adding the all the components in the order as mentioned in Table 2.2.9, in a 25 ml beaker, by keeping acrylamide concentration at 12% (w/v). Similarly, the stacking gel (4%, w/v) was prepared by dissolving all the components mentioned in Table 2.2.10.

Table 2.2.9 Composition of SDS-PAGE for preparation of resolving gel.

Components	12% gel volume (ml)
Acrylamide solution (30%, w/v)	4.0
Deionized water	0.7
SDS (10%, w/v)	1.0
Glycerol (50%, v/v)	1.0
1.5 M Tris-HCl (pH 8.8)	3.3
Amonium per sulphate (10%, w/v)	0.1
TEMED	0.01

Table 2.2.10 Composition of SDS-PAGE components for preparation of stacking gel.

Components	4% gel volume (ml)
Acrylamide solution (30%, w/v)	0.7
Deionized water	2.8
SDS (10%,w/v)	0.5
0.5 M Tris-HCl (pH 6.8)	1.0
Amonium per sulphate (10%, w/v)	0.05
TEMED	0.005

2.2.15.4 Preparation of SDS-PAGE running buffer

The SDS-PAGE gels were run using a 1x running or tank buffer prepared from the 10x stock solution as described in Table 2.2.11. The final pH of the buffer was adjusted to 8.3. The 5x buffer was filtered (Whatman, Filter No. 1) and stored at 4°C.

Table 2.2.11 Composition of 5x Tris-Glycine, running or tank buffer.

Components	Final concentration (5x buffer)
Tris base	0.250 M
Glycine	1.92 M
SDS	1.0 % (w/v)

2.2.15.5 Preparation of sample buffer

5x sample loading buffer was prepared by dissolving the components while keeping the concentration of components as described in Table 2.2.16 and the pH of the buffer was adjusted to 6.8. The components were dissolved in the order as mentioned in Table 2.2.12 to make 5x sample buffer. However, the final concentration while loading to a SDS-PAGE gel was always kept to 1x by mixing 4 volumes of sample (protein) with 1 volume of 5x sample buffer.

Table 2.2.12 Composition of 5x sample loading buffer (Laemmli, 1970).

Components	Final concentration (5X buffer)
Tris-HCl (pH 6.8)	62.5 mM
Glycerol	20.0 (% v/v)
SDS	2.0 (% w/v)
Bromophenol Blue	0.025 (% w/v)
β -mercaptoethanol	5.0 (% w/v)

2.2.15.6 Preparation of staining and destaining solutions

The staining solution (100 ml) was prepared by dissolving 250 mg or 0.25% (w/v), of Coomassie Brilliant Blue (CBB R-250) dye in 50 ml of deionized water in an amber colour bottle by keeping on a magnetic stirrer for overnight. The solution was filtered (Whatman, Filter No. 1), then 40 ml of methanol and 10 ml of glacial acetic acid were added to finally make the ratio 5: 4: 1 (deionized water: methanol: glacial acetic acid). The destaining solution was prepared by dissolving deionized water: methanol: glacial acetic in 5: 4: 1 ratio. The gels were destained by immersing it in destaining solution under gentle shaking condition with change of destaining solution every 30 min, until the protein bands were clear.

2.2.16 Purification of recombinant proteins

The *E. coli* BL-21(DE3) cells harbouring recombinant plasmids were grown in 100 ml LB medium (containing 50 μ g/ml kanamycin) at 37°C to mid-exponential phase (OD = 0.6). To induce recombinant gene expression, 1 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) was added to the culture that was further incubated at 24°C for 16 h. The cells were pelleted by centrifugation at 8,000 xg at 4 °C for 10 min. The cell pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl. The resuspended cells were subjected to ultra-sonication (Sonics, Vibra cell) for 10 min (10s on and 10s off pulse; 33% amplitude) and the lysed cells were centrifuged

at 15,000g, 4 °C for 50 min. The resulting supernatant was filtered through 0.45 µm membrane and loaded on to a 1 ml Ni²⁺ ion chelating column (HiTrap, GE Healthcare, USA). The column bound protein was washed with 30 ml washing buffer (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl and 60 mM Imidazole) and the protein was eluted (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl and 300 mM Imidazole). The eluted enzyme was dialysed against 50 mM Tris buffer (pH 7.5) to remove salt and imidazole. Purified CtLam81A was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% (w/v) polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R-250 following conventional procedures.

2.2.17 Protein estimation by Bradford method

The concentrations of the recombinant proteins were determined using the Bradford's method of estimating proteins at a wavelength of 595 nm (Bradford, 1976). Bovine serum albumin (BSA) purchased from Sigma-Aldrich Co. LLC, USA. was used as standard protein. A standard plot of OD at 595 nm versus different concentration of BSA in 10-100 µg/ml was prepared.

2.2.17.1 Preparation of Bradford's reagent

The Bradford assay estimates the amount of protein in a solution by using the spectral properties of Coomassie Brilliant Blue G-250 (Bradford, 1976). 100 mg 0.01% (w/v) Coomassie Brilliant Blue G-250 was weighed and dissolved in 50 ml 95% ethanol (in an amber colour bottle). 100 ml 85% (w/v) phosphoric acid was added to it. A magnetic bead was placed inside the bottle and the contents were mixed properly by keeping on magnetic stirrer until the dye completely dissolved. The dye was finally diluted to 1 liter with deionized water, filtered (Whatman, No. 1 paper) under dark conditions and stored at 4°C. Commercial Bradford reagent (Sigma-Aldrich Co. LLC,

USA) was also used for protein content determination. The amount of recombinant protein was estimated using the following equation,

$$[\text{Protein}] = \frac{\Delta A_{595} \times V \times C}{v}$$

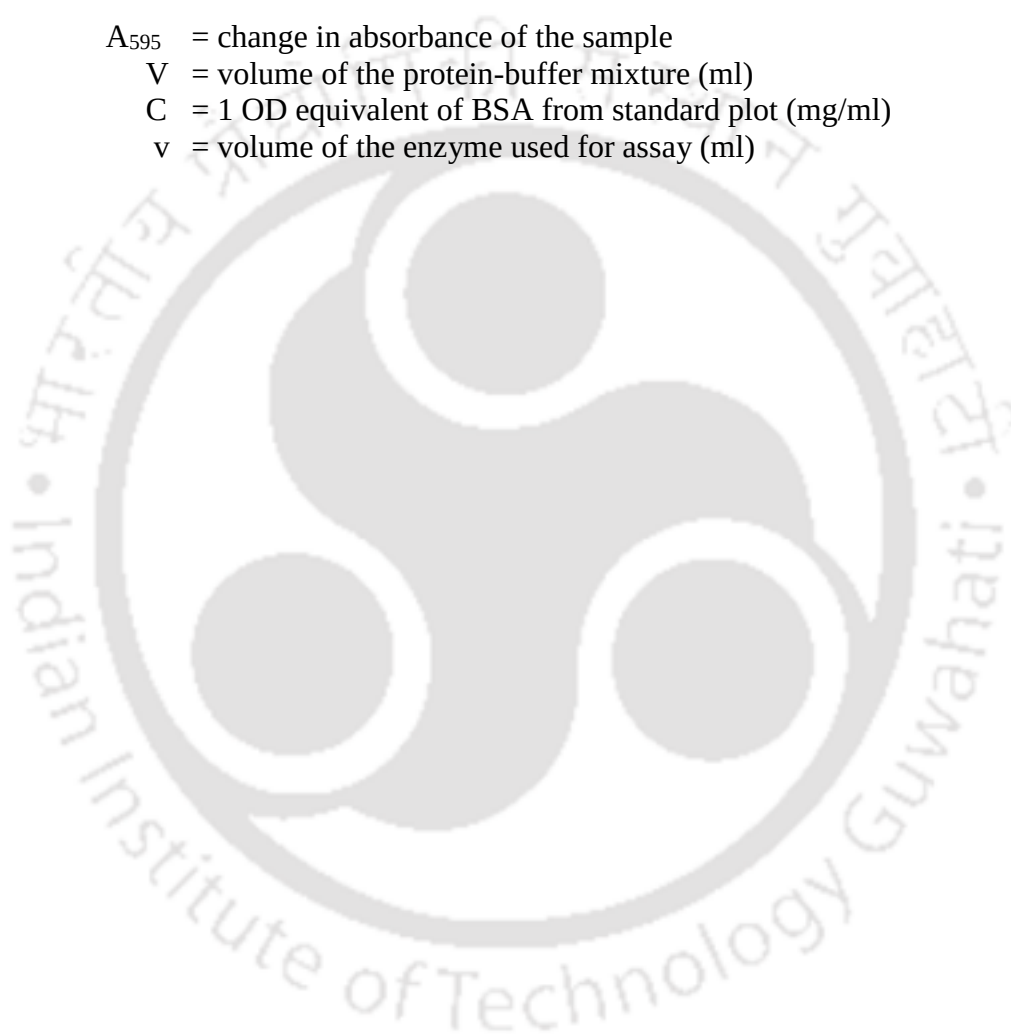
Where,

A_{595} = change in absorbance of the sample

V = volume of the protein-buffer mixture (ml)

C = 1 OD equivalent of BSA from standard plot (mg/ml)

v = volume of the enzyme used for assay (ml)



2.3 Results and Discussion

2.3.1 Molecular architecture of β -1,3-glucanase from *Clostridium thermocellum*

Sequence analysis of β -1,3-glucanase (locus tag *Cthe_0660*) from *Clostridium thermocellum* showed the presence of an N-terminal family 81 GH catalytic domain (CtLam81A) and a type I dockerin domain at the C-terminal connected via linker (Fig. 2.3.1). The dockerin domain binds to cohesion domain, located on the scaffoldin protein in cellulosome complex (Ding *et al.*, 2001).

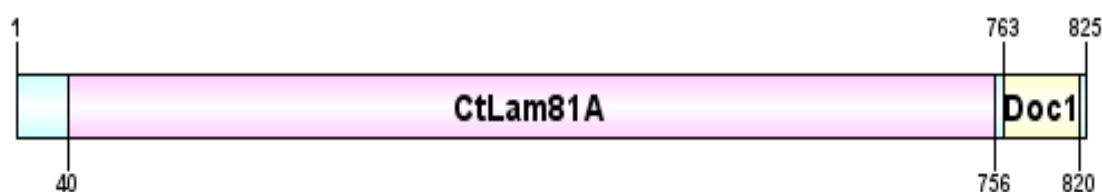


Fig. 2.3.1 Molecular architecture of *Cthe_0660*.

2.3.2 PCR amplification of CtLam81A

The 2151 bp DNA sequences encoding CtLam81A was amplified from genomic DNA of *Clostridium thermocellum* by PCR using the conditions as mentioned in Section 2.2.3. Agarose gel electrophoresis showed the PCR amplified product of size approximate 2.2 kb (Fig. 2.3.2). The PCR amplified product was purified from gel using gel extraction kit as mentioned in Section 2.2.5 and was stored at -20°C for subsequent cloning experiments.

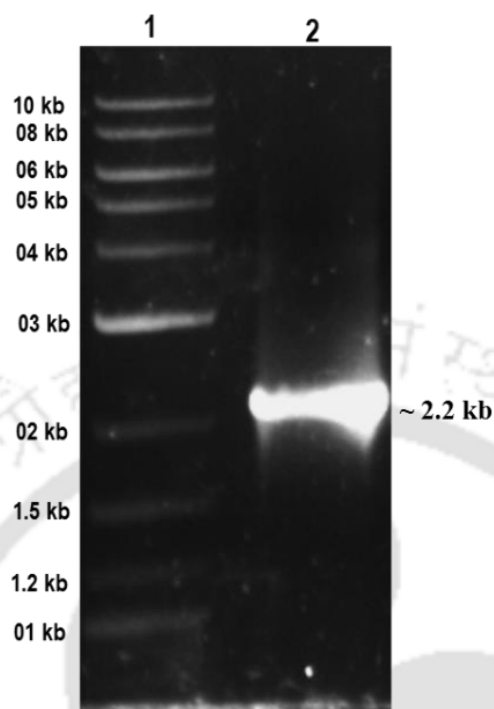


Fig. 2.3.2 Agarose gel showing PCR amplified fragment of *CtLam81A*. Lane 1: DNA marker (2-Log DNA Ladder, NEB), Lane 2: *CtLam81A*.

2.3.3 Restriction enzyme digestion of PCR amplified fragments of *CtLam81A*

The amplified fragments of *CtLam81A* was digested with *NheI* and *XhoI* restriction enzymes to prepare them for cloning into pET-28a(+) expression vector. Simultaneously, pET-28a(+) vector was also digested using the same restriction enzymes. *NheI-XhoI* digested pET-28a(+) and *CtLam81A* were visualized on 1.0 % agarose gel (Fig. 2.3.3). The digested pET-28a(+) and *CtLam81A* were further purified from gel using gel extraction kit as mentioned in Section 2.2.4 and used to clone *CtLam81A* in to pET-28a(+) vector.

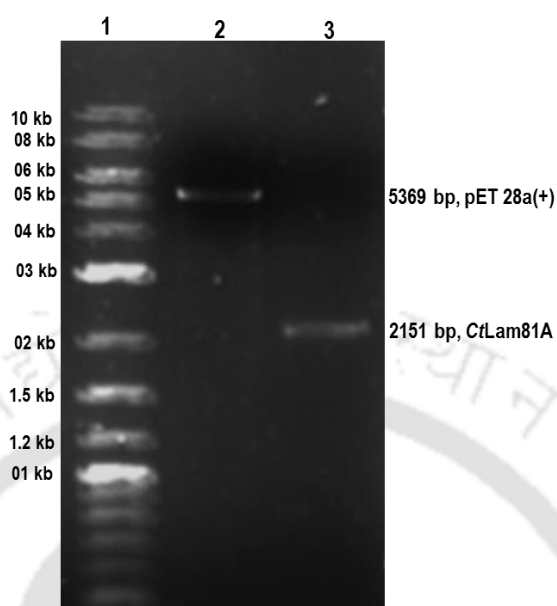


Fig. 2.3.3 Agarose gel showing *NheI-XhoI* digested fragments of pET-28a(+) and CtLam81A. Lane 1: DNA marker (2-Log DNA Ladder, NEB), Lane 2: pET-28a(+) and Lane 3: CtLam81A.

2.3.4 Cloning, expression and purification of recombinant CtLam81A protein

2.3.4.1 Ligation of *NheI-XhoI* digested CtLam81A fragments to pET-28a(+) expression vector

The digested fragments of CtLam81A were ligated in to pET-28a(+) vector using T4 DNA ligase enzyme and the resulted recombinant plasmid was confirmed after transformation in to *E. coli* (DH5 α) and digestion with *NheI* and *XhoI* restriction enzymes.

2.3.4.2 Transformation of *E. coli* (DH5 α) competent cells with recombinant plasmid DNA

E. coli (DH5 α) competent cells of transformation efficiency 4.8×10^5 was transformed with recombinant plasmid DNA of CtLam81A as described in Section 2.2.9. The transformed cells were grown on LB agar plates and incubated at 37°C for 12 h. Total 45 colonies were observed on LB agar plate showing the transformation

efficiency of 4.5×10^4 . 10 single colonies were picked up and grown on 5 ml LB medium respectively to purify the recombinant plasmids.

2.3.4.3 Isolation of plasmid DNA and screening of positive clones

The plasmid DNA of each 10 colonies was isolated from 5.0 ml LB medium supplemented with kanamycin, using the NID method as described in Section 2.2.10.1. The isolated plasmid DNA for each colony was shown on agarose gel (Fig 2.3.4). The undigested pET28a(+) vector was also run on the agarose gel to identify the recombinant plasmids. The isolated plasmid DNA of colonies 1,2,7,8 and 10 showed different kind of movement compared to the pET28a(+) vector which showed the recombinant form of plasmid. The recombinant plasmid of colony 2 was digested with *NheI* and *XhoI* restriction enzymes to confirm the cloning of *CtLam81A* in to pET28a(+) vector. The *NheI* and *XhoI* digested plasmid were run on agarose gel which showed the presence of two DNA band equal to the size of pET28-a(+) vector (5369bp) and *CtLam81A* (2151bp) confirming the clone of *CtLam81A* in to pET28-a(+) vector (Fig. 2.3.5). The colony 2 of *E. coli* (DH5 α) containing recombinant plasmid was again grown in 10 ml LB medium and the plasmid was isolated by plasmid miniprep kit (Sigma-Aldrich Co. LLC, USA) for storage and transformation in expression host.

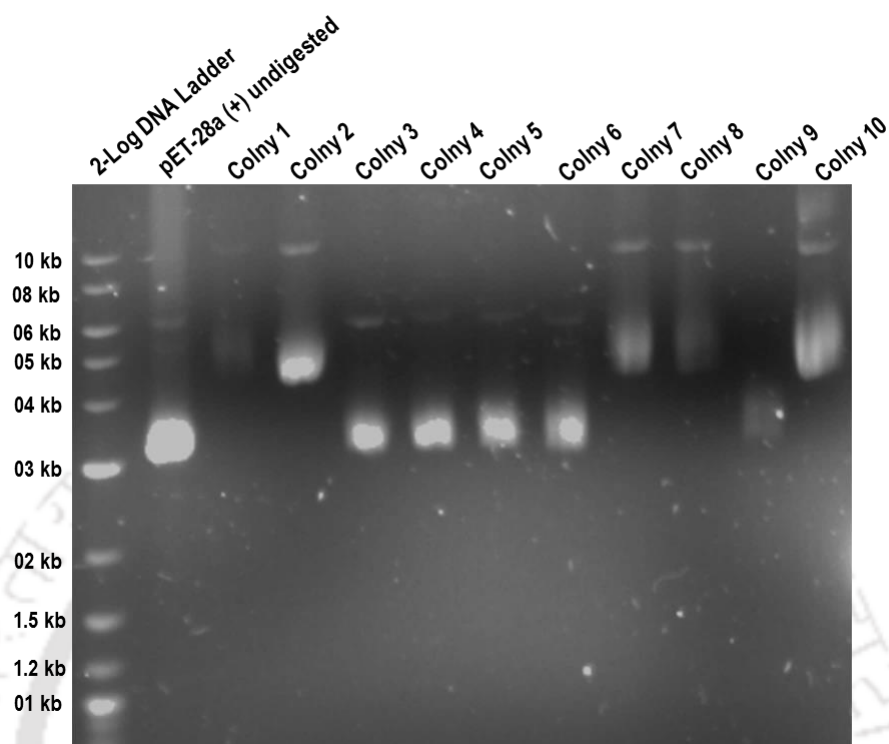


Fig. 2.3.4 Agarose gel showing the recombinant plasmid purified by NID method.

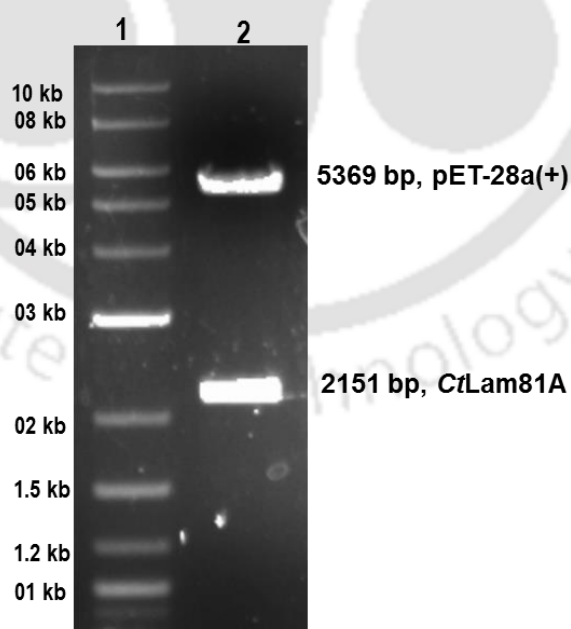


Fig. 2.3.5 Agarose gel showing *NheI-XhoI* digested recombinant plasmids containing *CtLam81A*. Lane 1: DNA marker (2-Log DNA Ladder, NEB) and Lane 2: digested 2151bp fragment of *CtLam81A* (insert) and vector (5369 kb, pET-28a).

2.3.5 Expression and purification of recombinant proteins CtLam81A

The purified recombinant plasmid was transformed in to *E. coli* BL-21(DE3) competent cells and CtLam81A was hyper-expressed and purified by immobilized metal ion affinity chromatography as described in Section 2.2.13. CtLam81A was expressed as soluble protein, as can be seen in cell free extract (Fig. 2.3.6). The purified CtLam81A showed the molecular size, approximately, 79 kDa, which is different from theoretically expected size (82.4 kDa). The observed molecular weight of a protein might differ from the expected size may be because of the migration pattern resulting from SDS interaction with the migrating protein as also reported earlier (Rath *et al.*, 2009). The purified CtLam81A obtained from 100 ml culture was 2.0 mg.

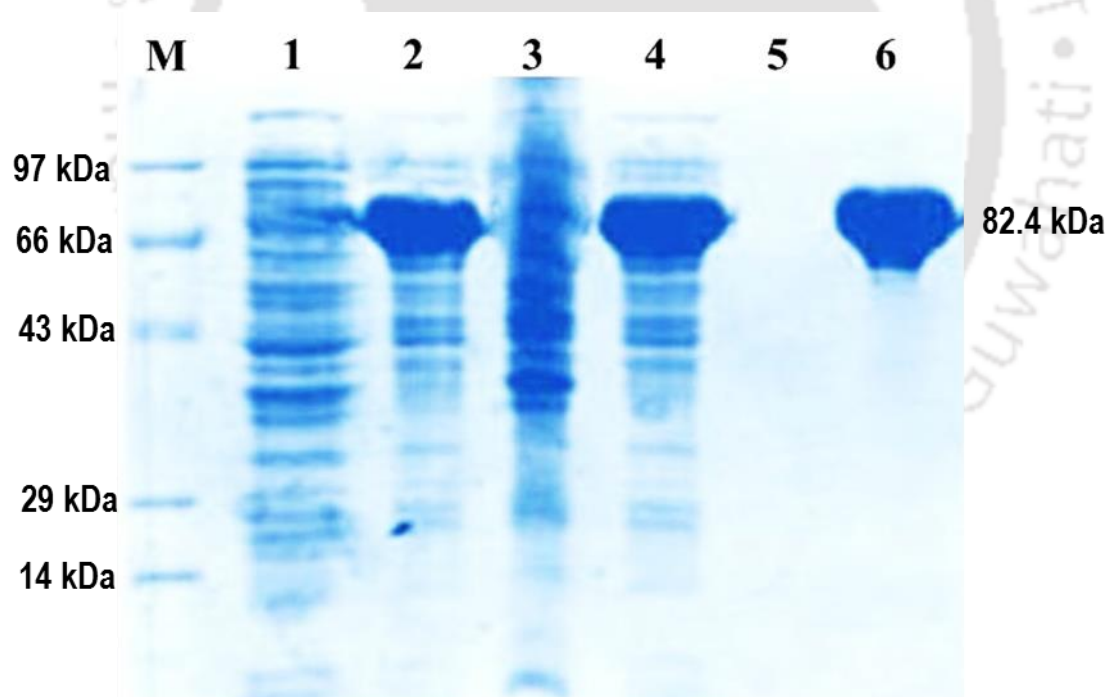


Fig. 2.3.6 Expression and purification recombinant CtLam81A on SDS-PAGE by using 12% (w/v) gel. Lane M: Molecular mass marker (14.3 - 97.4 kDa, Genei), lane 1: uninduced *E. coli* (DE3) BL-21 cells, lane 2: induced *E. coli* (DE3) BL-21 cells, lane 3: cell pellet after sonication, lane 4: cell free extract after sonication, lane 5: Last column wash, lane 6: purified CtLam81A.

2.4 Conclusions

Sequence analysis of β -1,3-glucanase (locus tag *Cthe_0660*) from *Clostridium thermocellum* showed the presence of an N-terminal family 81 GH catalytic domain (CtLam81A) and a type I dockerin domain at the C-terminal connected via a linker. The 2151 bp of DNA sequence encoding the CtLam81A was PCR amplified and cloned in to pET-28a(+) vector. The recombinant plasmid DNA was transformed using *E. coli* (DH5 α) competent cells. The positive clone of CtLam81A was confirmed by restriction digestion analysis of the recombinant plasmid DNA. *E. coli* BL-21 competent cells were transformed using the recombinant plasmid DNA for protein expression. The hyper-expression of recombinant protein was achieved by using IPTG as inducer at a final concentration of 1 mM. The hyper-expression of the recombinant CtLam81A was checked and confirmed by SDS-PAGE analysis. The recombinant CtLam81A was purified from the sonicated cell free extract by immobilized metal ion chromatography (IMAC) using HiTrap chelating column. The purified CtLam81A was expressed as a soluble protein and displayed molecular size of approximately, 82 kDa, which was similar to the theoretical value of 82.4 kDa. The amount of CtLam81A protein obtained from 100 ml of cultures after IMAC purification was 2.5 mg. This purified CtLam81A was used for further biochemical, structural and functional characterization.

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Chapter 3

Biochemical and functional characterization of CtLam81A from *Clostridium thermocellum* ATCC 27405

3.1 Introduction

β -1,3-Glucans are major carbohydrates found in bacteria, fungi and algae (McIntosh *et al.*, 2005; Volman *et al.*, 2008). β -1,3-Glucanases are the enzymes catalyse the hydrolysis of the β -1,3-glycosidic linkages in β -glucan chains. A wide variety of biotechnological applications has been explored for β -1,3-glucanases. In agriculture, β -1,3-glucanase is used in combination with chitinase to control fungal diseases (Nookaraju *et al.*, 2012; Gupta *et al.*, 2013). β -1,3-Glucanases are also major players in algal biomass conversion to bioethanol (John *et al.*, 2011). In addition, β -1,3-glucanase also helps in the production of yeast extract (Conway *et al.*, 2001). Furthermore, the partial hydrolysis of β -1,3-glucan produces oligosaccharides that can be used as prebiotics (Salyers *et al.*, 1977), as immune-activation precursors (Akramiene *et al.*, 2007) or anticancer agents (Miyanishi *et al.*, 2003, Jamois *et al.*, 2005). Optimization of the above-mentioned processes and development of novel applications for β -1,3-glucanases can be achieved if the mechanism of action of these enzymes are clearly understood.

β -1,3-Glucanases can display either an endo- or an exo- mode of action. Endo- β -1,3-glucanases are found in six glycoside hydrolase (GH) families: 16, 17, 55, 64, 81 and 128 (Sakamoto *et al.*, 2011) while, exo- β -1,3-glucanases are found in GH families 3, 5, 17 and 55. Thus, GH17 and GH55 families contain β -1,3-glucan specific enzymes that act by following exo- or endo- mechanisms. In contrast, families such as GH81 only contain endo- β -1,3-glucanases. Family 81 GH contains β -1,3-Glucanases that are widely distributed in bacteria, fungi and higher plants (<http://www.cazy.org/GH81.html>). Some of its members are biochemically characterized like *TfLam81* from *Thermobifida fusca* (McGrath & Wilson, 2006), *Eng2* from *Saccharomyces cerevisiae* (Martin-Cuadrado *et al.*, 2008) and β -glucan-binding protein (GBP) from *Glycine max* (Fliegmann *et al.*, 2005). Most of the family 81 GH characterized so far, are of fungal origin as per CAZy database, of which only two enzymes are from bacteria.

Clostridium thermocellum is an anaerobic thermophilic bacterium which is known to contain a multienzyme complex cellulosome (Fontes *et al.*, 2014). The mechanism used by highly diverse multi-enzyme complexes, such as the cellulosome, to hydrolyze β -1,3-glucans remains poorly understood. One such cellulosomal β -1,3-glucanase (locus tag *Cthe_0660*) belongs to family 81 GH was found in thermophilic bacterium *Clostridium thermocellum* (Wei *et al.*, 2014). To gain a deeper insight about the biochemical properties of GH81 enzymes, β -1,3-glucanase from *C. thermocellum* here termed *CtLam81A* was biochemically characterized and mode of action was determined by TLC analysis.

3.2 Materials and Methods

3.2.1 Substrates and reagents

Laminarin (from *Laminaria digitata*), curdlan (from *Alcaligenes faecalis*), birchwood xylan, beechwood xylan, carboxy methylcellulose (CMC), hydroxyethyl cellulose, β -D-glucan from barley and lichenan were purchased from Sigma-Aldrich Chemicals Co., USA. Mannan, carob galactomannan and laminari-oligosaccharides were purchased from Megazyme, Ireland. Thin layer chromatography plates (TLC Silica gel 60 F₂₅₄, 20×20 cm) were from Merck, India. Sodium carbonate, sodium potassium tartarate, sodium bicarbonate, sodium sulphate, sodium phosphate (monobasic), sodium phosphate (dibasic) and salts of metal ions viz. Ca²⁺, Mg²⁺, Ni²⁺, Zn²⁺, Mn²⁺, Cu²⁺, Co²⁺ and Al³⁺ were procured from HiMedia Laboratories Pvt. Ltd., India. Sodium arsenate, ammonium molybdate, sulphuric acid, ethyl acetate and acetic acid were purchased from Merck INC., USA.

3.2.2 Enzyme assay

The enzyme assay of CtLam81A was carried out in 100 μ l of a reaction mixture containing 1% (w/v) substrate (Laminarin, Curdlan or Carboxymethyl cellulose etc.) dissolved in 50 mM buffer (sodium phosphate, Tris-HCl or HEPES) and 10 μ l of purified CtLam81A (0.01 mg/ml). The reaction mixture was incubated at different temperatures for 15 min. The enzyme activity was measured by estimating the liberated reducing sugar by Nelson-Somogyi method (Nelson, 1944; Somogyi, 1945) as described in Section 3.2.2.4. 100 μ l of reagent D was added (Section 3.2.2.1) to 100 μ l of reaction mixture containing the reducing sugar. The solution was mixed and heated for 20 min in the boiling water bath. After 20 min of boiling, 100 μ l of reagent C (Section 3.2.2.1) was added. 700 μ l of water was added to the mixture to make up the

volume to 1 ml and the absorbance at 500 nm (A_{500}) was measured by UV-Visible spectrophotometer (GeneQuant 1300, GE Healthcare, UK). A standard plot of D-glucose was generated (Section 3.2.2.2) to quantify the reducing sugar present in the reaction mixture.

3.2.2.1 Preparation of reagents for reducing sugar estimation

Reagent A

Sodium carbonate anhydrous	6.25 g
Sodium potassium tatarate	6.25 g
Sodium bicarbonate	5.0 g
Sodium sulphate anhydrous	50.0 g

The above mentioned components were dissolved in 100 ml of deionized water and the final volume was adjusted to 250 ml. The solution was filtered (Whatman No. 1) and stored at 30°C.

Reagent B

Reagent B was prepared by dissolving 15 g of copper sulfate (CuSO_4) in 50 ml deionized water and one or two drops of concentrated sulphuric acid was added to it. The final volume was made up to 100 ml with deionized water and the solution was filtered (Whatman No. 1) and stored at 30°C.

Reagent C

Reagent C was prepared in two steps under dark. First, 2.5 g of ammonium molybdate was dissolved in 45 ml of deionized water in 100 ml beaker and 2.1 ml of concentrated sulphuric acid was added to it. In another beaker 0.3 g of sodium arsenate was dissolved in 2.5 ml of deionized water. Now, this solution was added to ammonium molybdate solution and the contents were mixed (total volume was around 50 ml). The

solution was filtered (Whatman No. 1) under dark conditions and stored at 37°C. The solution was used after 24h incubation.

Reagent D

Reagent D was prepared by mixing reagent A and reagent B in the ratio 25:1. Reagent D was freshly prepared for the assay.

3.2.2.2 Generation of standard plot of D-glucose

The standard plot using of D-glucose was prepared by varying the concentration from 10-100 µg/ml. 100 µl reaction mixture contained D-glucose in 50 mM sodium phosphate buffer pH 7.0 was incubated at 50°C for 15 min and then 100 µl of solution D (Section 3.2.2.1) was added to it. The reaction mixture was then heated in boiling water bath for 20 min and cooled. 100 µl of solution C (Section 3.2.2.1) was added and the contents were mixed. Then 700 µl of deionized water was added to make the final volume to 1 ml. The absorbance at 500 nm (A_{500}) was measured using UV-Visible spectrophotometer (Varian, Cary 100 Bio) against a buffer blank. A standard plot of A_{500} versus D-mannose concentration (µg/ml) was generated and 1 A_{500} equivalent of D-glucose (µg/ml) was calculated. 1 A_{500} equivalent of D-glucose (µg/ml) was converted into mg/ml for calculation of enzyme activity.

3.2.2.3 Calculation of enzyme activity

The activity of the enzyme was expressed as U/ml and the specific activity as U/mg of protein. One unit (U) of enzyme activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar per min. The enzyme activity was calculated as described below,

$$\text{Enzyme activity (U/ml)} = \frac{\Delta A_{500} \times C \times V}{180 \times t \times v} = (\mu \text{ mole/min/ml})$$

where,

ΔA_{500} = change in absorbance of the sample at 500 nm

C = 1 OD equivalent D-glucose concentration from standard plot

V = volume of the reaction mixture (ml)

t = time of reaction (min)

180 = molecular weight of D-glucose

v = volume of the enzyme taken in assay (ml) for reducing sugar estimation.

3.2.3 Substrate specificity of CtLam81A

The substrate specificity of CtLam81A was determined against various polysaccharides consisting of β -1,3-glucans (laminarin and curdlan), β -1,3;1,4 mixed linkage glucans (lichenan and β -D-glucan), β -1,4-glucans (carboxymethyl cellulose and hydroxyethyl cellulose), xylans (birchwood xylan and beechwood xylan), mannans and carob galactomannans. The 100 μ l reaction mixture contained 1% (w/v) substrate in 50 mM HEPES buffer (pH 7.0) and 10 μ l purified CtLam81A enzyme (0.01mg/ml). The reaction was incubated at 75°C for 10 min. The specific activity of CtLam81A enzyme was determined by quantifying reducing sugar produced in the mixture as described earlier (section 3.2.2).

3.2.4 Biochemical and kinetic properties of CtLam81A

The effect of pH on CtLam81A activity was studied by analyzing enzyme activity in different buffers: 50 mM citrate phosphate (pH 5.0-7.0), 50 mM phosphate (pH 5.8-8), 50 mM HEPES (pH 6.8-8.0), 50 mM Tris-HCl (pH 7.5-9.0) and 50 mM carbonate-bicarbonate (pH 9.5-10.5). The reaction mixture (100 μ l) contained 10 μ l enzyme (0.01 mg/ml) and 1% (w/v) Laminarin dissolved in different buffers was performed at 75°C for 10 min. The reducing sugar released was quantified as described in section 3.2.2. To determine the effect of temperature on CtLam81A activity, the

enzyme was assayed in a 100 μ l reaction mixture containing 1% (w/v) Laminarin in 50 mM HEPES buffer, pH 7.0 at different temperatures ranging from 30°C to 90°C for 10 min. The reducing sugar released was estimated as mentioned in section 3.2.2.

The effect of pH on the stability of CtLam81A was determined by incubating the enzyme in different buffers ranging from pH 5.0-10.5 at 25°C for 60 min followed by determination of specific activity at optimum conditions of pH 7.0 and 75°C. Similarly, the effect of temperature on stability of CtLam81A was determined by incubating the enzyme in HEPES buffer (pH 7.0) at different temperatures ranging from 30°C to 90°C for 60 min followed by determination of specific activity at optimum conditions of pH 7.0 and 75°C.

To determine the kinetic parameters (K_m and V_{max}), CtLam81A was incubated with different concentrations of laminarin and curdlan at pH 7.0 and 75°C. Kinetic constants (K_m and V_{max}) were determined by using Michaelis-Menten and Lineweaver-Burk plots in GraphPad Prism 6. The effects of various metal ions on the activity of CtLam81A were determined by analysing its activity in presence of 10 mM $CaCl_2$, $MgCl_2$, $CuSO_4$, $MnCl_2$, $CoCl_2$, $NiSO_4$ and $ZnSO_4$. The effects of Ca^{2+} and Mg^{2+} ions on the activity of CtLam81A were determined by varying the $CaCl_2$ and $MgCl_2$ concentration from 0.1 to 10 mM in the reaction mixture.

3.2.5 Protein melting curve of CtLam81A

Protein melting was analysed by subjecting CtLam81A (50 μ g/ml) in 50 mM HEPES buffer (pH 7) to different temperatures on a UV-Visible spectrophotometer (Varian, Cary 100 Bio) coupled with Peltier temperature controller. A curve was generated by plotting change in the absorbance at 280nm (A_{280}) with respect to the

temperature. The experiments were carried out by separately adding 10 mM CaCl₂, MgCl₂ or EDTA to examine their effect on the stability of protein.

3.2.6 Mode of action of CtLam81A

The hydrolyzed products of curdlan by CtLam81A were analyzed by thin layer chromatography (TLC) on silica gel-coated aluminium plate (TLC Silica gel 60 F254, 20×20 cm, Merck). A 100 µl reaction was set up by adding 10 µl of CtLam81A (0.02 mg/ml) to 90 µl of 0.55% (w/v) curdlan, dissolved in 50 mM HEPES buffer (pH 7.0) and incubated at 75°C for different time intervals. The resulting reaction mixture (100 µl) was treated with an equal volume of absolute ethanol and centrifuged at 13,000g for 10 min. The supernatant containing the released oligosaccharides was transferred to a clean micro-centrifuge tube and concentrated to 20 µl (for time interval 1 min - 5 min) and to 40 µl (for time interval 10 min - 24h) by evaporating in the hot-air oven at 80°C. After this, the amount of reducing sugar of curdlan oligosaccharides was measured by the method as described earlier (Nelson, 1944; Somogyi, 1945). Precisely 0.8 µl of concentrated samples and 0.6 µl of standards, glucose, laminaribiose and laminaritriose (each were 2.0 mg/ml) were loaded on the TLC plate. The TLC plate was dried for 5 min and kept in the developing chamber. The mobile phase consisted of ethyl acetate/acetic acid/water in the ratio 2:2:1 (Lee et al., 2014). The plate was dried and oligosaccharides were visualized by spraying the visualizing solution (sulphuric acid: methanol 5:95, v/v and α -naphthol 0.5%, w/v). The degree of polymerization of oligosaccharides formed in the 5 min reaction was analyzed by performing Matrix-Assisted Laser Desorption Ionization–Tandem time of Flight mass spectrometry (MALDI–TOF/TOF MS) in the positive ion reflectron mode by using an ultrafleXtreme system (Bruker Daltonics Inc., USA). The sample for MALDI-TOF analysis was

prepared by mixing 1 μL of concentrated 5 min sample with 0.3 μL of 0.01 M NaCl and 0.5 μL of 50 mg/mL 2,5-dihydroxybenzoic acid in 50% (v/v) acetonitrile and spotted onto a stainless steel target plate (Wang *et al.*, 2016).



3.3 Results and Discussion

3.3.1 Substrate specificity of CtLam81A

The hydrolytic capacity of CtLam81A against diverse plant cell wall polysaccharides was tested (Table 3.3.1). CtLam81A displayed the highest activity against laminarin (100 U/mg) followed by curdlan (65 U/mg), indicating that CtLam81A displayed activity against both soluble and partial soluble substrates. There is no report available on the activity of family 81 GH against curdlan till date. The enzyme displayed a residual activity against lichenan (1 U/mg) and β -D-glucan (0.5 U/mg) and was unable to depolymerize birchwood xylan, beechwood xylan, carboxy methylcellulose (CMC), hydroxyethyl cellulose, mannan and carob galactomannan. These results indicated that CtLam81A efficiently hydrolyses β -1,3-glucan polymer but is unable to recognize other type of glycosidic linkages. Thus, the substrate specificity of CtLam81A is similar to other GH81 enzymes (Fliegmann *et al.*, 2005; Martin-Cuadrado *et al.*, 2008; McGrath & Wilson, 2006).

Table 3.3.1 Activity of CtLam81A against various polysaccharides.

Substrate	Specific Activity (U/mg)
Laminarin	100 $\pm$ 1.2
Curdlan	65 $\pm$ 0.6
Lichenan	1.0 $\pm$ 0.2
β -glucan	0.5 $\pm$ 0.1
Other substrates ^a	ND

ND = Not detected.

^a Birchwood xylan, Beechwood xylan, Carboxymethyl cellulose, Hydroxyethyl cellulose, Ivory nut mannan, Carob galactomannan

3.3.2 Biochemical properties of CtLam81A

CtLam81A displayed an optimum pH of 7.0 and retained more than 80% activity at pH 5.5 and 90% of activity at pH 8.0 (Fig. 3.3.1a). Similar pH optimum was reported for Lam81 from *Thermobifida fusca* (McGrath & Wilson, 2006) whereas, SpEng2 from *S. pombe* and ScEng2 from *S. cerevisiae* showed optimum pH 6.0 and 6.5, respectively (Martin-Cuadrado *et al.*, 2008). CtLam81A displayed stability in a wide range of pHs (6.5-9.0) (Fig. 3.3.1b). However, CtLam81A rapidly lost stability at pHs below 6.5 and above 9.0 (Fig. 3.3.1b). The optimum temperature for CtLam81A activity was 75°C (Fig. 3.3.1c). In addition, CtLam81A retained 100% of its activity at 70°C and 93% of its activity at 75°C after 1h incubation (Fig. 3.3.1d), indicating the thermophilic nature of the enzyme. This is the first report on an enzyme from family 81 GH that shows the stability up to 75°C. The thermal tolerance revealed by CtLam81A makes it suitable for industrial application as several industrial processes require thermostable enzymes for easy mixing, better substrate solubility, high mass transfer rate and reduced risk of contamination (Zamost *et al.*, 1991).

The kinetic parameters of CtLam81A were determined by analyzing the Michaelis–Menten plot and Lineweaver-Burk plot. CtLam81A displays a $V_{\max}$ of 109.0 ± 1.8 U/mg and a K_m of 2.1 ± 0.12 mg/ml for laminarin and a $V_{\max}$ of 74.2 ± 2.5 U/mg and a K_m of 0.54 ± 0.06 mg/ml for curdlan (Fig. 3.3.2). The effect of various cations and EDTA on the activity of CtLam81A was also investigated. The result indicated that the enzyme activity of CtLam81A increased in the presence of Ca^{2+} and Mg^{2+} ions, while activity was inhibited in the presence of Mn^{2+} , Zn^{2+} and Cu^{2+} ions and EDTA and there was no effect of Co^{2+} and Ni^{2+} ions on enzyme activity (Table 3.3.2). However, this result differs from previous studies, which showed no effects of Ca^{2+} and

Mg²⁺ ions on the activity of SpEng2 from *S. pombe* and ScEng2 from *S. cerevisiae* (Martin-Cuadrado *et al.*, 2008). The observed decrease in activity resulting from incubation with EDTA indicated that CtLam81A requires Ca²⁺ ions for structural stability and efficient catalysis. To study the effect of Ca²⁺ and Mg²⁺ ions in details, CaCl₂ and MgCl₂ concentration was varied from 0.1 to 10 mM in the reaction mixture. The result revealed that the enzyme activity of CtLam81A increased by 31% and 33% in the presence only 1 mM of Ca²⁺ and Mg²⁺ ions, respectively (Fig. 3.3.3).

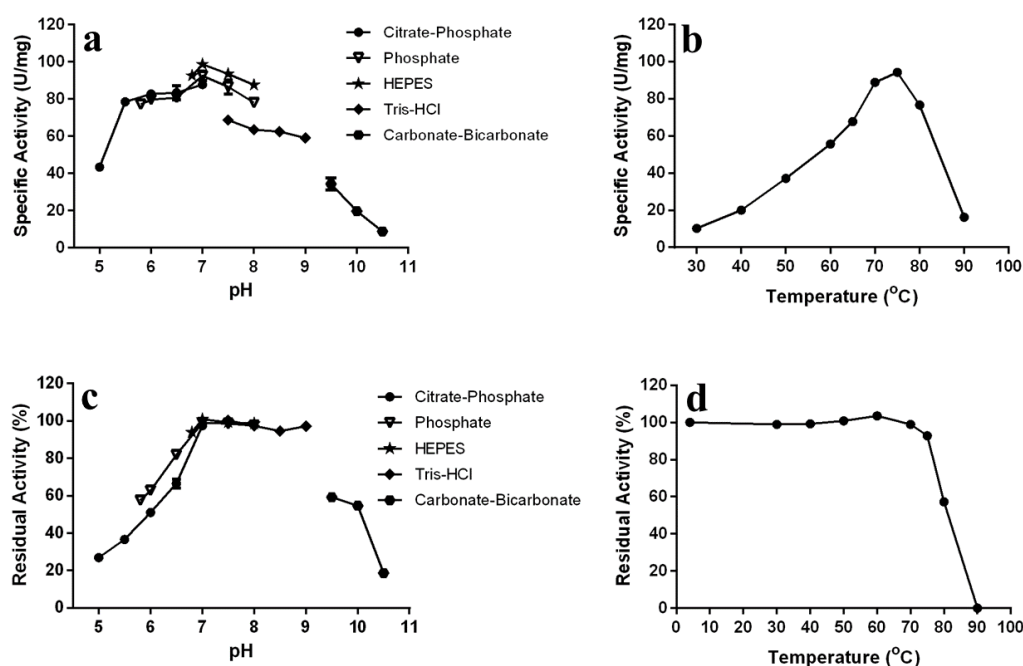


Fig. 3.3.1 Specific activity of CtLam81A was measured by incubating the enzyme with laminarin in different pH ranging 5.0-10.5 at temperature 75°C (a) or by incubating the enzyme with laminarin at different temperature ranging 30-90°C in pH 7.0 (b). Stability was assayed by incubating the CtLam81A for 60 min at different pH (c) and at different temperatures (d) before laminarin was added. In panel a and c, specific activity was measured in U/mg (1 Unit is defined as amount of enzyme in mg required to produce 1 μmol of glucose per min from laminarin). In panel b and d, activity at pH 7.0 and temperature 75°C was taken as 100%. The assay was performed in 100 μl reaction containing 1% laminarin. The reactions were carried out in triplicate sets.

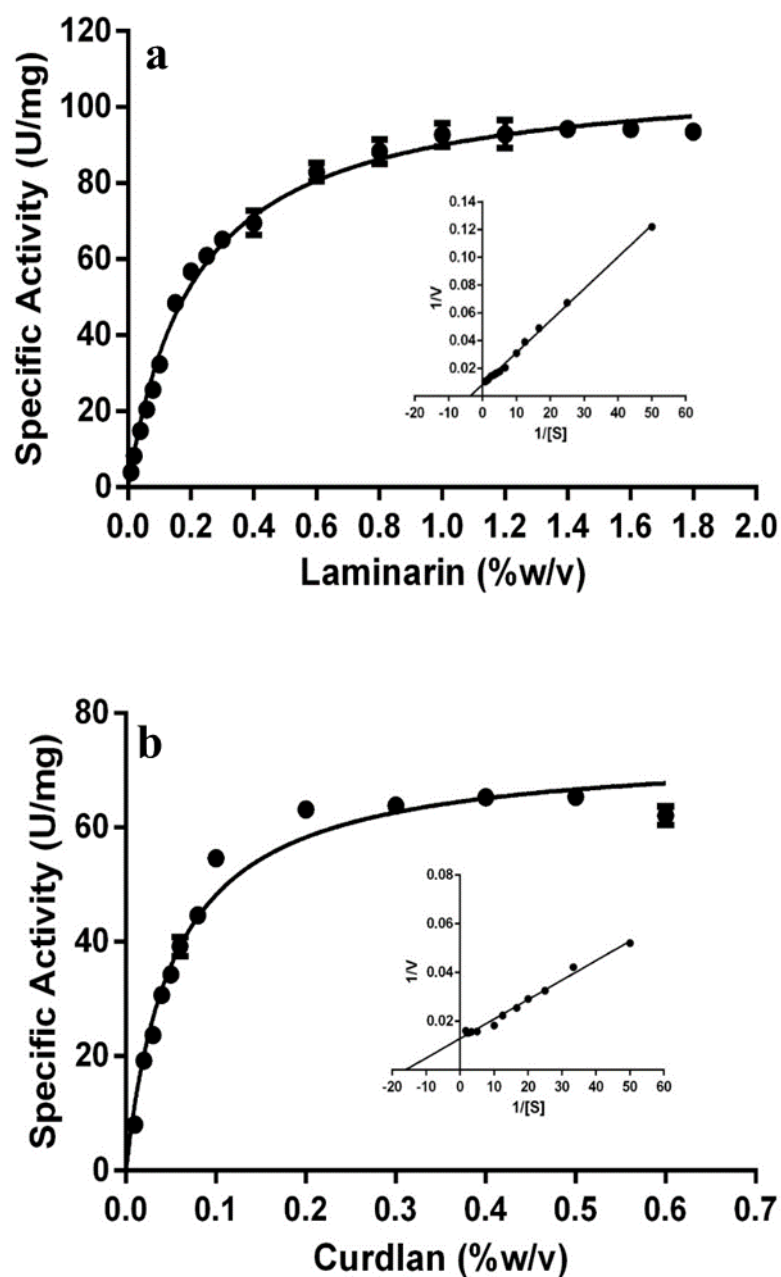
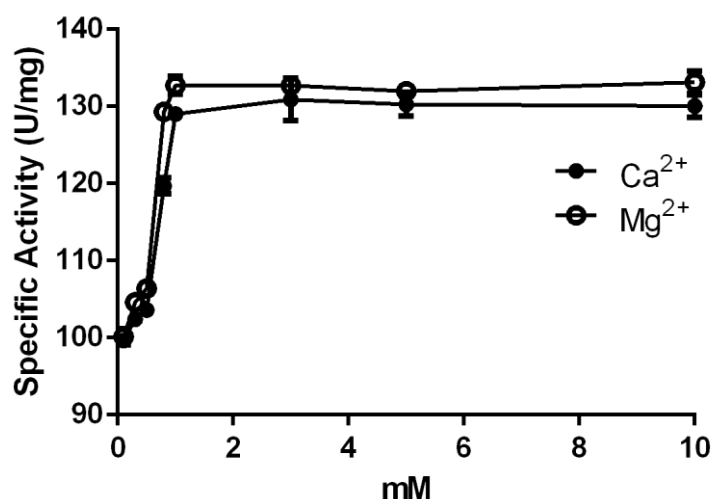


Fig. 3.3.2 Michaelis-Menten and Lineweaver-Burk (inset) plots for determining the kinetic parameters of *CtLam81A* for laminarin (**a**) and curdlan (**b**). The assay was performed in 100 μ l reaction mixture containing 10 μ l *CtLam81A* (0.01 mg/ml) with different concentrations of substrates at pH 7.0 and incubated at 75°C for 10 min as described in methods. The reactions were carried out in triplicate sets.

Table 3.3.2 Effect of metal ions on the activity of CtLam81A.

Metal ions/ Chelating agent (10 mM)	Specific Activity (U/mg)	Residual Enzyme Activity (%)
Control	100	100
Ca ²⁺	130.9±2.7	131
Mg ²⁺	133±1.2	133
Co ²⁺	93.6±0.9	94
Ni ²⁺	94.4±1	94
Zn ²⁺	26.2±0.5	26
Mn ²⁺	26.1±1.2	26
Cu ²⁺	14.2±0.5	14
EDTA	67.7±1	68

**Fig. 3.3.3** Effect of Ca²⁺ and Mg²⁺ ions on the activity of CtLam81A.

3.3.3 Protein melting curve of CtLam81A

The melting temperature of CtLam81A was found to be 92°C (Fig. 3.3.4a). This melting temperature was shifted towards a higher temperature, 96°C in the presence of 10 mM Ca²⁺ (Fig. 3.3.4b) or 10 mM Mg²⁺ ions (Fig. 3.3.4c). However, 10 mM EDTA shifted the melting temperature to a much lower temperature of 83°C (Fig. 3.3.4d). This observation suggested that Ca²⁺ and Mg²⁺ ions thermally stabilize CtLam81A and provide resistance to unfolding and denaturation at higher temperatures, whereas,

EDTA removes native metal ions and induces instability. These results are also corroborated by the *CtLam81A* activity profile in the presence of Ca^{2+} , Mg^{2+} ions and EDTA as described in the previous section. Taken together, data relating to protein melting and enzyme activity suggested that Ca^{2+} and Mg^{2+} ions are involved in maintaining enzyme structural integrity.

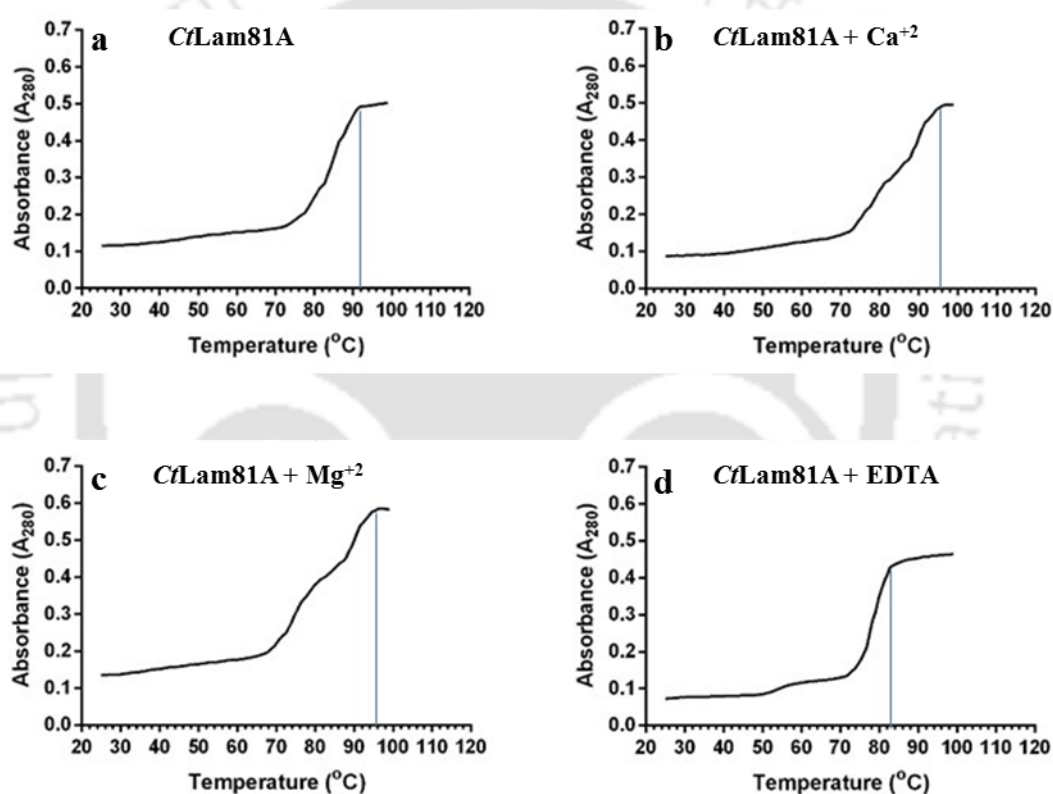


Fig. 3.3.4 Temperature melting curve of *CtLam81A* (a), *CtLam81A* + 10 mM Ca^{2+} ions (b), *CtLam81A* + 10 mM Mg^{2+} (c) and *CtLam81A* + 10 mM EDTA (d). 50 μg of *CtLam81A* protein was suspended in 1 ml 50 mM HEPES buffer (pH 7.0) and subjected to different temperatures and A_{280} was monitored on a UV-Visible spectrophotometer.

3.3.4 Hydrolysis of curdlan by CtLam81A and its mode of action

The amount of reducing sugar released during the hydrolysis of curdlan is displayed in Fig. 3.3.5a. This indicated that the reducing sugar release saturated after 12 h. The products generated by CtLam81A during the hydrolysis of curdlan were analysed by TLC (Fig. 3.3.5b). The chromatogram showed the presence of β -1,3-oligosaccharides of various degrees of polymerization (DP). Initially, upto first 5 min of the reaction, a wide range of oligosaccharides were formed, whereas towards the end of 24 h, only glucose, laminaribiose and laminaritriose were the major products. Thus, the data suggested that CtLam81A displays a typical endoglucanase activity. Further MALDI-TOF analysis of hydrolysed products of curdlan produced by CtLam81A enzyme in 5 min displayed peaks at m/z 203.7, 363.5, 526.7, 689.5, 852, 1014 and 1176.3 (as sodium adduct, $[M+Na^+]$) which corresponded to glucose (Glu), Laminaribiose (DP2), Laminaritriose (DP3), Laminaritetraose (DP4), Laminaripentaose (DP5), Laminarihexaose (DP6) and Laminariheptaose (DP7) (Fig. 3.3.5c). These oligosaccharides can be used as prebiotics (Salyers *et al.*, 1977) and also anticancer agents (Miyaniishi *et al.*, 2003, Jamois *et al.*, 2005).

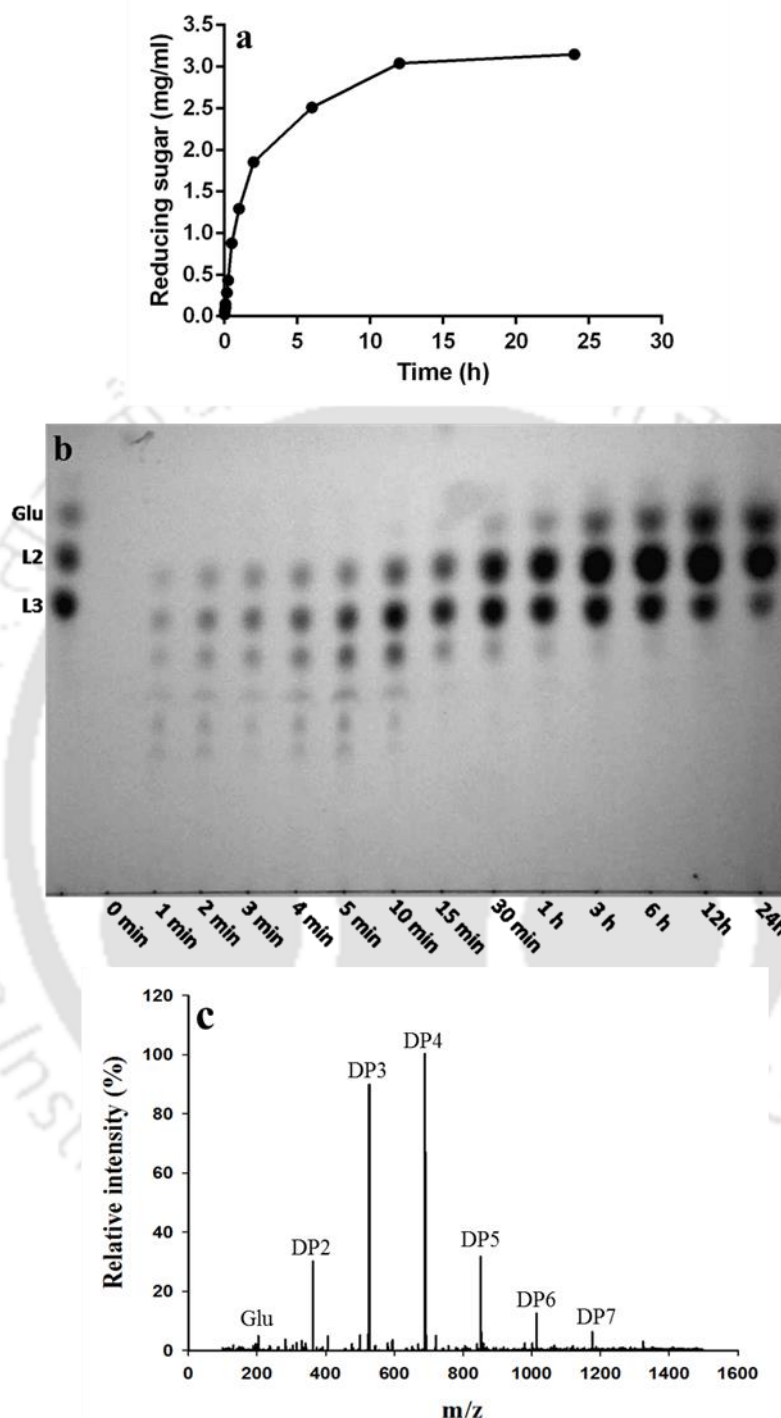


Fig. 3.3.5 Reducing sugar released by *CtLam81A* hydrolysis of curdlan at different time intervals (a), TLC analysis, Glu, L2 and L3 are glucose, laminaribiose and laminaritriose as standard (b) and MALDI-TOF analysis for mass confirmation (c). The assay was performed in 100 μ l reaction mixture containing curdlan (0.5%, w/v) at 75°C and pH 7.0 (HEPES) for different time intervals as described in methods.

3.4. Conclusions

The biochemical properties of recombinant CtLam81A from *Clostridium thermocellum* were studied. The enzyme is a major component of the cellulosome, a high molecular mass multi-enzyme complex that orchestrates the deconstruction of structural carbohydrates. CtLam81A displayed maximum activity against laminarin and therefore was named Laminarinase 81A (CtLam81A). CtLam81A displayed maximum activity at neutral pH and 75°C. The enzyme was stable in the pH range, 7.0 to 9.0 and was thermally stable up to 75°C for 1 h. CtLam81A displayed K_m , 2.1 ± 0.12 mg/ml and V_{max} , 109 ± 1.8 U/mg, against laminarin under optimized conditions. Ca^{2+} or Mg^{2+} ions enhanced the activity of CtLam81A and also raised the melting temperature by 5°C confirming their involvement in catalysis and also to provide protein stability. During the initial stages of hydrolysis of β -1,3-glucans, as analyzed by TLC and MALDI-TOF, CtLam81A released predominantly a range of oligosaccharides with different DP confirming an endo- β -1,3-glucanase activity. CtLam81A displays a large array of biotechnological applications including its use in agriculture to control fungal diseases. It can also be used in combination with exo- β -1,3-glucanase to produce fermentable sugar in bioethanol industry. The thermostability of CtLam81A makes it suitable for industrial applications as several industrial processes require thermostable enzymes for easy mixing, better substrate solubility, high mass transfer rate and lowering the risk of contamination. Finally, the capacity of CtLam81A to produce β -1,3-glucan oligosaccharides of different DP may be applied to the production of prebiotics, immune-activation precursors or anticancer agents.

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Chapter 4

Structural characterization of endo- β -1,3-glucanase of family 81 glycoside hydrolase (CtLam81A) from *Clostridium thermocellum* by *in silico* homology modeling and X-ray crystallography

4.1 Introduction

β -1,3-Glucans are linear polysaccharides of β -1,3-linked glucose units. These polysaccharides can be variably substituted with β -1,6-linked glucose in yeast, algae and plants (McIntosh *et al.*, 2005; Volman *et al.*, 2008). Polysaccharide turnover in biomass is largely performed by the action of microbes that contains a large group of specialized enzymes known as Carbohydrate Active enZymes (CAZY) to depolymerize these macromolecules. One of the predominant classes of degradative CAZymes is the glycoside hydrolases (GHs), which are presently grouped into 165 families based on sequence similarity (<http://www.cazy.org/Glycoside-Hydrolases.html>). The polysaccharides are accommodated within the active site of the GHs enzymes, which was described using the subsite nomenclature (Davies *et al.*, 1997). Each subsite accommodate a monosaccharide unit of the substrates. Subsites are numbered with increasingly negative numbers (-1, -2, -3, etc.) towards the non-reducing terminal, and with increasingly positive numbers (+1, +2, +3, etc.) towards the reducing terminal from the cleavage point. Exo-acting enzymes typically have a relatively small number of

subsites and usually have only one or two minus or plus subsites that accommodate the non-reducing or, more rarely, reducing end sugars, respectively, that are ultimately released from the polysaccharide by hydrolysis (Davies and Henrissat, 1995). Endo-acting enzymes usually have a larger number of subsites, and the active sites are open at both ends to allow internal binding on polysaccharide chains. The ability of individual active-site subsites to recognize particular monosaccharide substituents of the substrate, and to recognize these when linked in a specific fashion to neighbouring monosaccharides, provides GHs with their specificity (Davies and Henrissat, 1995). The efficient hydrolysis of β -1,3-glucans is typically achieved by the activity of both exo- β -1,3-glucosidases (EC 3.2.1.58), which fall into GH families 3, 5, 17, and 55, and endo- β -1,3-glucanases (EC 3.2.1.39) in GH families 16, 17, 55, 64, 81, and 128. Family 81 glycoside hydrolases are known for their endo- β -1,3-glucanase activity and are widely distributed in plants, yeast, fungi, bacteria, archaea and viruses. All of the proteins in family 81 GHs share a common size of around 650 amino acids. Some of its members are biochemically characterized like β -glucan-binding protein (GBP) from *Glycine max* (Fliegmann *et al.*, 2005), *TfLam81* from *Thermobifida fusca* (McGrath & Wilson, 2006) and Eng2 from *Saccharomyces cerevisiae* (Martin-Cuadrado *et al.*, 2008). ^1H NMR spectroscopic analysis of GBP hydrolysed product revealed the retaining hydrolytic mechanism of this family (Fliegmann *et al.*, 2005). So far only two enzymes, *RmLam81A* from *Rhizomucor miehei* (Zhou *et al.*, 2013) and *BhGH81* from *Bacillus halodurans* (Pluvinau *et al.*, 2017) provide information about crystal structure solved from family GH81. Crystal Structure analysis of *RmLam81A* depicted the presence of β -sandwich domain at N-terminal and $(\alpha/\alpha)_6$ domain at C-terminal connected via additional domain between them. Similar type of domain was also found

in *BhGH81* protein. Interaction study of laminarin with *BhGH81* revealed that the active site of this protein can accommodate maximum five glucose residues of β -1,3-glucan (Pluvinage *et al.*, 2017). *Clostridium thermocellum* contains multienzyme complex, known as cellulosome. Cellulosome complex is made by a high affinity interaction between dockerin modules of the cellulosomal catalytic proteins and cohesin modules on the scaffoldins (Fontes and Gilbert, 2010). One such cellulosomal enzyme of *Clostridium thermocellum* is β -1,3-glucanase (locus tag *Cthe_0660*) belongs to family 81 GH. In this study, the secondary structure elements of *CtLam81A* were determined by Circular Dichroism and also confirmed by PsiPred. The 3D structure of *CtLam81A* was modeled by homology modeling and ligand docking study was performed to identify the amino acid residues involved in the catalysis. The catalytically inactive mutant *CtLam81A-E515A* generated by site-directed mutagenesis was co-crystallized. The high resolution structure of the mutant *CtLam81A-E515A* obtained up to 1.4 Å, has allowed the understanding of how the individual components of high molecular mass multi-enzyme complexes attack recalcitrant carbohydrates.

4.2 Material and Methods

4.2.1 Retrieval of amino acids sequence of CtLam81A

The gene sequence of family 81 glycoside hydrolase protein of *Clostridium thermocellum* ATCC27405 having gene accession number ABN51895.1 and uniprot ID A3DD6 was retrieved from CAZy database (<http://www.cazy.org/>). The boundaries of the family 81 GH catalytic domain (CtLam81A) in the amino acid sequence of β -1,3-glucanase (locus tag Cthe_0660) protein was identified by using the conserved domain database (<http://www.ncbi.nlm.nih.gov/cdd/>). The 2151 bp gene encoding CtLam81A protein was cloned, expressed and purified earlier (Kumar *et al.*, 2018).

4.2.2 Secondary structure analysis of CtLam81A

Secondary structure of CtLam81A protein sequence for alpha helices, beta strands, turns and loops were predicted with the help of PsiPred v3.3 method (<http://bioinf.cs.ucl.ac.uk/psipred/>). The composition of secondary structure of CtLam81A was also determined by Circular dichroism (CD). The purified CtLam81 at a concentration 0.7 μ M in 50 mM phosphate buffer, pH 7.5 was used for CD analysis. The CD spectrum was recorded on a spectro-polarimeter (Jasco J-815, Japan) at 25°C using 1 nm bandwidth over far UV region between 190 to 250 nm at scanning rate of 50 nm/min. The CD data were presented by difference in molar extinction coefficient ($\Delta\epsilon$, deciliter mol⁻¹ cm⁻¹) as a function of wavelength (Kelly *et al.*, 2005). Percentage of α -helix and β -sheet were measured by web based K2D3 software package (<http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/>) (Andrade *et al.*, 1993).

4.2.2.1 Circular dichroism data evaluation

The CD data are recorded either as differences in the absorbance of left (Δ_L) or right (Δ_R) handed polarized light gives molar extinction coefficient ($\Delta\epsilon$) as given earlier by Andrade *et al.*, (1993).

$$\Delta\epsilon = \Delta_L - \Delta_R \quad (1)$$

or they can be expressed in terms of molar ellipticity $[\theta]$,

$$[\theta] = 3300 \times \Delta\epsilon. \quad (2)$$

Applying Beer-Lambert law to equation 1 yields,

$$\Delta\epsilon = \epsilon_L - \epsilon_R = \frac{\Delta_L - \Delta_R}{c \times l} \quad (3)$$

where, c is the concentration of protein in moles/litre and l is the path length in cm. Molar extinction coefficient of CtCBM35 in left hand polarized light (ϵ_L) and right hand light (ϵ_R).

So, $[\theta]$ can be calculated as

$$[\theta] = \frac{\theta \times 100 \times M_r}{c \times l} \quad (4)$$

where, M_r is the protein molecular weight.

Now if the protein concentration is in mg/ml, the concentration of amino acids can be calculated by assuming a mean residue weight (MRW) of 740 amino acid residues

The mean molar ellipticity $[\theta]_{MRE}$ can be calculated as,

$$[\theta]_{MRE} = \frac{\theta \times 100 \times M_r}{c \times l \times N_A} \quad (5)$$

where, N_A is the number of amino acids per protein. The molar ellipticity was expressed in terms of $\text{deg cm}^2 \text{ dmol}^{-1}$ (Greenfield *et al.*, 2006). The factor 100 is added in the

above equations for converting the molar concentration to dmol/cm^2 concentration units.

4.2.3 Homology modeling CtLam81A

The three dimensional structure of CtLam81A was modeled with the help of SWISS-MODEL server. SWISS-MODEL is a web based server, which automatically identify structural templates based on BLAST analysis and generate 3-D protein structure (<https://swissmodel.expasy.org/>). The best modeled structure was selected based on the alignment score with templates.

4.2.4 Model refinement and quality assessment

The best modeled structure was energy minimized by Swiss-PDB viewer. Swiss-PDB viewer comprises a version of GROMOS 43B1 force field (Van Gunsteren *et al.*, 1996). This force field allows the evaluation of the energy of structure as well as it repairs the distorted geometry through energy minimization. Then the quality of energy minimized structure was checked by plotting the Ramachandran plot using PROCHECK server (<https://servicesn.mbi.ucla.edu/PROCHECK/>). This modeled structure was also validated by using the Verify-3D program (http://services.mbi.ucla.edu/Verify_3D/), which analyzes the compatibility of atomic model with the amino acid sequence (Liithy *et al.*, 1992).

4.2.5 Prediction of active site and mechanism of action

The amino acid residues in the active site are generally conserved in the proteins of the same family. To identify the amino acid residues at active sites, CtLam81A was structurally aligned with the structure of BhGH81 (PDB ID- 5T49) using Pymol. To confirm whether these residues are conserved within family 81 GHs, multiple sequence alignment (MSA) was performed with the already characterized homologous proteins

of the family 81 GHs viz. *Bacillus halodurans* GH81 (*BhGH81*; Q9KG76), *Thermobifida fusca* Lam81A (*TfLam81A*, AAZ56163), *Glycine max* Gbp (*GmGbp*; BAA11407), *Aspergillus fumigatus* EngA (*AfEngA*; AAF13033), *Saccharomyces cerevisiae* Eng2 (*ScEng2*; AAB82378) and *Rhizomucor miehei* Lam81 (*RmLam81A*; KC847083). The percentage similarity between amino acid sequences of *CtLam81A* with its homologous proteins from family 81 GH was analysed by BLAST tool (Altschul *et al.*, 1990). MSA was performed with the help of CLUSTALW program (<http://www.genome.jp/tools-bin/clustalw>) and the conserved amino acid residues were viewed by ESPript 3.0 (<http://esprict.ibcp.fr/ESPript/ESPript/>). To study the mechanism of hydrolysis the distance between carboxyl group of catalytic amino acids (Asp438 and Glu515) of *CtLam81A* was measured in the molecular visualization system PyMol 2.0.

4.2.6 Molecular dynamic simulation of *CtLam81A* modeled structure

Molecular dynamic (MD) simulation of *CtLam81A* was performed by using GROMACS v 5.14 (Berendsen *et al.*, 1995) GROMOS96 53a6 force field was used to calculate the Protein forces, where the protein *CtLam81A* was placed within in a cubic box of single point charge (SPC) with water molecules. The *CtLam81A* protein charges were neutralized by adding the 15 numbers of counter ions (Na^+). Then the whole system was equilibrated for 500 ps in NVT ensemble (constant number of particles, volume and temperature) was carried out for restraining the solute atoms. This system was again equilibrated for 500 ps by NPT ensemble (constant number of particles, pressure and temperature) twice, once with restraints and then without restraints. Production run was performed for 30 ns with NPT ensemble adopting a 2 fs integration time. The linear constraint solver (LINCS) algorithm (Hess *et al.*, 1997) was employed

to constrain the bonds associated with hydrogen atoms and radius of gyration. Throughout the production run the modeled *CtLam81A* structure was analyzed as a time dependent function to ascertain its stability in the solvent system. The variation in the *CtLam81A* protein backbone (root mean square deviation, RMSD) was estimated by the least square fitting method.

4.2.7 Biding interaction analysis of *CtLam81A* with the ligands

Molecular docking of *CtLam81A* with the laminarioligosaccharides was performed by using SwisDock, the web based server (<http://www.swissdock.ch/docking>). Laminaribiose, laminaritriose and laminaritetraose were downloaded from PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Laminaripentaose and laminarihexaose were generated using the GLYCAM server (Kirschner *et al.*, 2008). The modeled *CtLam81A* was saved in PDB format and the ligands were saved in Mol2 file format for docking analysis in the SwissDock tool. SwissDock generates large number of ligand binding results. The enzyme-ligand docked complex showing the strongest binding with maximum of negative binding energy were selected. This ligand bound structure was downloaded and visualized in PyMol 2.0. The depiction of ligand interaction with the amino acid residues of the protein was generated using the PDBsum Generate tool (<https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html>).

4.2.8 Generation of mutant *CtLam81A*-E515A by site-directed mutagenesis

To study the mode of binding of *CtLam81A* with the substrate, the catalytic base, Glu515 was mutated to alanine. The mutant (*CtLam81A*-E515A) was generated by site-directed mutagenesis by using the NZYmutagenesis kit (NZYTech, Lisbon, Portugal) using the plasmid *CtLam81A*-pET28a as template. The primers used were as

follows: “forward 5’-GCCGACGGCAACAATCAAG**CG**TCATCATCGGAGGCCATCAAC-3’” and “reverse 5’-GTTGATGGCCTCCGATGATGAC**CG**CTTGATTGTTGCCGTCGGC-3’”. The details of PCR reaction mixture (50 µl) and PCR cycles for amplification are shown in Tables 4.2.1 and 4.2.2, respectively. The generated plasmid was sequenced to ensure that only the appropriate mutation had been incorporated into the amplified DNA. The recombinant pET28a derivative encoding *CtLam81A-E515A* was used to transform *E. coli* BL-21 cells, expressed and purified as described earlier (Chapter, section 2.2.9 & 2.2.13). The activity of mutant (*CtLam81A-E515A*) was determined in 100 µl reaction mixture containing 1% (w/v) laminarin in 50 mM HEPES buffer (pH 7.0) and 10 µl purified mutant (*CtLam81A-E515A*) (1 mg/ml). The reaction was incubated at 75°C for 10 min and activity was determined as described in chapter section 3.2.2).

Table 4.2.1 PCR mixture for site directed mutagenesis of *CtLam81A*.

PCR components	Volume (µl)
10x reaction buffer	5.0
dNTP mix	1.0
Forward primer (125 ng/µl)	1.0
Reverse primer (125 ng/µl)	1.0
Sigma water, pH 8.0	40.0
<i>CtLam81A</i> -pET28a(+) (50.0 ng/µl)	1.0
NZYProof DNA polymerase (2.5 U/ µL)	1.0
Total	50.0

Table 4.2.2 PCR cycles for generating site specific mutant of *CtLam81A*.

Steps	Time
I. Denaturation at 95°C	2 min
II. 18 cycles of	
i) Denaturation at 95°C	1 min
ii) Annealing at 60°C	1 min
iii) Extension at 68°C	8 min
III. Final extension at 68°C	15 min

4.2.9 Crystallization of CtLam81A-E515A, data collection and processing

The mutant CtLam81A-E515A (30 mg/ml) was co-crystallized in the presence of laminaritetraose (30 mM) and laminarihexaose (30 mM) supplemented with 0.5 mM CaCl₂, using the sitting drop vapor-diffusion method and drops were set at 277 K. The drops were prepared by adding an equal volume (1 µL) of protein-ligand mixture and reservoir solution on 96-well crystallization plates (SWISSCI 'MRC' 2-Drop Crystallization Plates, Douglas Instruments, Berkshire, UK) using a nanodrop robotic dispensing system (Oryx8, Douglas Instruments). Several commercial screens, namely the PEG/Ion HT and CS1/CS2 (Hampton Research, Aliso Viejo, CA, USA), the JCSG screen (Jena Bioscience, Jena, Germany) were used. CtLam81A-E515A crystallized only in one of the conditions of the JCSG screen containing 30% (w/v) 2-methyl-2,4-pentanediol, 0.1M sodium acetate pH4,6 and 0.02M calcium chloride. CtLam81A-E515A crystals were harvested from the crystallization drop and transferred into a cryoprotectant solution mimicking the mother liquor supplemented with 30% (w/v) glycerol. A complete dataset was collected at beamline ID23-1 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and the crystal diffracted up to a resolution of 1.4 Å. The dataset was processed with XDS (Kabsch, 1988) and scaled with AIMLESS (Evans, 2006). The crystal indexed in the body-centered orthorhombic space group $I2_12_12_1$ with unit-cell parameters $a = 61.92$ Å, $b = 139.03$ Å and $c = 197.91$ Å. The Matthews coefficient calculations suggested the presence of one molecule per asymmetric unit (Matthews coefficient of 2.61 Å³/Da) with a solvent content of 53%. The data-collection and processing statistics are presented in Table 4.3.3.

4.2.10 Structure solution and refinement

At the time of the structure determination process, the only structural model available for phasing was the family 81 glycoside hydrolase from *Rhizomucor miehei* (PDB: 4K3A) with a sequence identity of 27%. The phasing model was optimized by pruning non-conserved residues and domains by using Chainsaw (Stein 2008) prior to Phaser-MR (McCoy *et al.*, 2007) from the PHENIX package (Adams *et al.*, 2010). AutoBuild (Terwilliger *et al.*, 2008) from PHENIX (Adams *et al.*, 2010) was used for automated model building producing a model for CtLam81A-E515A with 684 residues with R_{work} and R_{free} of 0.198 and 0.213, respectively. Restrained refinement to structure completion was performed with PHENIX.REFINE (Afonine *et al.*, 2012), with iterative inspection of the electron density maps and manual rebuilding with COOT (Emsley *et al.*, 2004). In the final stages of refinement, the R_{work} and R_{free} converged to 10.8% and 13.3%. The geometrical validation and model improvement was carried out by using PDBREDO (Joosten *et al.*, 2009; Joosten *et al.*, 2012) and other validation programs such as PROCHECK (Laskowski *et al.*, 1993) and MOLPROBITY (Chen *et al.*, 2010). The analysis of the model showed that 97.6% of the protein residues are in the most favored or additionally allowed regions of the Ramachandran plot, while only 0.14% are in disallowed regions (Residue H79). Refinement statistics are summarized in Table 4.3.3.

4.2.11. Accession Codes

Coordinates and observed structure factor amplitudes for CtLam81A-E515A have been deposited in the Protein Data Bank under the accession code 6FOP.

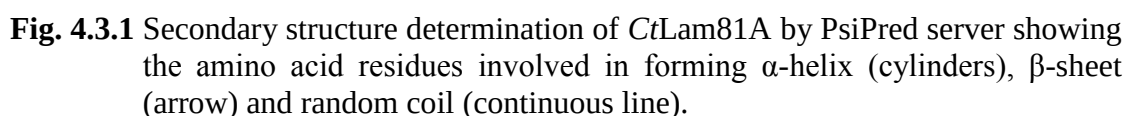
4.3 Results and Discussion

4.3.1 Molecular architecture of CtLam81A

CtLam81A from *Clostridium thermocellum* contains an N-terminal catalytic domain and a type I dockerin domain at the C-terminal connected via linker. The dockerin domain binds to cohesion domain, located on the scaffoldin protein in cellulosome complex (Ding *et al.*, 2001). The 2151 bp gene encoding family 81 glycoside hydrolase (CtLam81A) and linker was cloned in pET-28a(+) vector, expressed in *E. coli* BL21 cells and purified (Kumar *et al.*, 2018). Total length of the purified protein (CtLam81A) contains 740 amino acids, of which 23 amino acids are of pET28a(+) vector and 717 amino acids are of catalytic domain. The theoretical molecular mass of CtLam81A is 82.4 kDa.

4.3.2 Secondary structure analysis of CtLam81A

Secondary structure of CtLam81A predicted by PsiPred tool showed the presence of 14 α -helices (23.1%), 31 β -strands (23.2%) and 45 random coils (53.7%) (Fig. 4.3.1). CtLam81A showed almost an equal amount of β -strands and α -helices. The N-terminal of CtLam81A showed the predominance of β -strands and the C-terminal showed the abundance of α -helices. The predicted secondary structure was also confirmed by circular dichroism analysis of purified CtLam81A. The CD spectrum of CtLam81A was analysed with the help of K2D3 server, which gives the percentage of α helix and β strand by comparing the available secondary structures of known proteins (Louis-Jeune *et al.*, 2012). This analysis showed that, CtLam81A contains 23.6% α -helices and 23.6% β -strands (Fig. 4.3.2). The results of CD analysis corroborated with the secondary structure predicted by PsiPred method. This distribution of secondary structure elements is common in family 81 GH (Zhou *et al.*, 2013).



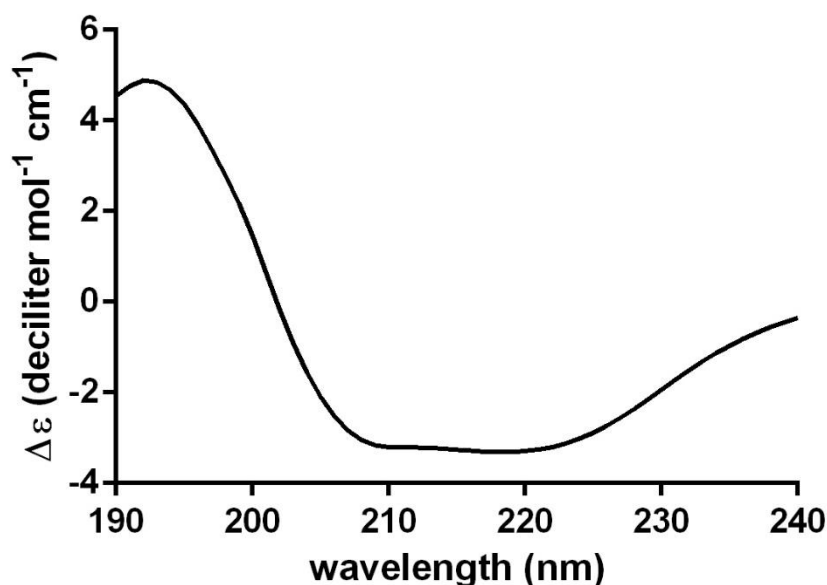


Fig. 4.3.2 Circular dichroism (CD) spectrum of *CtLam81A* for determining the percentage of secondary structure elements. The CD data were presented by difference in molar extinction coefficients ($\Delta\epsilon$, decilitre mol⁻¹ cm⁻¹) as a function of wavelength. The purified *CtLam81* at a concentration 0.7 μ M in 50 mM phosphate buffer, pH 7.5 was used for CD analysis. The CD spectrum was recorded at 25°C using 1 nm bandwidth over far UV region between 190 to 250 nm at scanning rate of 50 nm/min.

4.3.3 Homology modeling and structure validation

The web based server SWISS-MODEL generated the 3-D model structure of *CtLam81A* by using the structural template of protein *BhGH81* (PDB ID- 5T49) having 42% sequence identity with query coverage 100%. The overall structure of the modeled *CtLam81A* (Fig. 4.3.3A) consisted of three domains, N- terminal β -sandwich domain I (green colour), an $(\alpha/\alpha)_6$ barrel domain II (red colour) and a short β -sandwich domain III (yellow colour) at C-terminal. This modeled structure was energy minimized and validated before docking analysis. Ramachandran plot of *CtLam81A* modeled protein showed 86.1 % of the residues found in the favourable region, 12.3 % residues found in additional allowed region, 1.1 % residues found in generously allowed region and

only 0.5 % residues (Phe177, Ser183 and Glu276) was found in outlier region (Fig. 4.3.3B). This indicated that the amino acid residues in the modeled *CtLam81A* occupied favourable phi (ϕ) and psi (ψ) backbone dihedral angles. Verify_3D result showed that 95.25% of the residues in modeled structure had an averaged 3D-1D score ≥ 0.2 , which indicated the compatibility of amino acids with the modeled structure (Fig. 4.3.3C). ProSA result of modeled *CtLam81A* indicated that the protein is error free and reside in the x-ray zone with Z-scores of -9.88 (Fig. 4.3.3D).

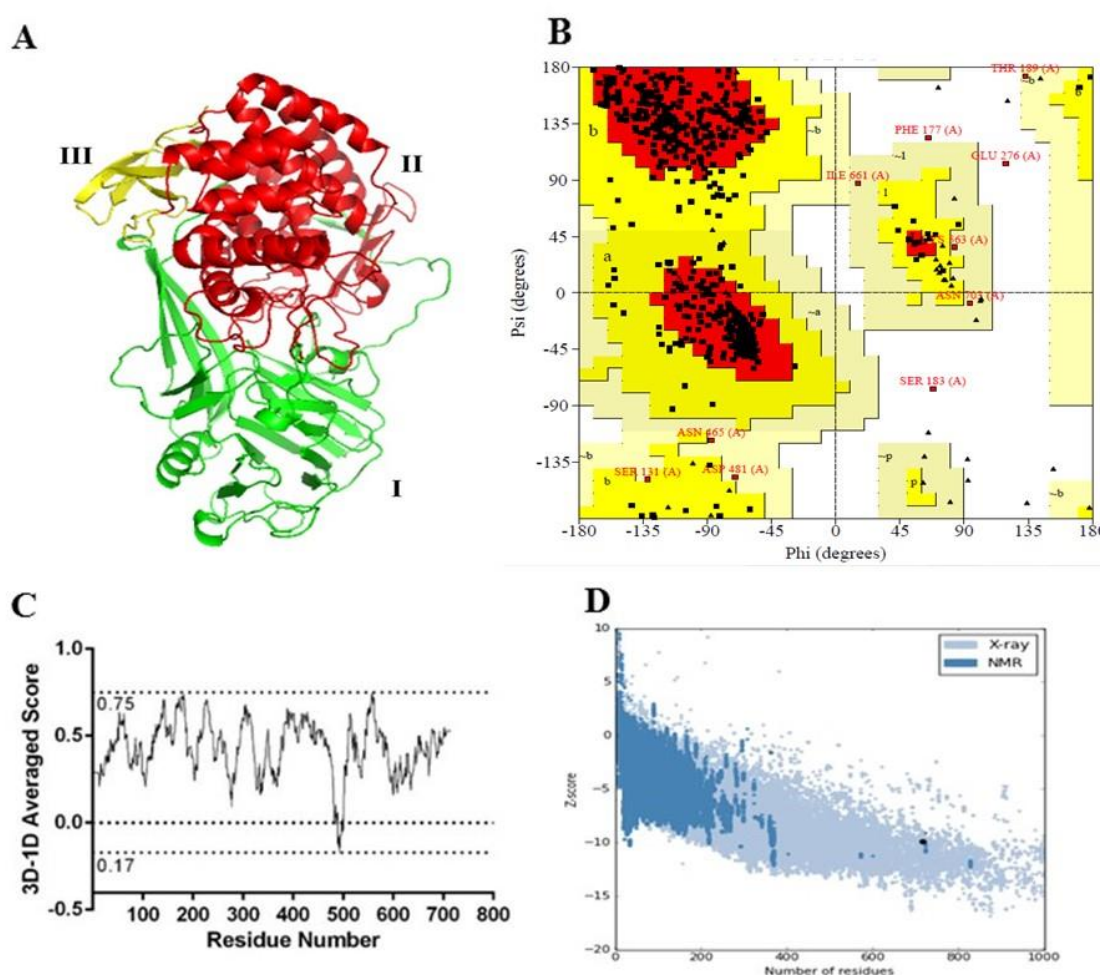


Fig. 4.3.3 (A) Cartoon representation of modeled structure of *CtLam81A* displaying N-terminal β -sandwich domain I (red colour), an $(\alpha/\alpha)_6$ barrel domain II (green colour) and a short β -sandwich domain III (yellow colour) at C-terminal. Quality assessment of modeled *CtLam81A* by (B) Ramachandran plot of modeled *CtLam81A*, (C) Verify-3D and (D) Prosa web server.

4.3.4 Catalytic cleft and mechanism of catalysis of CtLam81A

The amino acids residues involved in the catalysis and in substrate binding were explored by superimposing the CtLam81A modeled structure with crystal structure of BhGH81 (PDB ID – 5T49) having RMSD, 0.495 Å as shown in Fig. 4.3.4A. The catalytic core of CtLam81A existed within (α/α)₆ barrel domain of the protein. The superposition of modeled structure of CtLam81A (shown in green colour) with BhGH81 (shown in cyan colour) showed that β -sheet and α -helix of CtLam81A aligned well with the β -sheet and α -helix of BhGH81 (Fig. 4.3.4A). However, some loop regions (43-65 amino acids, 96-105 amino acids, 214-222 amino acids and 296-302 amino acids) of the CtLam81A modeled structure do not align with the BhGH81 structure. The analysis of superimposition showed that Glu515 acts as catalytic base and Asp438 acts as acid catalyst while Trp588 and Trp589 helps in binding the ligand (Fig. 4.3.4B). CtLam81A shared amino acid sequence similarity with homologous proteins from *Bacillus halodurans* GH81 (BhGH81; Q9KG76), *Thermobifida fusca* Lam81A (TfLam81A, AAZ56163), *Glycine max* Gbp (GmGbp; BAA11407), *Aspergillus fumigatus* EngA (AfEngA; AAF13033), *Saccharomyces cerevisiae* Eng2 (ScEng2; AAB82378) and *Rhizomucor miehei* Lam81 (RmLam81A; KC847083) as shown in Table 4.3.1. The multiple sequence alignment of CtLam81A showed that the catalytic amino acids are conserved in all family 81 glycoside hydrolases. Trp588 is conserved in both bacterial and plant proteins, while Trp589 is conserved in bacteria only (Fig. 4.3.5). Glycoside hydrolases catalyse the glycosidic bond, either by inverting or retaining hydrolytic mechanism. It has been found that, in the inverting mechanism, the average distance between two carboxyl groups of acid/base catalytic residues is 10.5 Å and in the retaining mechanism the average distance is only 5.5 Å (Zechel & Withers, 1999). The

superimposition of *CtLam81A* structure with crystal structure of *BhGH81* indicated that Glu515 is catalytic base and Asp438 is catalytic acid. The distance between these two catalytic residues was found to be 8.3Å (Fig. 4.3.6), indicating the inverting hydrolytic mechanism for *CtLam81A*. The same inverting hydrolytic mechanism was also reported for β -glucan-binding protein (GBP) a member of family 81 glycoside hydrolases from soybean (Fliegman *et al.*, 2005).

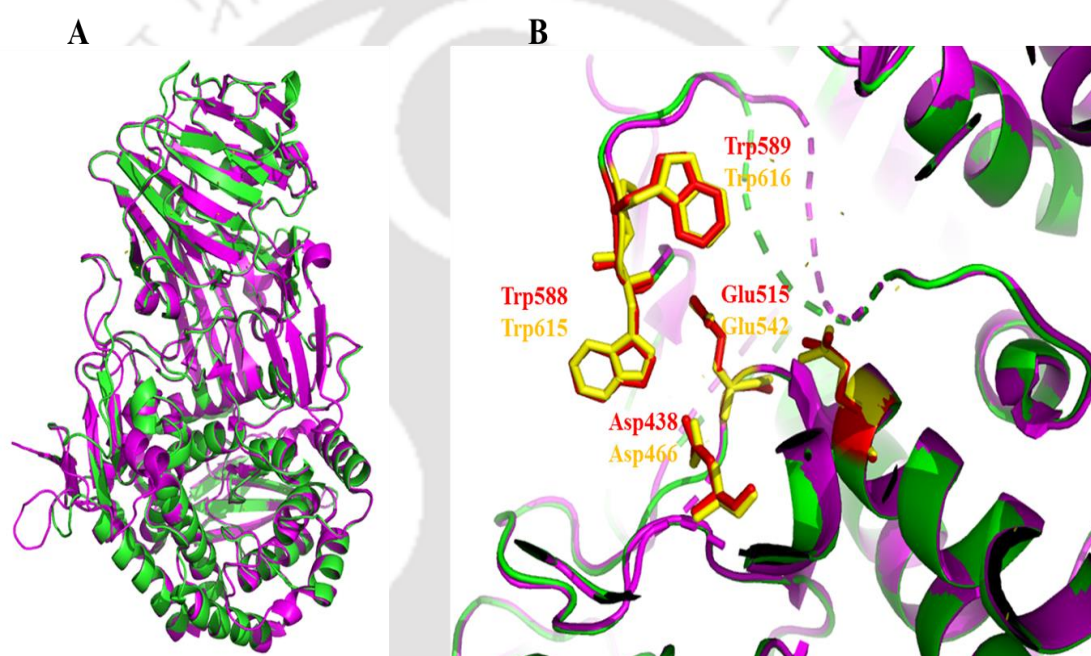


Fig. 4.3.4 (A) Superimposition of *CtLam81A* modeled structure (green) with *BhGH81* crystal structure (cyan) and (B) Superimposition of active site residues of *CtLam81A* (red colour) with active site residues of *BhGH81* (yellow colour) by molecular visualization system PyMol 2.

Table 4.3.1 Blast analysis of CtLam81A with its homologues from family 81.

Organism	PDB ID	Query coverage (%)	Identity (%)	e-Value	Total Score
<i>Bacillus halodurans</i> C-125	5T4A	100	42	0	553
<i>Thermobifidafusca</i> YX-ER1	-	96	43	0	570
<i>Glycine max</i>	-	83	24	4e-35	130
<i>Aspergillus fumigatus</i> Af293	-	71	26	5e-35	130
<i>Saccharomyces cerevisiae</i> YPA84	-	64	27	2e-30	116
<i>Rhizomucormiehei</i>	4K3A	44	27	2e-25	112

1 CtLam81A		1	MGSSHHHHHHSS...	10	GLVPRGSHMASQYYREG.	20
2 BhGh81			XGSSHHHHHHSS...		GLVPRGSHXASHAVSVG.	
3 RmLam81						MRFOV
4 TfLam81A	MS.....		HASRRRWRRRA...		TTSAATAALLCG...	ALLTFPSAPAAAQVRLG.
5 AfEngA						
6 ScEng2	MCYSRQATPPPPV		PNNRPGGTTNRGPP		PLPPRANVQPPVCSS	ENSSSKPRENRVAGESLRTPS
7 GmGbp						

1 CtLam81A		30	TCSTVVLPP	40	AKVPQ.....	50	AIY.....	K
2 BhGh81			KGSYATIEFPE		IDFGGI.....		NDPGFRDQQGEP	TIYR
3 RmLam81			IVAAATITMIT		SYIPQVASQSSDGD		DLFVPVSNFDPKS	IFPEIKHPFEP
4 TfLam81A			SGSYTTTTLPP				ASGSPS.....	D.....
5 AfEngA			MGIVSFLKPVV		L..ANGQDVFVPVS			TGPN SQDDWLE.....
6 ScEng2	SSNPLADSQVN		SDNIFQSPVL		SNLKAPPSVFNKV			QHPVPK.....
7 GmGbp			MVNIQTNTSY				IFPQTQSTVLPDP	SKFFS

1 CtLam81A	TSNLQGA	60	AVF	70	TNSWESSIL	80	WNQ.....	90	YSLPIYA	100	HPLTFKFKAE	110	GIE...V	120	GKPA...	130	LGG
2 BhGh81	SDRVITG		PXQ		TNSWGSLLA		VDR.....		FSXNQYP		HPFSVHRAE		GLH...V		FYDAPHMXV		
3 RmLam81	NTENGKIV		ENSWISNLFYPSA				DNLAFTPD		PTLTL		LDQYGGN...		PLTROP		SAK		
4 TfLam81A	TADFTQ		PVV		TNDWSSLI		FQRYPGNPYGEN		LYA		HPLSFKQA		AGLE...V		GYPDTPELVA		
5 AfEngA	SVNNTDT		PVE		TNKFFYCGFLG				TQNTNT		FTHPYSVAWVK		GGTSQSGMA		ISHVESN		
6 ScEng2	DDQSVDP		PLE		TNKFFYTNML		LD.....		DNTQPIWT		HPYSIWF		SRD...		ELFGLA		NHTLAS
7 GmGbp	SNLLSS		PLP		TNSFFQNFV		LKN.....		GDQOEYI		HPYLIK		SSNSL...		SLSYPS		RQASSA

1 CtLam81A		100	SGI...	110	AYFGAHKNDF	120	VGHSS...	130	VYTFPD	140	ARADKISDFA	150	VDVAVM...
2 BhGh81	H...ENREA		..GTW...		HIHGAIGTDT		IKHSG...		TANFEQ		AVDDYNDWY		VRGLLM...
3 RmLam81	VLGSYPP		TNDVPY		TADAGYMIN		SVVDLR.		LTSSSEW		SDVVPDR.		QVTDWDHLS
4 TfLam81A			DGL...		KYQYTHSP		DFV...		LGIHG...		L.NAPA		KVAGYSDWT
5 AfEngA	IVAHG		PVNTAIP		CSPISYVNP		IGTHSV		LSASE...		LGPTVT		TENPLFSA
6 ScEng2	Q.....		RVFDTT		TNPPRFY		FNPTNKS		SVFFKARE				FVSSND
7 GmGbp	VI.....		FQ.....		VFNPD...		LT.		ISAPQGP		KQGPPGKHL		ISSYSDLS

1 CtLam81A	ASG	140	SGS	150	KATLMKGS	160	PYAFVFTG	170	GNFRID	180	FSGTPTVFY	190	GDSDG...	200	SQCLGV	210	TINGVN
2 BhGh81	ENGAH		QXAIT		YGVGS		PYIFV		YEDGSA		VLDFDIAP		DVWEXN...		GHVIGF		STHDKH
3 RmLam81	PQDS		NSYID		DFP		IVRGMA		YITAN		YNNLT		PQFLSQHAI		ISVEADE		KKSDNTST
4 TfLam81A	SDGT		RLQRT		IGQGL		PFVYAD		VSGGF		IRVEFTAP		PTVWRRS...		GNNAVST		VNGHK
5 AfEngA	SASS		SQITIP		VVQGM		GFVTGI		YSLNLP		QPKIQSGV		FFTKMVTAGS				PR.TGIFK
6 ScEng2	SLSS		SQFIEFF		LVIQGM		GFVTGI		YSLNLP		QPKIQSGV		FFTKMVTAGS				PR.TGIFK
7 GmGbp	DFP		SSNLS		FFLV		GS		PYLT		TVSVTQ		TFPL.		SITTIHS		ILSFS

1 CtLam81A	YGLFAPS	200	GSKW	210	QGIGTGTI	220		230	TCILPAG	240	KNYFS	250	IAVLP	260	DNTVSTLT
2 BhGh81	YAAFA		APP		GQNW		SGIGSKTL				TNNADY		IAIAKL		PEKDGXLA
3 RmLam81	ITMND		DPPT		STFI		IYISLGDK				PLELRKQD		NSNLVASK		PKPYTG
4 TfLam81A	YALFAPS		GTTW		SES		DTVF.....				TADVGS		SGYAS		VALLPSP
5 AfEngA	YSLSL		LEDG		TSWLL		YATPDD		GSQP		QLASNSE		IIGPAGW		SGTIQ
6 ScEng2	YSLSL		LEDG		TSWLL		YATPDD		GSQP		QLASNSE		IIGPAGW		SGTIQ
7 GmGbp	YTFQF		NN		GQTW		LLYAT		TSPI.....		KLNHTL		SEITSNAF		SGIIRIAL

1 CtLam81A	YYKDYAYC	240	FVTD	250	TKVEWS	260	YNETES	270	TLTTF	280	TAESVK	290	EGTNKG	300	ITILALY	310	PHQWRN
2 BhGh81	KFEQYAYS		VVRDAV		ADWT		YDEATG		TVTTT		FEVTTEAK		VQVQAPD		G.TIFALY		PHQYRHLA
3 RmLam81	LLDASRAV		WPTGGD		ISARSDD		NGGAS		SYTIK		WKT...		N...SNEA		PLTYAY		AHLTSLID
4 TfLam81A	RYAPYAYS		FVSTST		LTLYD		YDPASAT		LTSTY		RVTTTEAR		EGTAQG		TLLALY		PHQWKETT
5 AfEngA	FYDNSSG		VAVEGA		VMGVS		VSESTGT		YSLMWAK				AGKDAQNT		PLLMFAL		PHHMFESD
6 ScEng2	SIDMAAG		CVYCDL		LSGQVSE		DEHTNY		RFNYTV...		AGY.SQS		GTTLMYAL		PHHKAFT		
7 GmGbp	VLDKYSSC		YFVSGK		AVFR...		EPFF		CEVY		NWEK...		K...DS		GDLILL		HAHPTLVQQLR

1 CtLam81A		300	HIPLPYTYS	310	TLRGIMKT	320	QCTSF	330	FKTVYRYH	340		350	GLIF...	360	NLPDKGT	370	YDREAL
2 BhGh81	SSS.ENQ		LQNYQYEI		IRGTXIG		LGKRF		TTTELTY		P.....		CVLP...		SLPDLGDY		DRERL
3 RmLam81	DSNVK		RTDM		TLOSAT		KGPM		TALVGN		EDFTLRE		TSLPV		EWLPLQ		AAP.....
4 TfLam81A			TALT		DLSYAS		PRCP		MRVVEG		DRFTTEL		TLTH...		GILF...		SLPTVSDA
5 AfEngA	ASTQSRAT		NI		TLRTTT		KGQATA		VEY		WTMVEPE		LPISM		GFAFWSV		SGGSIDKIS
6 ScEng2	PEMQERE		IAS		SLDST		TVKGL		MTGYLT		NSFDM		QVQVPQEL		GFEFVAL		SLNKKADYS
7 GmGbp	NGDNDV		K		LEDLKYK		SID		GLVGV		VDSD		WVLKTDPL				FVTHWSIKG

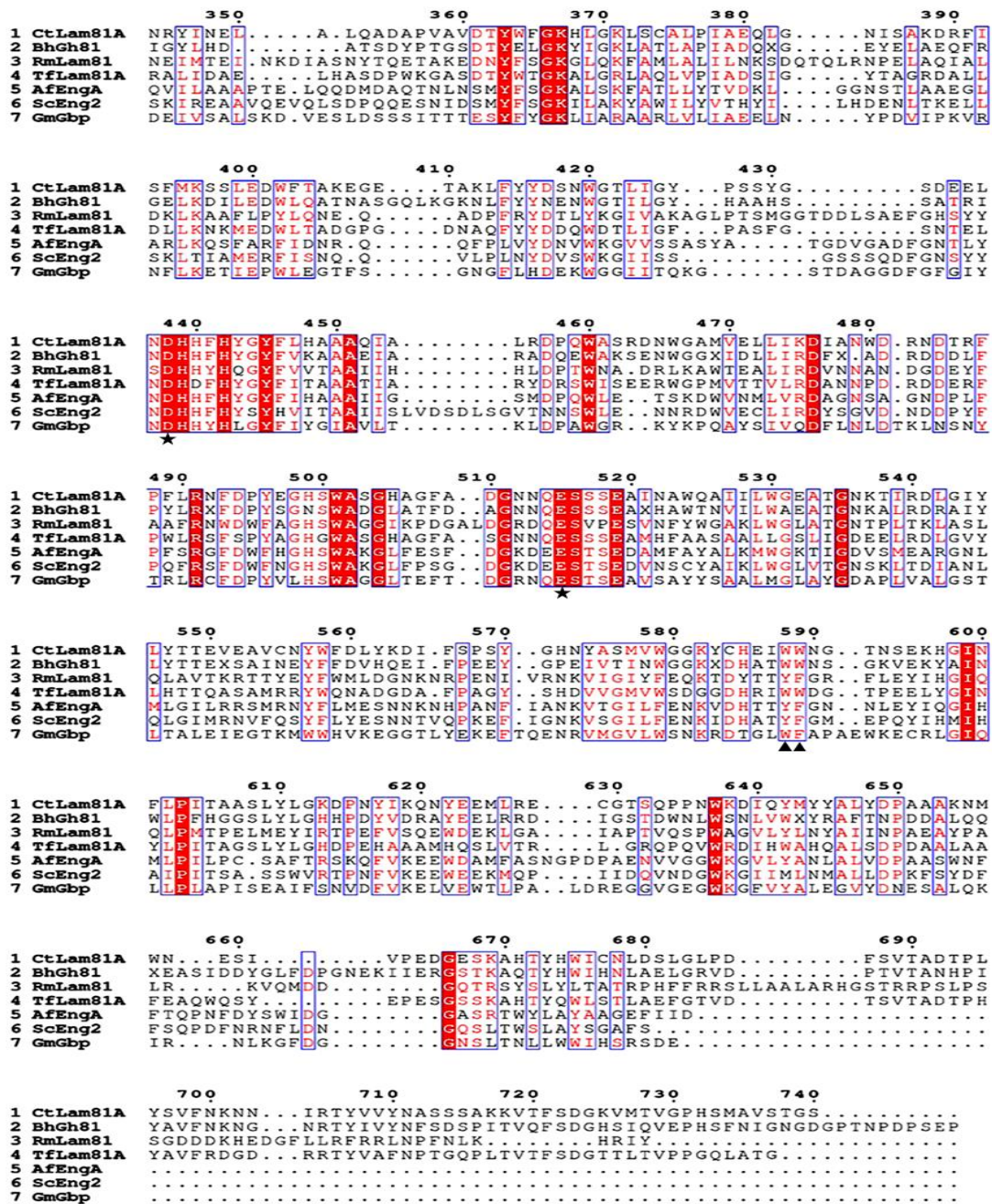


Fig. 4.3.5 Multiple sequence alignment of CtLam81A with the homologous proteins of the family 81 glycoside hydrolase viz. *Bacillus halodurans* GH81 (BhGH81; Q9KG76), *R. miehei* Lam81 (RmLam81A; KC847083), *T. fusca* Lam81A (TfLam81A, AAZ56163), *A. fumigatus* EngA (AfEngA; AAF13033), *S. cerevisiae* Eng2 (ScEng2; AAB82378) and *G. max*Gbp (GmGbp; BAA11407). The conserved amino acid residues are shown in red background and semi conserved residues are shown in box. This figure was generated by EsPrIPT3.0 (<http://espript.ibcp.fr/>).

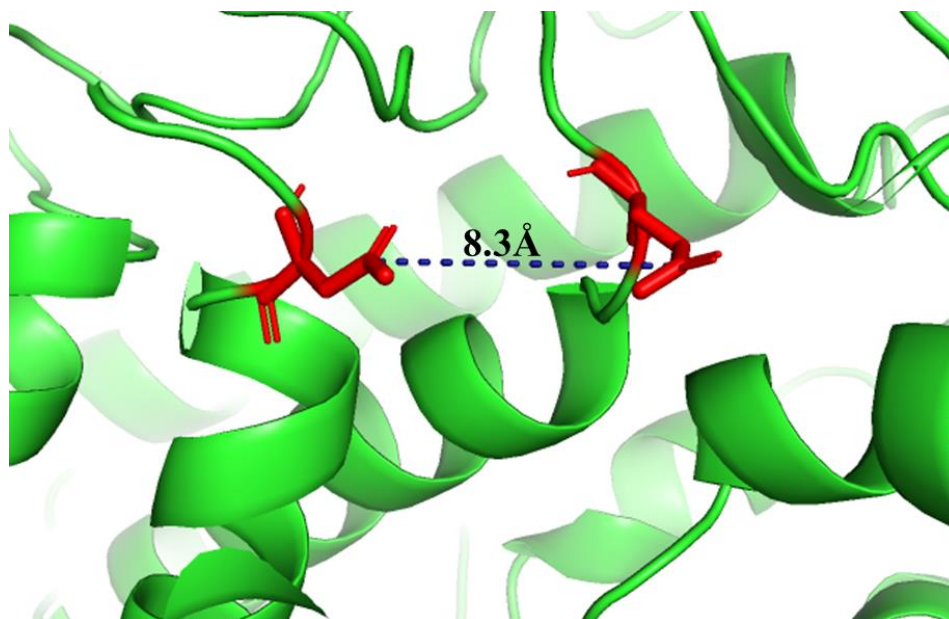


Fig. 4.3.6 Elucidation of inverting hydrolytic mechanism of *CtLam81A* enzyme by measuring the distance between carboxyl group of the acid catalyst (Asp438) and catalytic base (Glu515) of *CtLam81A* by molecular visualization system PyMol 2.

4.3.5 Molecular dynamics simulation of *CtLam81A* modeled structure

Molecular dynamics simulation of modeled *CtLam81A* was performed to analyse the structural stability and compactness of the structure over a 30 ns duration. The MD simulation results showed the fluctuation in RMSD value of *CtLam81A* up to 5 ns and thereafter the structure was completely stable till 30 ns. The overall deflection was 0.32 nm RMSD (Fig. 4.3.7A). The radius of gyration (Rg) of *CtLam81A* structure remained between 2.31 and 2.39 nm till the end of the run (Fig. 4.3.7B). Based on the MD simulation result of *CtLam81A*, it was concluded that the modeled structure of *CtLam81A* structure has a stable conformation.

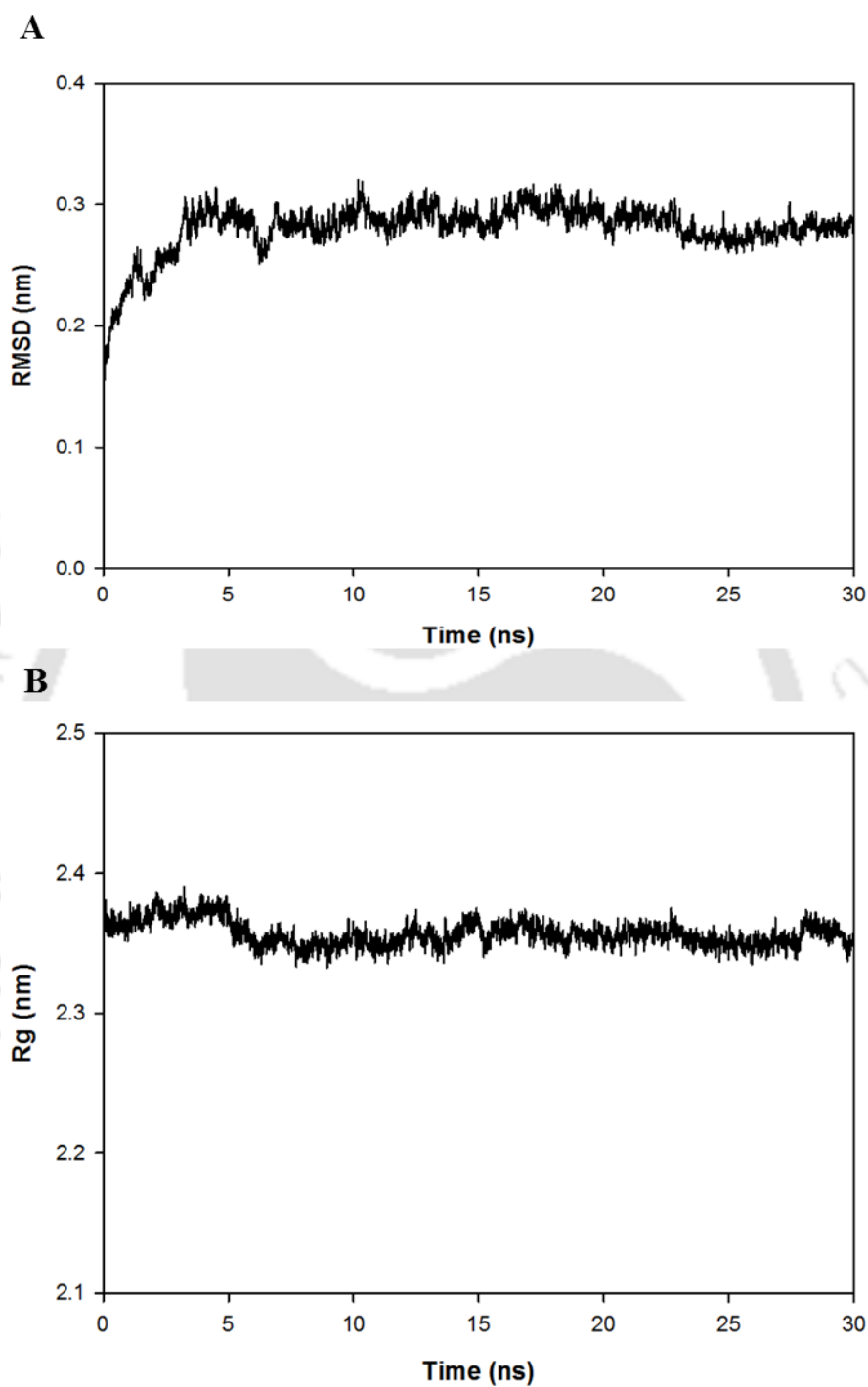


Fig. 4.3.7 Molecule Dynamics (MD) simulation of modeled *CtLam81A* showing (A) RMSD plot and (B) Radius of gyration plot

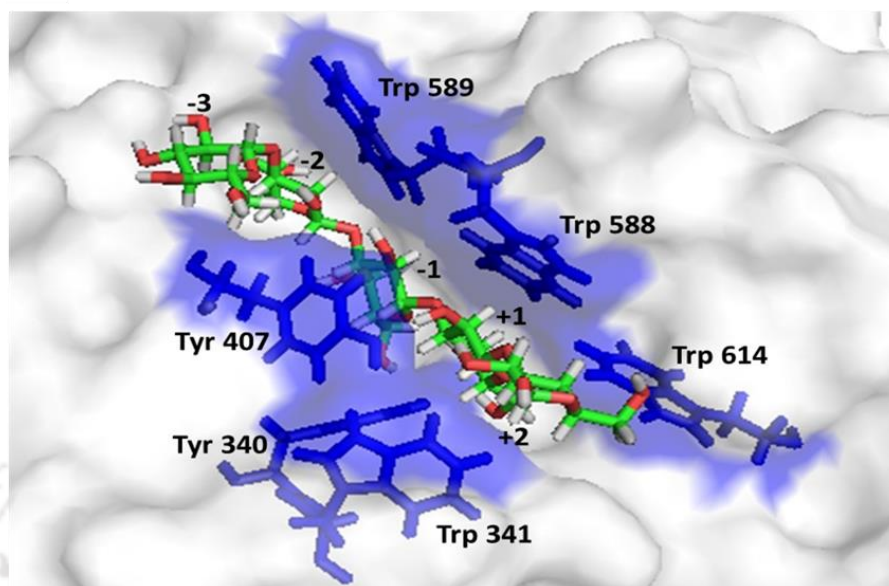
4.3.6 Docking study of CtLam81A with ligands

The docking study of CtLam81A was performed in order to analyse the interaction of ligands with its active site. The binding energies of laminari-oligosaccharides with CtLam81A are shown in Table 4.3.2. Laminaripentaose showed the strongest binding with maximum of negative binding energy of -11.47 kcal/mol. The docking of laminaripentaose on surface of CtLam81A is shown in Fig. 4.3.8A. The non-reducing end of laminaripentaose is expressed as $-n$ subsite and the reducing end as $+n$ subsite. This result revealed that the catalytic cavity of CtLam81A can occupy maximum of five glucose residues of β -1,3 glucan. Similar results were reported for β -1,3-glucanase (BhGH81) from *Bacillus halodurans* (Pluvinage *et al.*, 2017). The aromatic amino acid residues viz. Tyr363, Trp364, Tyr430, Trp588, Trp589 and Trp637 in the catalytic pocket are involved in the holding of laminaripentaose. Trp 589 of CtLam81A lies near the -2 subsite of the laminaripentaose and Trp 588 lies near the +1 subsite, which helps in holding the ligand, as also reported for BhGH81 from *Bacillus halodurans* (Pluvinage *et al.*, 2017). The catalytic core residues, Tyr363, Lys367, Tyr430, Asp438, Asp513 and Glu519 of CtLam81A are making polar interactions with laminaripentaose (Fig. 4.3.7B), while, the residues Trp637, Asp665, Ile599, Leu602, Trp364, Glu515, Trp588, Gly431, Ala502 and Phe441 are involved in hydrophobic interaction with the ligand at the catalytic site.

Table 4.3.2 Binding analysis of CtLam81A with Laminari-oligosaccharides.

Ligand	Binding free energy, ΔG (kcal/mol)	Residue making Polar interactions	Residue making Hydrophobic interactions
Lamaribiose	-7.29	Asp438, Glu515	Tyr430, Gly431, Glu435, Asn437, Phe 441, Trp588
Lamaritriose	-8.24	Glu435, Asp438, Asn 513, Trp588	Tyr430, Asn 437, Ala502, Ser503, Phe508, Trp589
Lamaritetraose	-7.01	Tyr430, Trp588, Trp637	Tyr353, Trp364, Trp589, Asp665
Lamaripentaose	-11.47	Tyr363, Lys367, Tyr430, Asp438, Asn513, Glu519	Trp364, Gly431, Phe441, Ala502, Glu515, Trp588, Ile599, Leu602, Trp637, Asp665,
Lamarihexaose	-10.18	Tyr363, Tyr430, Asp438, Asn513, Glu519	Lys367, Asn437, Phe441, Ala502, Phe508, Glu515, Trp588, Trp589, Asn590, Leu602, Asn636, Trp637

A



B

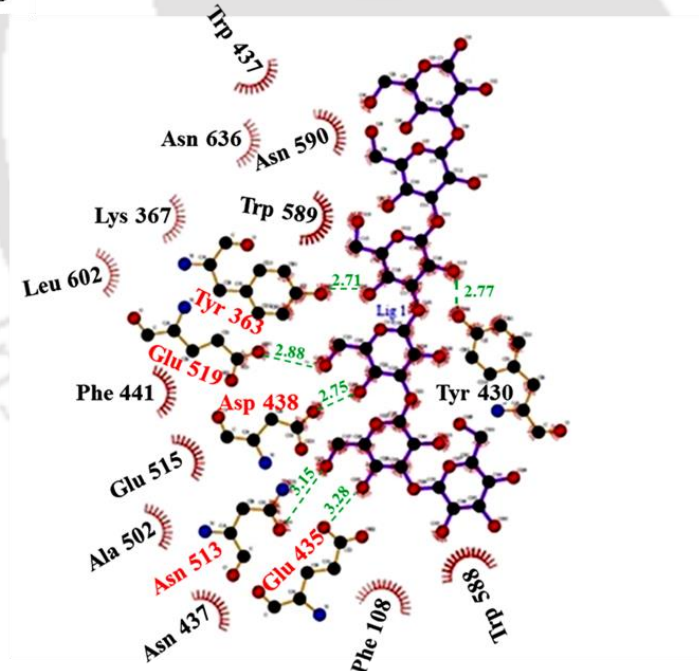


Fig. 4.3.8 (A) Surface view of CtLam81A showing docking of Laminaripentaose with the amino acid residues of the catalytic cleft and (B) 2D Schematic presentation of Laminaripentaose interaction with the amino acid residues at active site of CtLam81A. Dashed lines show the hydrogen bonds and the amino acid residues shown in arc with spokes are making hydrophobic interactions with the ligand.

4.3.7 3D structure analysis of CtLam81A-E515A by X-ray Crystallography

In order to study the mode of interaction of CtLam81A with its ligand, its catalytically inactive mutant (CtLam81A-E515A) was constructed by site-directed mutagenesis. CtLam81A-E515A gave no activity against β -1,3-glucan substrates confirming that the residue E515 acts as a catalytic base. Tetragonal crystals containing one molecule of CtLam81A-E515A per asymmetric unit were obtained by using a solution of 0.1M sodium acetate pH 4.6, 0.02M calcium chloride and 30% (w/v) 2-methyl-2,4-pentanediol as the precipitating agent. The crystals diffracted up to 1.4 Å resolution (Fig. 4.3.9) and the data collection statistics are presented in Table 4.3.3. To solve the structure of CtLam81A-E515A, sequence alignments were performed to find the highest similarity models that could lead to good initial phases obtained by molecular replacement (MR). Structure determination was performed by MR using as search model, the family 81 glycoside hydrolase from *Rhizomucor miehei* (PDB code 4K3A), the highest similarity structure available at the time of structure solution that showed 27% sequence identity to CtLam81A-E515A. In the initial attempts to solve the phase problem, the non-conserved amino acids were pruned to facilitate the rotational and translational searches. Nevertheless, an MR solution could only be obtained while searching for small sections of the protein separately: Domain I, from residues 1 to 310; Domain II from residues 311 to 654. This procedure is normally used for large, multi-domain or oligomeric proteins, where a high degree of flexibility can be expected between the different domains/subunits. A solution was obtained for domain II. Automated model building was used, producing a preliminary model with 684 residues and a R_{work} and R_{free} of 0.198 and 0.213. The preliminary inspection of the electron density map, calculated after structure solution by MR, showed no evidence of the

presence of the ligand bound at the active site of CtLam81A-E515A. The electron density for the mutated residue 515 was confirmed. In the final stages of refinement, the R_{work} and R_{free} converged to 10.8% and 13.3%. The structure solution details and refinement statistics are listed in Table 4.3.3. Fig. 4.3.10 shows the overall structure obtained and its superposition with the recently solved structure of family 81 GH from *Bacillus halodurans* (Pluvinage *et al.*, 2017), that shows 41% sequence similarity with CtLam81A, revealing their structural conservation (rmsd of 1.19 Å for 697 C_{α} atoms). The structure of CtLam81A-E515A, presented in Fig. 4.3.10 (PDB code 6FOP), comprises one polypeptide chain (residues 38 to 740), 714 water molecules and has approximate dimensions of 45.2 x 41.2 x 83.7 Å³. The accessible surface area of the structure to solvent is about 23748 Å². The CtLam8-E515A structure consists of 15 α -helices and 38 β -strands, comprising a four-domain organization starting from the N-terminus of a β -sandwich domain (I, green color), an α/β -domain (II, orange), an $(\alpha/\alpha)_6$ barrel domain (III, blue), and finally a small 5-stranded β -sandwich domain (IV, yellow) (Fig. 4.3.10). With the exception of a small defined secondary structure in the N-terminal and a C-terminal domain, this overall structure has been described in detail by Zhou *et al.*, (2013) for the RmGH81 structure from *Rhizomucor miehei* and more recently by Pluvinage *et al.*, (2017) for BhGH81 from *Bacillus halodurans*. Similar to CtLam81, BhGH81 also has the C-terminal domain that appears to be unique for these two enzymes from *C. thermocellum* and *B. halodurans* organisms. This domain appears to separate the $(\alpha/\alpha)_6$ barrel domain from the following domain, which is a dockerin module in CtLam81 and a CBM6 module in BhGH81. In RmGH81, these 50 amino acids from the C terminus of the crystallized protein are not present and may comprise

another uncharacterized domain similar to that seen in *CtLam81* and *BhGH81*. Several solvent ions and molecules could be identified in the electron density, including three calcium ions, four chloride ions, two sodium ions, four magnesium ions, two nickel ions and twelve MPD (methyl pentanediol) molecules. The Ca (3) atom is coordinated in an octahedral geometry by residues D401, T404, E409 and K412 (Fig. 4.3.11A) and the Mg(1) coordinated by residues I67, N70, Q71, Y72 and A125 (Fig. 4.3.11B). This high number of cations has not been reported for other members of GH81 family and may be related to the higher thermal stability and higher catalytic activity observed for *CtLam81A* in the presence of Ca^{2+} and Mg^{2+} , reported above. These structural ions have a probable protective role, conferring structural stability to the enzyme.

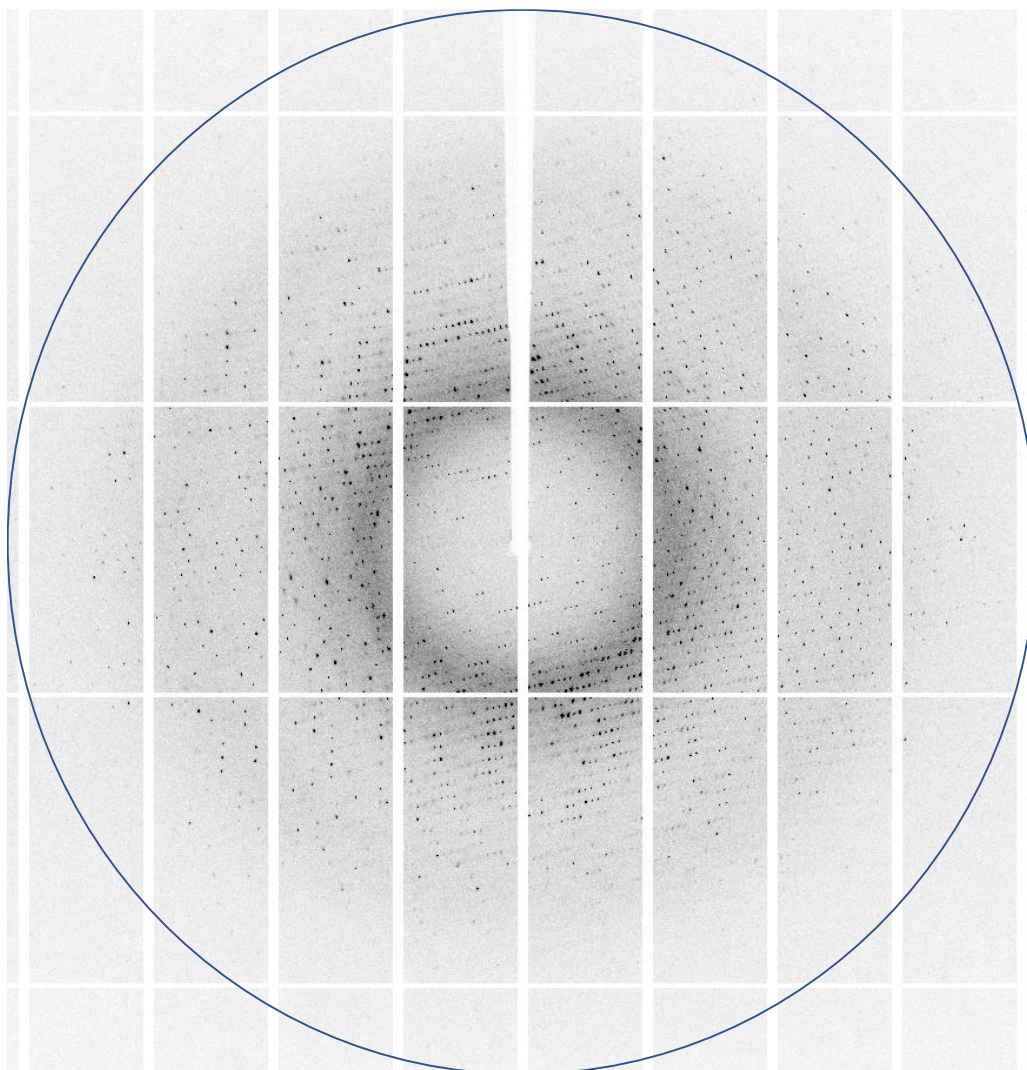


Fig. 4.3.9 Representative diffraction pattern of a CtLam81A-E515A crystal at 100K (the outer circle corresponds to 1.4 Å resolution), collected on a PILATUS 6M-F detector with a crystal-to-detector distance of 202.2 mm. The X radiation wavelength was 0.9918 Å. The crystal indexed in space group $I2_12_12_1$ with the unit-cell parameters: $a = 61.92$ Å, $b = 139.03$ Å, and $c = 197.91$ Å.

Table 4.3.3 Data collection and processing statistics for the CtLam81A-E515A crystal. Values in parentheses correspond to the highest resolution shell.

Data set	CtLam81A-E515A
Source	ID23-1, ESRF
Detector	PILATUS 6M-F
Wavelength (Å)	0.9918
<i>a</i> (Å)	61.92
<i>b</i> (Å)	139.03
<i>c</i> (Å)	197.91
Space group	<i>I</i> 212121
Molecules per ASU	1
Matthews coefficient (Å ³ .Dalton ⁻¹)	2.68
Mosaicity (°)	0.10
Resolution range (Å)	45.15 -1.40 (1.42 – 1.40)
<1/σ _I >	14.7 (2.8)
Wilson B-factor	15.8
<i>R</i> _{merge} (%) [*]	6.6 (60.8)
<i>R</i> _{pim} (%) ⁺	2.8 (24.8)
Half-dataset correlation CC1/2	0.998 (0.904)
Multiplicity	6.6 (6.9)
No. of observed reflections	1103933 (55838)
No. of unique reflections	166379(8147)
Completeness (%)	99.5 (99.0)
Anomalous completeness (%)	97.9 (97.4)
Anomalous multiplicity	3.3 (3.5)
Solution method	MR
Refinement Statistics	
<i>R</i> _{work} ^{**}	0.108
<i>R</i> _{free}	0.133
Rmsd bonds (Å)	0.007
Rmsd angles (°)	0.937
Average B (Å)	20.0
Main chain	18.2
Ligands	33.7
Water molecules	33.8
Residues per ASU	717
Ligands per ASU	12 MPD, 2 ACT, 2 Na ⁺ , 4 Cl ⁻ , 3 Ca ²⁺ , 4 Mg ²⁺ , 2 Ni ²⁺
No. of water molecules	714
Total No of atoms	6652
Ramachandran favoured (%)	97.5
Ramachandran outliers (%)	0.14
PDB accession code	6FOP

^{*} where *I* is the observed intensity, and $\bar{I}$ is the statistically weighted average intensity of multiple observations.

⁺ a redundancy-independent version of *R*_{merge}.

^{**} where $|F_{calc}|$ and $|F_{obs}|$ are the calculated and observed structure factor amplitudes, respectively (*R*_{free} is calculated for a randomly chosen 5% of the reflections).

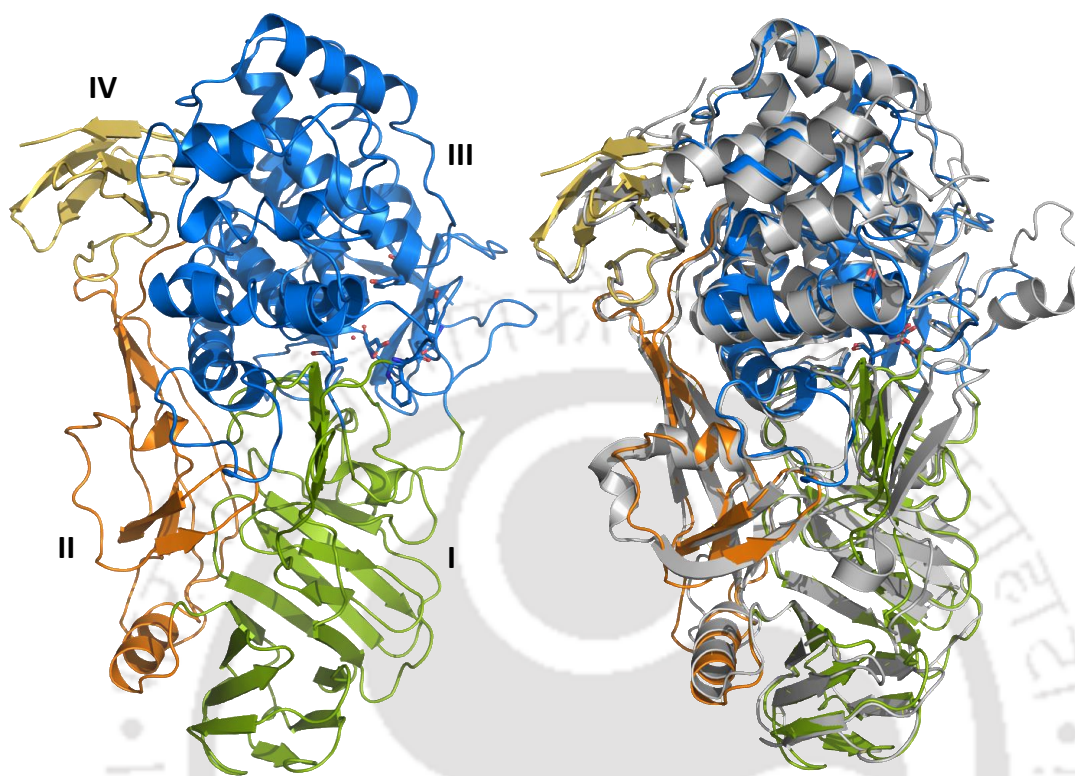


Fig. 4.3.10 The overall structure of *CtLam81-E515A*. The composite domains I–IV are labelled and coloured green, orange, blue and yellow, respectively. In the right panel, the superposition of the *BhGH81* is shown as a grey cartoon. All figures were produced using PyMOL v.2.0 ([http:// www.pymol.org](http://www.pymol.org); Schrodinger LLC).

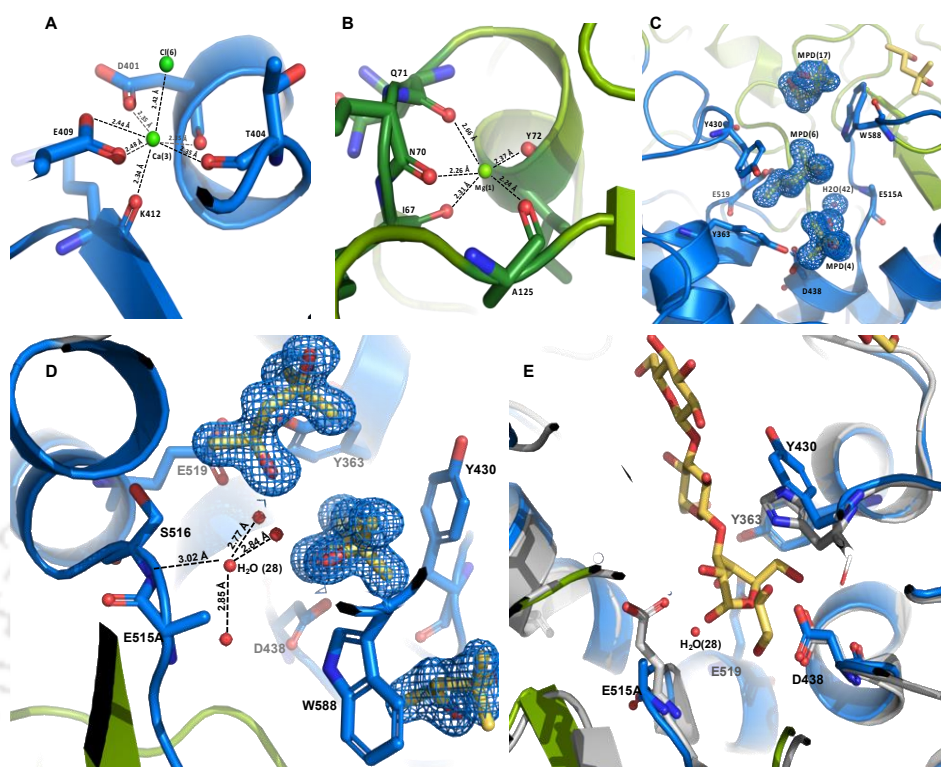


Fig. 4.3.11 Ligands interaction with *CtLam81-E515A*. **A)** Calcium interaction with domain I of *CtLam81-E515A* **B)** Magnesium interaction with domain III of *CtLam81-E515A* **C)** MPD interaction in *CtLam81-E515A* active site. The $2mF_o-DF_c$ electron density map is contoured at 1σ . **D)** Active site of *CtLam81-E515A* with the $2mF_o-DF_c$ electron density map contoured at 1σ . **E)** Active site of *CtLam81-E515A* superposed with the active site of *BhGH81* complex with products resulting from L6 cleavage. *CtLam81-E515A* (pdb: 6FOP) is shown in green cartoon and sticks for domain I and blue for domain III, water from *CtLam81-E515A* as red spheres and magnesium and calcium as green spheres. In the superposition, *BhGH81* (pdb: 5T4G) is shown in grey cartoon and sticks and L3 from *BhGH81* as yellow sticks. The figure was produced by using PyMOL v.2.0 (<http://www.pymol.org>; Schrodinger LLC).

4.3.8 The substrate binding site

As revealed by the crystal structure and its superposition with *BhGH81*, the carbohydrate-binding pocket of *CtLam81A* is very similar to *BhGH81* (Fig. 4.3.11E). In *RmGH81* and *BhGH81* the residues E553, E557, D475 and E542, E546, D466, respectively, were proposed to be the potential acid and base catalytic residues. The

corresponding conserved residues in CtLam81 are E515, E519, and D438, which are located in the base of a cleft present near the center of the $(\alpha/\alpha)_6$ barrel. These residues provide strong evidence for holding and the retention of the substrate. The residues Y363 and Y445 are involved in the formation of hydrogen bonds with E519 and are highly conserved in all GH family 81 members.

There are twelve MPD molecules found in the CtLam81E515A crystal structure. These were probably imported during the crystallization and cooling since MPD was present in high concentrations in the crystallization and cryoprotectant solutions. The aromatic side chain of W588, that provides the classic CH- π interactions to the sugar unit, is making the π interactions with an MPD molecule. A similar interaction with MPD is observed for residue W589. Other two molecules of MPD are present in the vicinity of catalytic residues E515A (proton donor), E519 (basic catalyst) and D438 (acid catalyst) (Fig. 4.3.11D). One of the MPD molecules contributes to a network of two hydrogen bonds with two neighboring water molecules and interacts with residues Y363 and K367. The second MPD molecule makes a network of three hydrogen bonds with residue D438 and two neighboring water molecules. The N atom of S516 hydrogen bonds to one water molecule, itself bound by three other water molecules (Fig. 4.3.11D). As reported by Pluvinaud *et al.*, (2017), if A515 was a glutamate, as in the wild type, it would not be well positioned, to directly attack the substrate. Therefore, this arrangement suggests that this water molecule is activated by E515, becoming a catalytic water, since it is reasonably well positioned to attack the substrate (Fig. 4.3.11E).

4.4 Conclusion

The amino acid sequence of *CtLam81A* was analysed for secondary structure elements by PsiPred tool and CD. CD analysis showed 23.6% of α -helices and 23.6% of β -strands. Modeled structure of *CtLam81A* showed the presence of an N-terminal β -sandwich domain, a $(\alpha/\alpha)_6$ domain and a short β -sandwich domain at C-terminal. The active site lies in the $(\alpha/\alpha)_6$ domain of the protein. Superimposition of *CtLam81A* with *BhGH81* and MSA confirmed that Glu515 is catalytic base and Asp438 is catalytic acid, which are 8.3 Å apart and involved in hydrolysing β -1,3-glucan by inverting mechanism. The docking study of *CtLam81A* with ligands showed the strongest binding energy against laminaripentaose, because the active site pocket of *CtLam81A* can occupy 5 glucose residues of β -1,3-glucan. The aromatic amino acid residues Tyr363, Trp364, Tyr430, Trp588, Trp589 and Trp637, Trp 589 and Trp 588 create a binding pocket for holding the ligand at the catalytic site. The mutant *CtLam81A*-E515A was co-crystallized and tetragonal crystals diffracted up to 1.4 Å resolution. The protein crystal structure was solved by Molecular Replacement using glycoside hydrolase family 81 from *Rhizomucor miehei* (PDB code 4K3A) as search model. Like on *Rhizomucor miehei* and *Bacillus halodurans* GH81 the predicted catalytic base is E515 and D438 might be the acid catalyst.

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Chapter 5

***In vitro* prebiotic and biocompatibility properties of Laminarioligosaccharides**

5.1 Introduction

Prebiotics are compounds which are non-digestible by human or animal and provide beneficial affect to host by selectively stimulating the growth of beneficial bacteria such as lactobacillus and bifidobacteria in gut (Gibson *et al.*, 1995). A large population in the world faces health issues related to digestion such as constipation or irritable bowel movement (Asadi *et al.*, 2016). This problem can be overcome by using the dietary intake that is rich in non-digestible fibres (prebiotics) or probiotics bacteria (such as lactobacillus and bifidobacteria) or mixture of both (Pandey *et al.*, 2015). A prebiotic must tolerate the acidic pH of stomach and the enzymes present in small intestines so that it can reach the colon of the large intestine. In colon, it can enhance the growth of certain specific bacteria like *Lactobacilli* and *Bifidobacteria* (Gibson *et al.*, 2004). Prebiotics fibre may also have several health-promoting benefits such as; immune system modulation (Akramiene *et al.*, 2006), antitumor (Miyanishi *et al.*, 2003; Jamois *et al.*, 2005) and cholesterol-lowering activities (Kumar *et al.*, 2012). In addition, the fermentation of prebiotics by probiotic bacteria generates the short chain

fatty acids (SCFA) and lactate which help in the suppression of non-desirable pathogenic bacteria in the intestine (Wong *et al.*, 2006; Jacobs *et al.*, 2009). A number of prebiotics are already commercially available in the market, such as inulin, gluco-oligosaccharides, fructo-oligosaccharides and galacto-oligosaccharides (Roberfroid *et al.*, 2010). It is therefore of great interest to explore new prebiotic compounds from easily and cheaply available carbohydrate sources.

Curdlan is an extracellular polysaccharides produced by bacteria (*Alcaligenes faecalis*, *A. rhizogenes*, *A. radiobacter*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia* etc.). Curdlan is a homopolysaccharide composed of linear β -1,3-linked glucose units (Xu & Zhang, 2016). Curdlan is used as a gelling agent for desserts and starch jelly and as thickener or food stabilizer for low caloritic foods (Spicer *et al.*, 1999). The supplementation of β -1,3-glucan (curdlan and laminarin) stimulated the growth of *Bifidobacterium* sp. with production of short chain fatty acids and lactate in rat (Shimizu *et al.*, 2001; Kuda *et al.*, 2005). The feeding of laminarin (β -1,3-glucan) to weaning pig reduced the population of the *E. coli* in faecal samples and enhanced the weight (O'Doherty *et al.*, 2010). The ingestion of laminarin by rat modulates the intestinal metabolism by affecting the mucus composition, intestinal pH and SCFA concentration (Deville *et al.*, 2007). Curdlan is insoluble in water due to its high molecular size and gelling properties (Xiao *et al.*, 2017). This property of curdlan makes it less suitable for food industry applications. Curdlan can be hydrolysed to shorter chains by using β -1,3-glucanase enzyme which can make it water soluble (Kumar *et al.*, 2018). The β -1,3-linked glucose oligomers (GOS) produced by hydrolysis of laminarin (β -1,3-glucan) induced monocytes to release the tumor necrosis factor alpha (TNF- α) for inhibition of human leukemic U937 cells (Miyanishi *et al.*, 2004) and stimulated

phagocytosis of macrophages and granulocytes (Jamois *et al.*, 2005). In tobacco plants, β -1,3-glucan oligosaccharides provide defense against tobacco mosaic virus (TMV) (Klarzynski *et al.*, 2000; Fu *et al.*, 2011). The oligosaccharides produced from hydrolysis of curdlan showed prebiotic activity by stimulating the lactobacillus growth (Shi *et al.*, 2018). In this study the controlled hydrolysis of curdlan was carried out to produce mixed laminarioligosaccharides (LOS) from curdlan. LOS were evaluated for prebiotic properties such as their resistance against artificial gastric juice and intestinal fluid compared with commercially available inulin. The effect of LOS was also analysed by *in vitro* proliferation assay on human embryonic kidney (HEK-293) cell line.

5.2 Material and methods

5.2.1 Chemicals, media and microorganisms

The MRS medium components, TGY medium components and the chemical components for estimation of total carbohydrate and reducing sugar were procured from Hi-Media Pvt. Ltd, India. The amylase enzyme and bile salts were purchased from Sigma–Aldrich, USA. *Lactobacillus plantarum* DM5 isolated from ethnic fermented beverage Marcha of Sikkim as reported earlier (Das *et al.*, 2014) was used. The *Lactobacillus acidophilus* NRRL B-4495 was obtained from Agricultural Research Service Culture Collection (Peoria, USA). The pathogenic bacterial strain *Enterobacter aerogenes* MTCC 7016 was obtained from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology, Chandigarh, India. Thin layer chromatography plate (TLC Silica gel 60 F₂₅₄, 20×20 cm) was purchased from Merck, India. The normal cells, Human embryonic kidney (HEK-293) and colon cancer (HT29) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. DMEM low glucose medium was purchased from Sigma–Aldrich, USA.

5.2.2 Production of laminarioligosaccharides by hydrolysis of curdlan

20 reaction mixture of each 1.0 ml containing 900 µl curdlan (11 mg/ml) dissolved in 50 mM HEPES buffer (pH 6.5) was mixed with 100 µl β-1,3-endoglucanase, CtLam81A (0.4 mg/ml) produced from *Clostridium thermocellum* (Kumar *et al.*, 2018). The reaction mixture was incubated at 75°C for 5 min. The reaction was stopped by incubating the reaction mixture in boiling water for 5 min. The 1.0 ml reaction mixture was concentrated to 100 µl by evaporating in hot air oven. The 0.6 µl of concentrated reaction mixture and 0.6 µl of the standards, glucose, laminaribiose and laminaritriose each of 2 mg/ml were loaded on TLC plate. The mobile phase for TLC contained ethyl

acetate/acetic acid/water: 2/2/1 (Lee *et al.*, 2014). The spots on the TLC were visualized by dipping TLC plate in staining solution containing 0.5% (w/v) α -naphthol in methanol/sulphuric acid: 95/5 solution. The degree of polymerization (DP) of LOS produced in the reaction mixture were identified by performing MALDI-TOF/TOF MS in the positive ion reflectron mode by using an ultrafleXtreme system (Bruker Daltonics). 1 μ l of sample was mixed with 0.5 μ l of 50 mg/mL 2,5-dihydroxybenzoic acid in 50% (v/v) acetonitrile and 0.3 μ l of 0.01 M NaCl. The mixed sample was spotted onto a stainless steel target plate.

5.2.3 Growth of probiotic bacteria in presence of laminari-oligosaccharides

The growth of probiotic bacteria (*L. plantarum* DM5 and *L. acidophilus* NRRL B- 4496) was observed in presence of laminari-oligosaccharides (LOS) and commercially available inulin as a standard prebiotic. The freshly grown culture of *L. plantarum* DM5 (1.6×10^7 CFU/ml) or *L. acidophilus* NRRL B-4495 (2.1×10^6 CFU/ml) were inoculated to 1.0 ml basal MRS medium (pH 6.5) comprising 1% (w/v) glucose (as positive control), inulin or laminarioligosaccharides (LOS). These cultures were grown under anaerobic conditions, at 37°C for 12h and 24 h. The growth of nonprobiotic bacteria *E. coil* and *E. aerogenes* MTCC 7016 was also observed in the presence of LOS. *E. coil* (2.2×10^7 CFU/ml) or *E. aerogenes* MTCC 7016 (2.3×10^7 CFU/ml) was inoculated in 1 ml TGY medium comprising 1% (w/v) glucose, inulin or laminarioligosaccharides and grown at 37°C for 12h and 24 h. The growth of both probiotic and non-probiotic bacteria was observed by spreading 10 μ l of grown culture on MRS agar plate (for probiotic bacteria) and on TGY agar plate (for nonprobiotic bacteria) and incubating plates at 30°C and 37°C for 18h. The number of colonies were counted as CFU/ml for each culture as reported earlier (Das *et al.*, 2014).

5.2.4 LOS utilization by probiotic bacteria

The freshly grown culture of *L. plantarum* DM5 (3.2×10^7 CFU/ml) or *L. acidophilus* NRRL B-4495 (4.2×10^6 CFU/ml) were inoculated to 1.0 ml MRS medium containing 0.5% (w/v) laminarioligosaccharides (LOS). These cultures were grown under anaerobic conditions, at 37°C for 24 h. 50 µl of grown culture was taken out periodically at 6h, 12h and 24h. Then each culture was centrifuged at 13,000g for 10 min. The supernatant of each sample was transferred to fresh microcentrifuge tube. The 1.0 µl of each supernatant sample and 0.6 µl of standards, glucose, laminaribiose and laminaritriose (each of 2 mg/ml) were loaded on TLC plate. TLC was run and visualized as described in section 5.2.2.

5.2.5 Hydrolysis of LOS by α -amylase

The α -amylase enzyme (100 U/ml) was added to 1×PBS buffer and its pH was adjusted 5, 6, 7 or 8 (Al-Sheraji *et al.*, 2012). 0.5%, w/v LOS or inulin mixed with 2 ml of α -amylase enzyme (200 U/ml) in 1×PBS buffer of each pH was incubated at 37°C for 30 min to 5h. 20 µl of sample was taken out periodically from each pH sample containing LOS or inulin. The reducing sugar content was measured by Nelson and Somogyi method (Nelson *et al.*, 1944; Somogyi *et al.*, 1945) and total carbohydrate content present in the sample by phenol-sulphuric acid method (Dubois *et al.*, 1956) using glucose as standard. The percentage hydrolysis of LOS and inulin was estimated by using the formula;

$$\text{Hydrolysis (\%)} = \frac{\text{Reducing sugar released} \times 100}{\text{Total sugar content} - \text{Initial sugar content}}$$

5.2.6 LOS digestibility by artificial gastric juice

Artificial human gastric juice was prepared by using phosphate buffer saline (PBS) containing (in g/L): NaCl, 8; Na₂HPO₄·2H₂O, 8.25; KCl, 0.2; NaHPO₄, 14.35; MgCl₂·6H₂O, 0.18 and CaCl₂·2H₂O, 0.1. The pH of artificial gastric juice was adjusted to 1, 2, 3 and 4 (Korakli et al. 2002) and supplemented with 1000 U/ml of pepsin. 0.5%, (w/v) LOS or inulin mixed with 2 ml of artificial gastric juice of each pH was incubated at 37°C for 30min to 5h. 20 µl of sample was taken out periodically from each pH of gastric juice sample containing LOS or inulin. The percentage hydrolysis of LOS and inulin was estimated by method described in section 5.2.5.

5.3.7 Digestibility of LOS by the intestinal fluid

The artificial intestinal fluid contained 0.5% (w/v) bile salt and 1,000 U/ml of trypsin dissolved in 1×phosphate buffer (PBS) saline and pH was adjusted to 8.0 (Fernandez *et al.* 2003). 0.5% (w/v) LOS or inulin was mixed with 2 ml of artificial intestinal fluid and incubated at 37°C for 30 min to 5h. 20 µl of sample was taken out periodically from intestinal fluid containing LOS or inulin. The percentage hydrolysis of LOS and inulin was estimated by method described in previous section 5.2.5.

5.2.8 *In vitro* biocompatibility assay of LOS on mammalian cells

The effect of LOS on viability of normal Human Embryo Kidney (HEK-293) and colon cancer (HT-29) cells were studied by a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) cell viability assay method (Mosmann et al. 1983). HEK-293 (1×10⁴ cells) and HT-29 (1×10⁴ cells) were seeded in 200 µl of complete DMEM low glucose medium and RPMI 1640 medium, respectively and incubated in 5% (v/v) CO₂ atmosphere at 37°C for 12h. After the incubation, the medium was completely removed and washed with 1×PBS buffer (pH 7.2). 200 µl of

complete medium containing varying concentration of LOS (100-1000 µg/ml) was added and incubated in 5% (v/v) CO₂ atmosphere at 37°C for 48 and 72 h. After the incubation, the medium containing LOS from each well were removed gently and 100 µl of MTT solution (0.5 mg/ml in PBS) was added into each well and incubated at 37°C for 4 h. After 4 h, the MTT solution of each well was replaced with equal volume of dimethyl sulfoxide (DMSO). The plate was then vortexed gently to dissolve the precipitate completely and the absorbance at 570 nm was detected with reference wavelength of 690 nm using a microplate reader (Tecan, Infinite 200 Pro). The percentage of cell viability was calculated as described below.

$$\text{Cell viability (\%)} = (N_t/N_c) \times 100$$

Where, N_t is absorbance of treated cells and N_c is the absorbance of untreated cells.

5.3 Result and Discussion

5.3.1 Production of laminarioligosaccharides

The laminarioligosaccharides generated by *CtLam81A* after the hydrolysis of curdlan were run on TLC (Fig. 5.3.1A). The chromatogram showed the presence of laminaribiose, laminaritriose and higher laminarioligosaccharides. MALDI-TOF analysis of laminarioligosaccharides displayed peaks at m/z 363.5, 526.7, 689.5, 852, 1014 and 1176.3 (as sodium adduct, $(M+Na^+)$ which corresponded to Laminaribiose (DP2), Laminaritriose (DP3), Laminaritetraose (DP4), Laminaripentaose (DP5), Laminarihexaose (DP6) and Laminariheptaose (DP7), respectively (Fig. 5.3.1B).

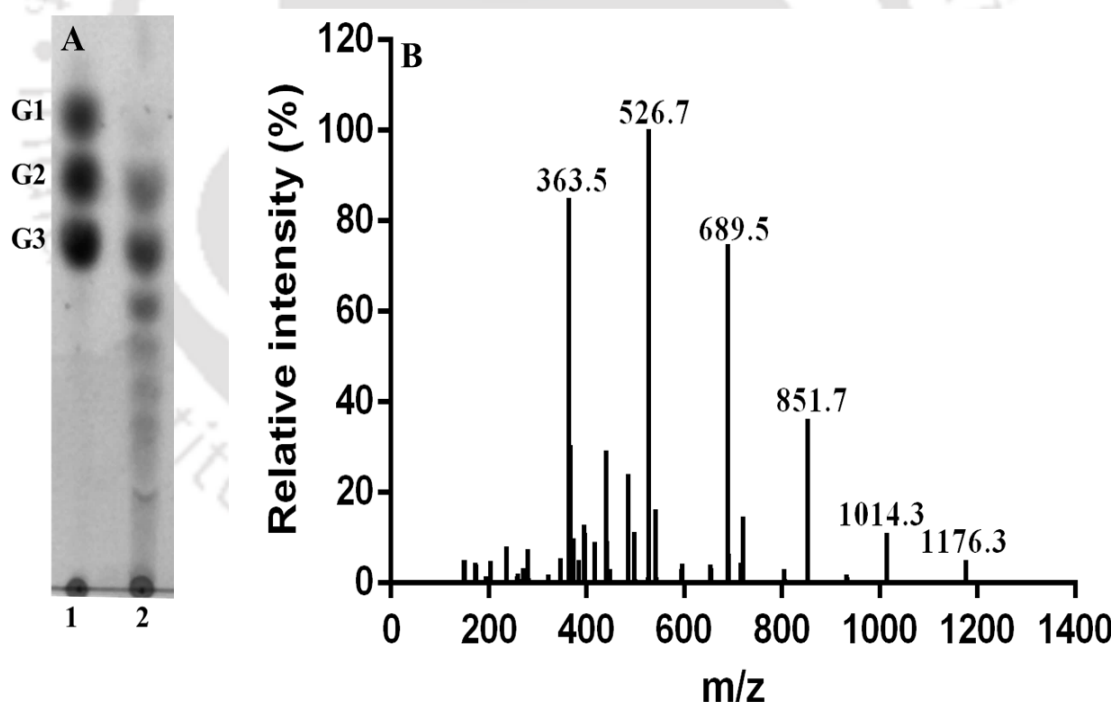


Fig. 5.3.1 TLC analysis of (A) reducing sugar released by β -1,3-glucanase (*CtLam81A*) hydrolysis of curdlan, lane 1 (G1, G2 and G3 are glucose, laminaribiose and laminaritriose as standards) and lane 2 (hydrolysed curdlan) and (B) MALDI-TOF analysis for mass confirmation.

5.3.2 Growth of probiotic and 4pathogenic bacteria in presence of laminarioligosaccharides

A compound is considered as prebiotic if it tolerates acidic pH of stomach and enzymes of the small intestines and increases the growth of selective bacteria, such as *Lactobacilli* and *Bifidobacteria*. The growth of probiotic bacteria (*L. plantarum* DM5 and *L. acidophilus* B-4496) in the presence of glucose, inulin or laminarioligosaccharides was studied at 37°C for 24h. The growth of probiotic bacteria in the presence of laminarioligosaccharides was similar to the growth of bacteria in presence of glucose or inulin (Table 5.3.1). LOS stimulated the growth of *L. plantarum* DM5 and *L. acidophilus* NRRL B-4496, by increasing the number of cells from 7.2 ± 0.02 to 10 ± 0.02 and 6.3 ± 0.02 to 8.0 ± 0.2 Log₁₀ CFU/ml, respectively in 24 h. The growth of non-probiotic bacteria (*E. coli* and *E. aerogenes* MTCC 7016) was not supported by the LOS (Table 5.3.1). The probiotic bacteria grow more in the presence of prebiotic because probiotic bacteria have necessary enzymes to hydrolyze the prebiotic oligosaccharides (Swennen et al., 2006). However, the enteric bacteria such as *E. coli* and *Enterococcus aerogenes* lack the enzymes to hydrolyze the prebiotic oligosaccharides so they are unable to utilize prebiotic for their growth.

Table 5.3.1 Growth of probiotic and non-probiotic bacteria in presence of prebiotics. (number of cells expressed as log₁₀ CFU/ml)

Bacterium	Glucose			Inulin		Laminarioligosaccharides	
	0h	12h	24h	12h	24h	12h	24h
<i>L. plantarum</i> DM5	7.2 ± 0.02	9.7 ± 0.15	10.1 ± 0.03	9.0 ± 0.07	9.5 ± 0.05	9.6 ± 0.05	10.0 ± 0.02
<i>L. acidophilus</i> 4495	6.3 ± 0.02	8.1 ± 0.17	8.8 ± 0.19	7.8 ± 0.08	8.1 ± 0.17	7.3 ± 0.10	8.0 ± 0.20
<i>E. coli</i> DH5α	7.2 ± 0.04	9.6 ± 0.05	9.9 ± 0.11	7.2 ± 0.24	7.0 ± 0.0	7.1 ± 0.17	7.2 ± 0.24
<i>E. aerogenes</i> 3030	7.3 ± 0.04	9.7 ± 0.06	10 ± 0.06	7.2 ± 0.17	7.1 ± 0.21	7.5 ± 0.11	7.4 ± 0.15

5.3.3 LOS utilization by probiotic bacteria

The fermentation of LOS by *L. plantarum* DM5 and *L. acidophilus* NRRL B-4495 was carried out at 37°C for different time intervals. The utilization pattern of LOS by *L. plantarum* DM5 and *L. acidophilus* NRRL B-4495 was analysed by running the supernatant (unutilized LOS) on TLC (Fig. 5.3.2A). The TLC analysis of *L. plantarum* DM5 fermented LOS showed the consumption of laminaribiose and laminaritriose within 6h and laminaritetraose in 24h (Fig. 5.3.2A). However, *L. acidophilus* NRRL B-4495 utilized LOS at slower rate than *L. plantarum* DM5 and consumed only laminaribiose and laminaritriose in 24h and did not utilize laminaritetraose within 24h (Fig. 5.3.2B). LOS of DP2 and DP3 were consumed readily by both *L. plantarum* DM5 and *L. acidophilus* NRRL B-4495 within 12h. This indicated that probiotic bacteria prefers to utilize LOS of DP2 and DP3 more favourably than higher DPs as a carbon source. Therefore, LOS of DP2 and DP3 can be used as an additive for functional foods.

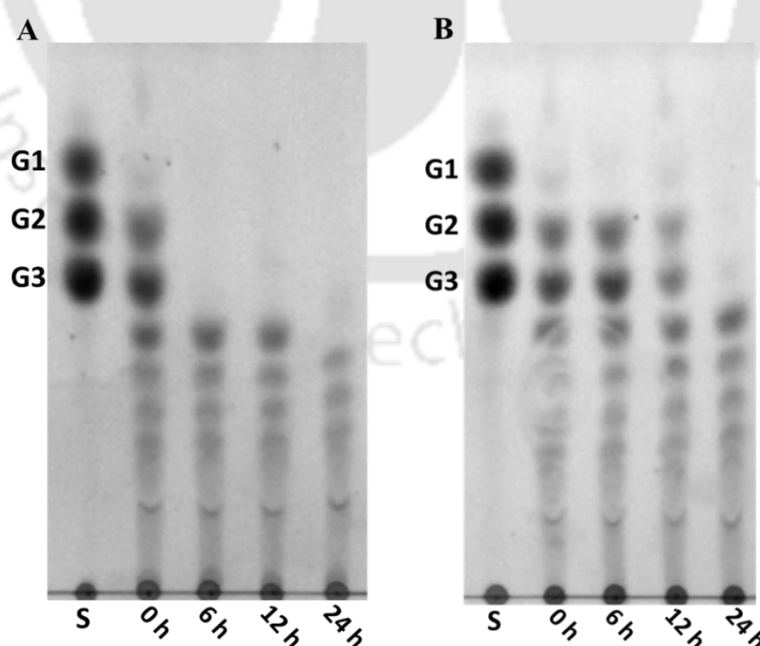


Fig. 5.3.2 The utilization pattern of LOS by probiotic bacteria (A) *Lactobacillus plantarum* DM5 and (B) *Lactobacillus acidophilus* NRRL B-4495. G1, G2 and G3 are glucose, laminaribiose and laminaritriose as standards.

5.3.4 Hydrolysis of laminarioligosaccharides by α -amylase

The hydrolysis of LOS and inulin was increased with increase in time period and also with the increase in pH. LOS showed only 2.3, 2.6, 2.9 and 3.1% hydrolysis at pH 5, 6, 7 and 8, respectively after 5 h of incubation (Fig. 5.3.3A). However, inulin showed 8.9, 10.4, 12.6, and 18.6% hydrolysis at pH 5, 6, 7 and 8, respectively in 5h (Fig. 5.3.3B). Therefore, higher resistance (over 97%) of LOS against α -amylase, gastric juice and intestinal fluid confirmed that it can act as a potential prebiotic that can reach the colon and can stimulate the growth of probiotic bacteria.

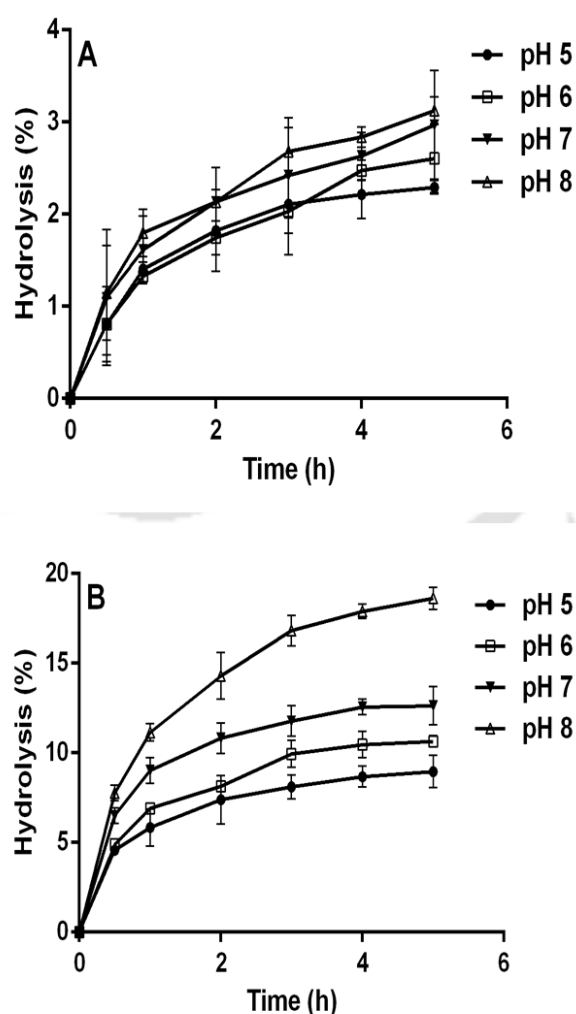


Fig. 5.3.3 Effect of α -amylase on hydrolysis of (A) laminari-oligosaccharides and (B) standard prebiotic inulin (B) at pH 5, 6, 7 and 8 for 5 h.

5.3.5 LOS digestibility by artificial gastric juice

The percentage of hydrolysis of LOS or inulin by gastric juice at pH 1, 2, 3 and 4 was estimated at different time intervals (30 min-5h) (Fig. 5.3.4A & B). LOS was hydrolysed only by 4.0, 2.5, 1.9 and 1.3% at pH 1, 2, 3 and 4 respectively in 5h of incubation (Fig. 5.3.4A). While, the inulin was hydrolysed by 42.3, 19.6, 6.3, and 1.5% at pH of 1, 2, 3, and 4, respectively, after 5 h of incubation (Fig. 5.3.4B). LOS showed 10.6, 7.84, 3.3 and 1.2% higher resistance to hydrolysis against gastric juice at pH 1, 2, 3 and 4, respectively as compared with inulin. This result indicated that laminarioligosaccharides will be available in the small intestine of gastrointestinal tract in human as it showed greater resistance to hydrolysis at low pH of gastric juice from the stomach.

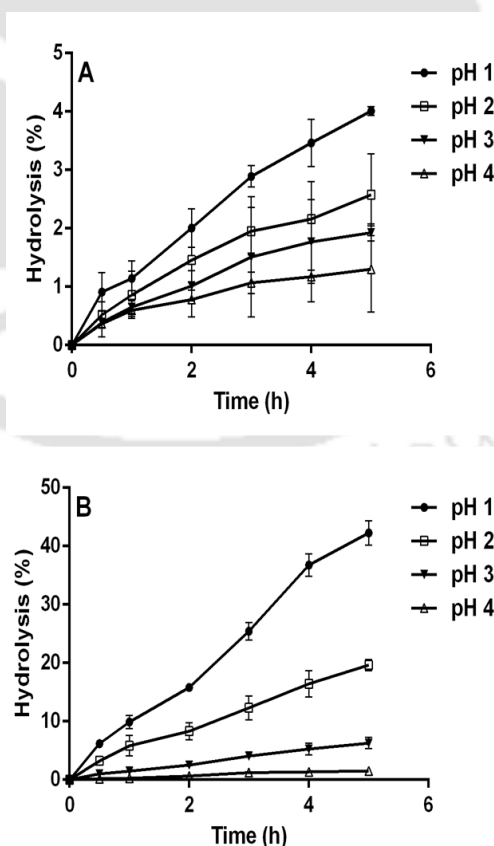


Fig. 5.3.4 Effect of artificial gastric juice on hydrolysis of (A) mixed laminarioligosaccharides and (B) standard prebiotic inulin at pH 1, 2, 3, and 4 at 37°C for 5 h.

5.3.6 Effect of artificial intestinal fluid on LOS

A prebiotic must tolerate the bile juice and enzyme of the intestinal fluid so that it can reach in intact form to the probiotic bacteria present in the colon. The percentage of hydrolysis of LOS and inulin was increased with time in the intestinal fluid. The maximum hydrolysis of LOS was 0.8% in intestinal fluid after 5 h of incubation at 37°C (Fig. 5.3.5). However, standard prebiotic inulin showed hydrolysis of 8.9% after 5 h of incubation at 37°C (Fig. 5.4.5). LOS showed 11 fold higher resistant to inulin. This result indicated that LOS possesses high resistance to intestinal fluid of small intestine and 99.2% will be accessible in intact form as carbon source for probiotic bacteria.

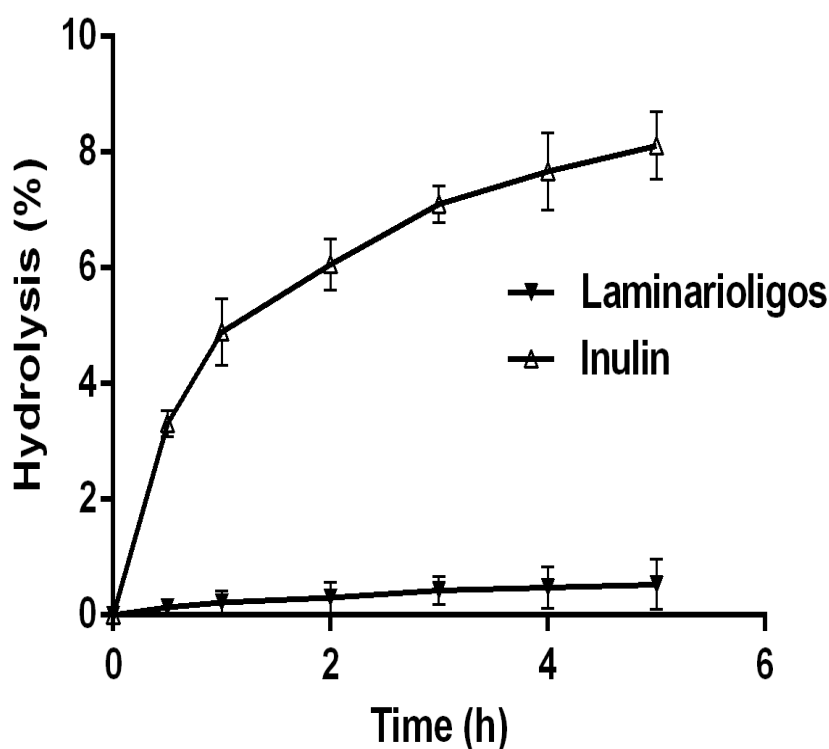


Fig. 5.3.5 Effect of an artificial intestinal juice on hydrolysis of mixed laminari-oligosaccharides and standard prebiotic inulin at pH 8 for 5 h.

5.3.7 *In vitro* biocompatibility assay of LOS on mammalian cells

The effect of LOS (with varying concentrations from 100 µg/ml to 1000 µg/ml) on viability of mammalian cells (HEK-293) and cancer cells (HT-29) was analysed by incubating for 48h and 72h. The viability of both HEK-293 and HT-29 cells was studied by the MTT assay (Fig. 5.3.6). The viability of both HEK-293 and HT-29 cells was not significantly affected even at higher concentrations of LOS (1000 µg/ml) for 72h (Fig. 5.3.6A & B). The nontoxic effect of LOS on human embryonic kidney cells (HEK-293) displayed its biocompatible nature. Hence, the LOS was considered as biocompatible and safe for consumption as functional food. LOS did not show any direct inhibitory effect on proliferation of cancer HT-29 cells (Fig. 5.3.6B). However, it has been reported that when monocytes were treated with laminarioligosaccharides, resulted in induction of TNF α production and displayed anticancer activity (Miyanishi et al. 2003).

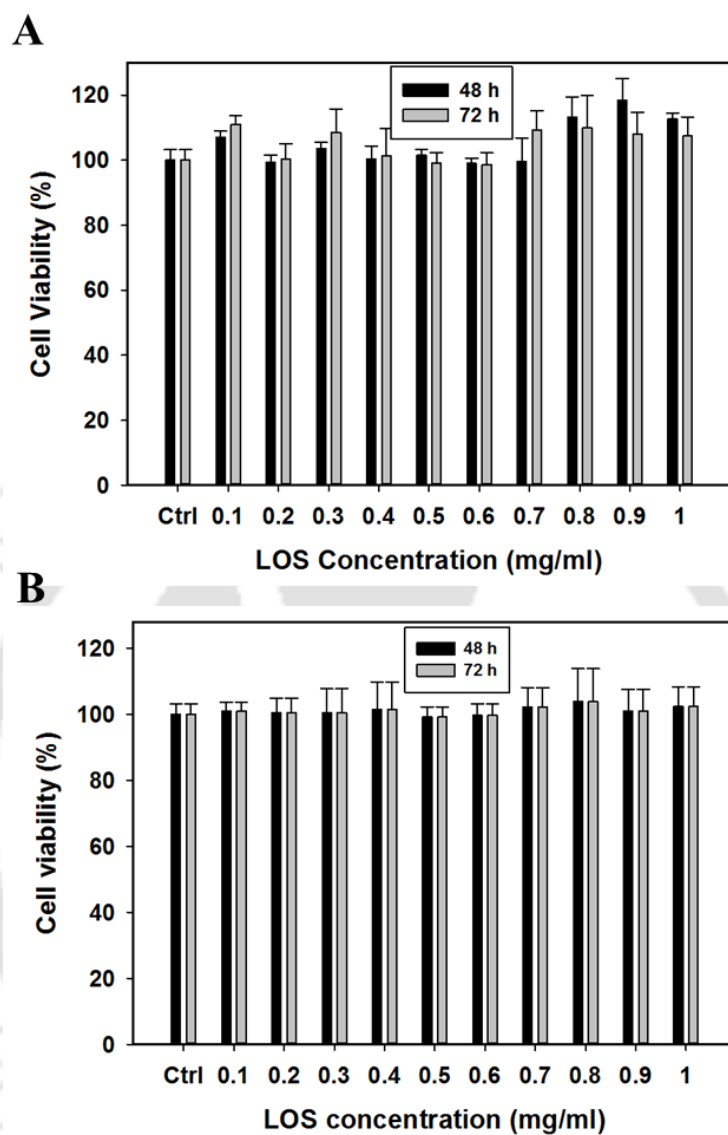


Fig. 5.3.6 The cell viability test of LOS treated (A) HEK-293 cells and (B) HT-29 cells for 48h and 72h by MTT assay.

5.4 Conclusion

Curdlan, a β -1,3-glucan was hydrolysed by β -1,3-endoglucanase (CtLam81A) to produce laminarioligosaccharides (LOS). TLC and MALDI–TOF analysis of hydrolysed curdlan showed the presence of laminarioligosaccharides of degree of polymerization, DP2-DP7. This mixture of laminarioligosaccharides was explored for *in vitro* prebiotic activities. LOS showed resistance or stability in lower pH environment of gastric juice, intestinal juice and digestive enzymes than inulin indicating their bioavailability to the probiotic bacteria present in the gastrointestinal tract of human. LOS stimulated the growth of probiotic bacteria such as *Lactobacillus acidophilus* and *Lactobacillus plantarum* DM5, while, they did not promote the growth of non-probiotic bacteria (*Escherichia coli* and *Enterobacter aerogenes*). The probiotic bacteria consumed laminaribiose and laminariotriose more readily than higher laminarioligosaccharides as carbon source for their growth. The *in vitro* cytotoxicity assay of laminarioligosaccharides (1 mg/mL) on human embryonic kidney (HEK 293) cells did not affect their viability even after 72 h indicating the biocompatible nature of the laminarioligosaccharides. The overall findings indicated that laminarioligosaccharides can serve as a potential prebiotic additive for functional food products.

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FUTURE PROSPECTS

The characterized enzyme *CtLam81A* displayed activity against β -1,3-glucan substrates. β -1,3-glucan is the main component of the yeast cell wall. The endo- β -1,3-glucanase (*CtLam81A*) can be used to hydrolyze yeast cell wall glucan to release yeast cell extract. Chitin and β -1,3-glucan are the major constituents of fungal cell wall. So, the cocktail of *CtLam81A* along with chitinase can hydrolyze the cell wall components and will lyse the fungus.

β -1,3-glucan is also found as storage in brown algae and as an extracellular polysaccharide in some bacteria. *CtLam81A* can be used to produce laminarioligosaccharides which can be used as food additive as they showed potent prebiotic activity. Laminarioligosaccharides can stimulate macrophage to release cytokines for inhibiting the cancerous cells proliferation. The use of endo- β -1,3-glucanase (*CtLam81A*) along with exo- β -1,3-glucanase can produce glucose from storage β -1,3-glucan of brown algae for bioethanol production. The stable activity of *CtLam81A* at wide pH range and at high temperature makes it a good candidate for use in high temperature industrial operations.



Journal publications**From Thesis**

1. **Kumar, K.**, Correia, M. A., Pires, V. M., Dhillon, A., Sharma, K., Rajulapati, V., Fontes C. M., Carvalho A. L. and Goyal, A. (2018). Novel insights into the degradation of β -1, 3-glucans by the cellulosome of *Clostridium thermocellum* revealed by structure and function studies of a family 81 glycoside hydrolase. *International Journal of Biological Macromolecules*, 117, 890-901.
2. **Kumar, K.**, Nath, P., and Goyal, A. (2018). Structural characterization of an endo β -1, 3-glucanase of family 81 glycoside hydrolase (CtLam81A) from *Clostridium thermocellum*. *Journal of Proteins & Proteomics*, 9(3).
3. **Kumar, K.**, Rajulapati, V. and Goyal, A. *In vitro* prebiotic and biocompatibility properties of laminari-oligosaccharides. (Submitted)

Other Publications

1. Khade, S. M., Srivastava, S. K., **Kumar, K.**, Sharma, K., Goyal, A., & Tripathi, A. D. (2018). Optimization of clinical uricase production by *Bacillus cereus* under submerged fermentation, its purification and structure characterization. *Process Biochemistry*, 75, 49-58.
2. Nath, P., Dhillon, A., **Kumar, K.**, Sharma, K., Jamaldheen, S. B., Moholkar, V. S., & Goyal, A. (2019). Development of bi-functional chimeric enzyme (CtGH1-L1-CtGH5-F194A) from endoglucanase (CtGH5) mutant F194A and β -1, 4-glucosidase (CtGH1) from *Clostridium thermocellum* with enhanced activity and structural integrity. *Bioresource Technology*, 282, 494-501.
3. **Kumar, K.**, Singal, S., & Goyal, A. (2019). Role of carbohydrate binding module (CBM3c) of GH9 β -1, 4 endoglucanase (Cel9W) from *Hungateiclostridium thermocellum* ATCC 27405 in catalysis. *Carbohydrate Research*, 484, 107782.
4. Goyal, D., **Kumar K.**, Centeno Maria S. J., Thakur A., Pires V. M., Bule P., Fontes C. M., and Goyal, A. (2019). Molecular cloning, expression, purification and biochemical characterization of a family 5 glycoside hydrolase first endo-mannanase (RfGH5_7) from *Ruminococcus flavefaciens* FD-1 v3. *Molecular Biotechnology*, 61(11), 826-835.
5. Goyal, D., **Kumar K.**, Sharma, K., Goyal, A. (2019). SAXS based structure, modeling and molecular dynamics analyses of a family 5 glycoside hydrolase first endo-mannanase (RfGH5_7) from *Ruminococcus flavefaciens* FD-1 v3. *Journal of Biomolecular Structure and Dynamics*, 1-14.
6. Nath, P., Sharma, K., **Kumar, K.**, & Goyal, A. (2020). Combined SAXS and computational approaches for structure determination and binding characteristics of Chimera (CtGH1-L1-CtGH5-F194A) generated by assembling β -glucosidase (CtGH1) and a mutant endoglucanase (CtGH5-F194A) from *Clostridium thermocellum*. *International Journal of Biological Macromolecules*.

7. **Kumar K.**, Singh, S., Sharma, K., Goyal, A. Computational modeling and Small-angle X-ray scattering based structure analysis and identifying ligand cleavage mechanism by processive endocellulase of family 9 glycoside hydrolase (*HtGH9*) from *Hungateiclostridium thermocellum* ATCC 27405 (Submitted).

Book Chapter

1. Baruah, R., **Kumar, K.**, & Goyal, A. (2019). Functional Foods and Their Health Benefits. *High Value Fermentation Products: Human Welfare*, 2, 127-145.

Conferences

1. Presented a poster entitled “Purification and characterization of a thermostable endo- β - 1,3-glucanase (*CtGH81*) from *Clostridium thermocellum*” in 57th International Annual Conference of “Association of Microbiologists of India” (AMI), in Gauhati University, Guwahati, Assam November 24-27, 2016.
2. Present a poster entitled “Purification and characterization of a thermostable endo- β -1,3-glucanase (*CtGH81*) from *Clostridium thermocellum*” in “Research Conclave” organized by IIT Guwahati, Assam during March 16-19, 2017.
3. Presented a poster entitled “*In silico* and CD based structural characterization of endo- β -1,3-glucanase (*CtLam81*) of family 81 glycoside hydrolase from *Clostridium thermocellum*” in 58th International Annual Conference of “Association of Microbiologists of India” (AMI), in B.B. Ambedkar Central University, Lucknow-226025, Uttar Pardesh during November 16-19, 2017.
4. Presented a poster entitled “*In vitro* prebiotics and biocompatibility evaluation of Laminari-oligosaccharides” in 33rd International Annual Conference of “Association of Carbohydrate Chemists and Technologists, India” (ACCTI), in Department of Chemical Sciences, IISER Kolkata during December 19-21, 2018.
5. Presented a poster entitled "In vitro prebiotic potential, digestibility and biocompatibility assay of Laminari-oligosaccharides produced from curdlan by β -1,3-endoglucanase (*CtLam81A*)" in 4th international conference on "Nutraceuticals and Chronic Diseases", in IIT Guwahati, Assam during September 23-25, 2019

Awards

1. Best poster award on the work entitled “Purification and characterization of a thermostable endo- β -1,3-glucanase (*CtGH81*) from *Clostridium thermocellum*” in “Research Conclave” organized by IIT Guwahati, Assam during March 16-19, 2017.

VITAE

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Novel insights into the degradation of β -1,3-glucans by the cellulosome of *Clostridium thermocellum* revealed by structure and function studies of a family 81 glycoside hydrolase

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ARTICLE INFO

Article history:

Received 9 May 2018

Received in revised form 31 May 2018

Accepted 1 June 2018

Available online 2 June 2018

Keywords:

Clostridium thermocellum

β -1,3-Glucanase

Laminarin

Thin layer chromatography

X-ray crystallography

ABSTRACT

The family 81 glycoside hydrolase (GH81) from *Clostridium thermocellum* is a β -1,3-glucanase belonging to cellulosomal complex. The gene encoding GH81 from *Clostridium thermocellum* (CtLam81A) was cloned and expressed displaying a molecular mass of ~82 kDa. CtLam81A showed maximum activity against laminarin (100 U/mg), followed by curdlan (65 U/mg), at pH 7.0 and 75 °C. CtLam81A displayed K_m , 2.1 ± 0.12 mg/ml and V_{max} , 109 ± 1.8 U/mg, against laminarin under optimized conditions. CtLam81A activity was significantly enhanced by Ca^{2+} or Mg^{2+} ions. Melting curve analysis of CtLam81A showed an increase in melting temperature from 91 °C to 96 °C by Ca^{2+} or Mg^{2+} ions and decreased to 82 °C by EDTA, indicating that Ca^{2+} and Mg^{2+} ions may be involved in catalysis and in maintaining structural integrity. TLC and MALDI-TOF analysis of β -1,3-glucan hydrolysed products released initially, showed β -1,3-glucan-oligosaccharides degree of polymerization (DP) from DP2 to DP7, confirming an *endo*-mode of action. The catalytically inactive mutant CtLam81A-E515A generated by site-directed mutagenesis was co-crystallized and tetragonal crystals diffracting up to 1.4 Å resolution were obtained. CtLam81A-E515A contained 15 α -helices and 38 β -strands forming a four-domain structure viz. a β -sandwich domain I at N-terminal, an α/β -domain II, an $(\alpha/\alpha)_6$ barrel domain III, and a small 5-stranded β -sandwich domain IV.

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1. Introduction

β -1,3-Glucans are major carbohydrates found in the cell walls of bacteria (such as *Alcaligenes faecalis*, *A. radiobacter*, *A. rhizogenes*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*), fungi (such as *Candida albicans*, *Saccharomyces cerevisiae*, *Lentinus edode*, *Sclerotium rolfsii*, *Schizophyllum commune*) and algae (such as *Laminaria digitata*) [1, 2]. β -1,3-Glucan is a part of the callose-containing substructures of plant cell walls, required in pollen development and cell plate formation [3, 4]. In bacteria, β -1,3-glucan is found as a linear chain of β -1,3-linked glucose [1], whereas, in fungi and algae, the β -1,3-glucan main chain contain branches of glucose linked by β -1,6-glycosidic bond [2]. However, in yeast the long linear main chain of β -1,3-glucan contains branches of short chain of β -1,3-glucan linked to the main chain by β -1,6-glycosidic linkages [2]. Thus, a variety of β -1,3-glucans exists in

nature, displaying a structural role in general but assuming a storage function in algae.

β -1,3-Glucanases catalyse the hydrolysis of the β -1,3-glycosidic linkages in β -glucan chains. A wide variety of biotechnological applications has been explored for β -1,3-glucanases. In agriculture, β -1,3-glucanase is used in combination with chitinase to control fungal diseases [5, 6]. β -1,3-Glucanases are also major players in algal biomass conversion to bioethanol [7]. In addition, β -1,3-glucanase also helps in the production of yeast extract [8]. Furthermore, the partial hydrolysis of β -1,3-glucan produces oligosaccharides that can be used as prebiotics [9], as immune-activation precursors [10] or anticancer agents [11, 12]. Optimization of the above-mentioned processes and development of novel applications for β -1,3-glucanases is hampered by our current lack of knowledge on the mechanism of action of these enzymes.

β -1,3-Glucanases can display either an *endo*- or an *exo*-mode of action. *Endo*- β -1,3-glucanase (EC 3.2.1.39) hydrolyzes β -1,3-glucan randomly with in the chain and releases a mixture of oligosaccharides. While, *exo*- β -1,3-glucanase (EC 3.2.1.58) hydrolyzes the terminal β -1,3-linkage and releases predominantly the glucose monomer or dimers

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(laminaribiose) [13]. According to the classification of Carbohydrate-Active enZymes (CAZy; <http://www.cazy.org/>, [14]), *endo*- β -1,3-glucanases are found in six glycoside hydrolase (GH) families: 16, 17, 55, 64, 81 and 128 [15]. *Exo*- β -1,3-glucanases are classified in GH families 3, 5, 17 and 55. Thus, GH17 and GH55 families contain β -1,3-glucan specific enzymes that act following an *exo*- or *endo*-mechanism. In contrast, families such as GH81 only contain *endo*- β -1,3-glucanases. Presently (April 2018), family GH81 contains 662 members widely distributed in plants, fungi, bacteria, archaea and viruses (<http://www.cazy.org/GH81.html>). GH81 enzymes have been shown to display diverse physiological roles. β -1,3-Endoglucanase (Eng1p) from *Saccharomyces pombe* plays a critical role in cell cycle by helping in the dissolution of the septum while another β -1,3-endoglucanase (Eng2p) is involved in actin assembly [16]. In addition, β -1,3-endoglucanase (CAENG1) from *Candida albicans* was found to be involved in cell wall separation [17], and β -1,3-endoglucanase from *Pneumocystis* regulates cell wall β -glucan processing during its life cycle [18]. In *S. pombe*, β -1,3-endoglucanase (Eng2) is required for the hydrolysis of the ascus wall that resulted in the release of ascospores [19]. In plants, β -1,3-endoglucanase provides part of the enzymatic defense mechanism against pathogenic fungi [20, 21]. Some of the members of the family 81 GH have been biochemically characterized such as Eng2 from fungus *Saccharomyces cerevisiae* [22], Tflam81 from bacterium *Thermobifida fusca* [23] and β -glucan-binding protein (GBP) from plant *Glycine max* [21]. According to the CAZy database, within family 81 GHs, the structural characterization has been reported for β -1,3-glucanase from *B. halodurans* C-125 in complex with various β -1,3-glucooligosaccharides [24] and *endo*- β -1,3-endoglucanase (Lam81A) from *Rhizomucor miehei* CAU432 [25].

The mechanism used by highly diverse multi-enzyme complexes, such as the cellulosome, to degrade β -1,3-glucans remains poorly understood. GH81 from *Clostridium thermocellum* is a typical β -1,3-glucanase, here termed CtLam81A, that belongs to the high molecular mass multi-enzyme cellulosome complex produced by this thermophilic bacterium [26]. Therefore, the enzymes produced by this bacterium may also be thermostable, which are on current demand for industrial applications [27]. Moreover, most of the family 81 GH characterized so far, are of fungal origin as per CAZy database, of which only two enzymes from bacteria are characterized. To gain a deeper insight about the biochemical properties of GH81 enzymes, the gene encoding family 81 GH from *Clostridium thermocellum* was amplified, cloned and the corresponding recombinant enzyme expressed, purified and crystallized. CtLam81A is a thermostable enzyme that specifically cleaves β -1,3-glucans following an *endo*-mode mechanism. The high resolution structure of the mutant CtLam81A-E515A obtained up to 1.4 Å, has allowed the understanding of how the individual components of high

Table 1
Substrate specificity of CtLam81A and CtLam81A-E515A.

Substrate	CtLam81A (U/mg)	CtLam81A-E515A (U/mg)
Laminarin	100 $\pm$ 1.2	0.03 $\pm$ 0.01
Curdlan	65 $\pm$ 0.6	ND
Lichenan	1.0 $\pm$ 0.2	ND
β -glucan	0.5 $\pm$ 0.1	ND
Other substrates ^a	ND	ND

ND = not detected.

^a Birchwood xylan, beechwood xylan, wheat arabinoxylan, xylan, carboxymethyl cellulose, hydroxyethyl cellulose, ivory nut mannan, carob galactomannan.

molecular mass multi-enzyme complexes attack recalcitrant carbohydrates.

2. Material and methods

2.1. Bacterial strain, plasmid and chemicals

Escherichia coli strains DH5 α (Novagen) and BL-21 (Novagen) were used as hosts for cloning and expression, respectively. The expression vector pET28a(+) was used for both cloning and expression of the gene encoding CtLam81A. Laminarin (from *Laminaria digitata*), curdlan (from *Alcaligenes faecalis*), birchwood xylan, beechwood xylan, carboxy methylcellulose (CMC), hydroxyethyl cellulose, β -D-glucan from barley and lichenan were purchased from Sigma-Aldrich Chemicals Co., USA. Mannan and carob galactomannan and laminari-oligosaccharides were purchased from Megazyme, Ireland and Thin layer chromatography (TLC Silica gel 60 F₂₅₄, 20 $\times$ 20 cm) from Merck, India.

2.2. Gene amplification, cloning and transformation

The genomic DNA of *Clostridium thermocellum* ATCC 27405 was obtained from DSMZ, Germany. The 2151 bp gene of *Cthe_0660* encoding the CtLam81A protein (Gen Bank Accession No: ABN51895.1) was amplified by PCR using 0.025 U/ μ l of Supreme NZYProof DNA polymerase (NZYTech, Portugal) using the following primers: forward primer 5'-CTCGTAGCCAGTATTACCGTGAG-3', which contains a *NheI* restriction site; and reverse primer 5'-CACCTCGAGTTAACTGCCGTTGAACTGC-3', which contains a *XhoI* restriction site. The PCR amplification conditions used were initial denaturation at 95 °C for 4 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 60 s, extension at 72 °C for 75 s and final extension at 72 °C for 10 min. The amplified product was electrophoresed on a 0.8% agarose gel and the DNA fragment corresponding to the Clostridial sequence was extracted and purified using the Qiagen Gel Extraction Kit. The purified PCR products and pET28a(+) expression vector were digested by *NheI* and *XhoI* restriction enzymes (Promega, USA) and ligated by T4 DNA ligase (Promega, USA). After ligation, the recombinant plasmid was used to transform *E. coli* DH5 α cells and positive recombinant clones were confirmed by restriction digestion of the isolated plasmid DNA.

2.3. Expression and purification of CtLam81A

The recombinant pET28a derivative encoding CtLam81A was used to transform *E. coli* BL-21 cells. Recombinant *E. coli* BL-21 cells were grown in Luria Broth (containing 50 μ g/ml kanamycin) at 37 °C to mid-exponential phase (OD = 0.6). To induce recombinant gene expression, 1 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) was added to the culture that was further incubated at 24 °C for 16 h. The cells were pelleted by centrifugation at 8000 $\times$ g at 4 °C for 10 min. The cell pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl. The resuspended cells were subjected to ultrasonication (Sonic, Vibra cell) for 10 min (10 s on and 10 s off pulse; 33% amplitude) and the lysed cells were centrifuged at 15,000 $\times$ g, 4 °C for 50 min. The resulting supernatant was filtered through 0.45 μ m

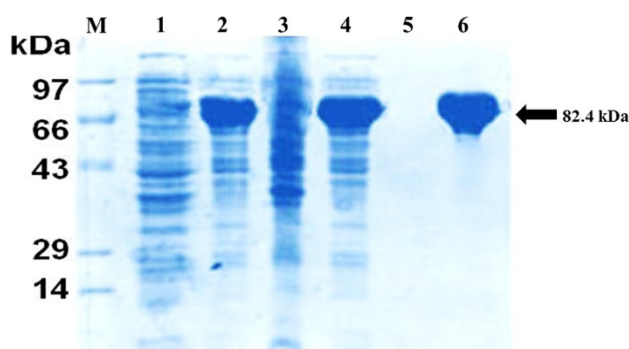


Fig. 1. Expression and purification of recombinant CtLam81A on SDS-PAGE gel (12%). Lane M - molecular mass marker (14.3–97.4 kDa, Genei), lane 1 - uninduced *E. coli* (DE3) BL-21 cells, lane 2 - induced *E. coli* (DE3) BL-21 cells, lane 3 - cell pellet after sonication, lane 4 - cell free extract, lane 5 - last column wash, lane 6 - purified CtLam81A. The reactions were carried out in triplicate sets.

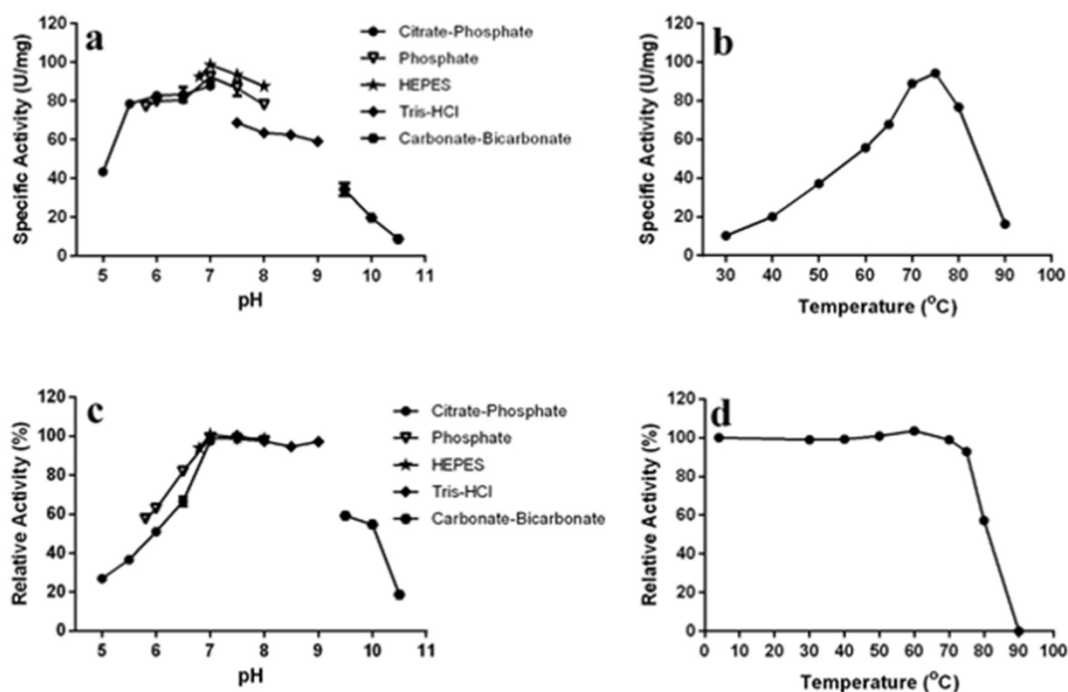


Fig. 2. Specific activity of CtLam81A was measured by incubating the enzyme with laminarin in different pH ranging 5.0–10.5 at temperature 75 °C (a) or by incubating the enzyme with laminarin at different temperature ranging 30–90 °C in pH 7.0 (b). Stability was assayed by incubating the CtLam81A for 60 min at different pH (c) and at different temperatures (d) before laminarin was added. In panel a and c, specific activity was measured in U/mg (1 unit is defined as amount of enzyme in mg required to produce 1 μ mol of glucose per min from laminarin). In panel b and d, activity at pH 7.0 and temperature 75 °C was taken as 100%. The assay was performed in 100 μ l reaction containing 1% laminarin. The reactions were carried out in triplicate sets.

membrane and loaded on to a 1 ml Ni^{2+} ion chelating column (HiTrap, GE Healthcare, USA). The column bound protein was washed with 30 ml washing buffer (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl and 60 mM Imidazole) and the protein was eluted (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl and 300 mM Imidazole). The eluted enzyme was dialysed against 50 mM Tris buffer (pH 7.5) to remove salt and imidazole. Purified CtLam81A was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% (w/v) polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R-250 following conventional procedures.

2.4. Substrate specificity of CtLam81A

The substrate specificity of CtLam81A was determined against various polysaccharides consisting of β -1,3-glucans (laminarin and curdlan), β -1,3;1,4 mixed linkage glucans (lichenan and β -D-glucan), β -1,4-glucans (carboxymethyl cellulose and hydroxyethyl cellulose), xylans (birchwood xylan and beechwood xylan), mannans and carob galactomannans. The 100 μ l reaction mixtures contained 1% (w/v) substrate in 50 mM HEPES buffer (pH 7.0) and 10 μ l purified CtLam81A enzyme (0.01 mg/ml). The reaction was incubated at 75 °C for 10 min. The specific activity of CtLam81A enzyme was determined by quantified reducing sugar produced in the mixture as described previously [28, 29].

2.5. Biochemical and kinetic properties of CtLam81A

The effect of pH on CtLam81A activity was studied by analyzing enzyme activity in different buffers: 50 mM citrate phosphate (pH 5.0–7.0), 50 mM phosphate (pH 5.8–8), 50 mM HEPES (pH 6.8–8.0), 50 mM Tris-HCl (pH 7.5–9.0) and 50 mM carbonate-bicarbonate (pH 9.5–10.5). The reaction mixture (100 μ l) contained 10 μ l enzyme (0.01 mg/ml) and 1% (w/v) Laminarin and reaction was performed at 75 °C for 10 min. The reducing sugar released was quantified

as described above [28, 29]. To determine the effect of temperature on CtLam81A activity, the enzyme was assayed in a 100 μ l reaction mixture containing 1% (w/v) Laminarin in 50 mM HEPES buffer, pH 7.0 at different temperatures ranging from 30 °C to 90 °C for 10 min. The reducing sugar released was estimated as mentioned earlier.

The effect of pH on the stability of CtLam81A was determined by incubating the enzyme in different buffers ranging from pH 5.0–10.5 at 25 °C for 60 min followed by determination of specific activity at optimum conditions of pH 7.0 and 75 °C. Similarly, the effect of temperature on stability of CtLam81A was determined by incubating the enzyme in HEPES buffer (pH 7.0) at different temperatures ranging from 30 °C to 90 °C for 60 min followed by determination of specific activity at optimum conditions of pH 7.0 and 75 °C.

To determine the kinetic parameters (K_m and V_{max}), CtLam81A was incubated with different concentrations of laminarin and curdlan at pH 7.0 and 75 °C. Kinetic constants (K_m and V_{max}) were determined using Michaelis-Menten and Lineweaver-Burk plots in GraphPad Prism 6. The effects of various metal ions on the activity of CtLam81A were determined by analyzing its activity in presence of 10 mM CaCl_2 , MgCl_2 , CuSO_4 , MnCl_2 , CoCl_2 , NiSO_4 and ZnSO_4 . The effects of Ca^{2+} and Mg^{2+} ions on the activity of CtLam81A were determined by varying the CaCl_2 and MgCl_2 concentration from 0.1 to 10 mM in the reaction mixture.

2.6. Protein melting curve of CtLam81A

Protein melting was analysed by subjecting CtLam81A (50 μ g/ml) in 50 mM HEPES buffer (pH 7) to different temperatures on a UV-Visible spectrophotometer (Varian, Cary 100 Bio) coupled with Peltier temperature controller. A curve was generated due to change in the absorbance at 280 nm with respect to change in temperature due to protein unfolding. The experiments were done by separately adding 10 mM CaCl_2 , MgCl_2 and EDTA to examine their effect on the stability of protein.

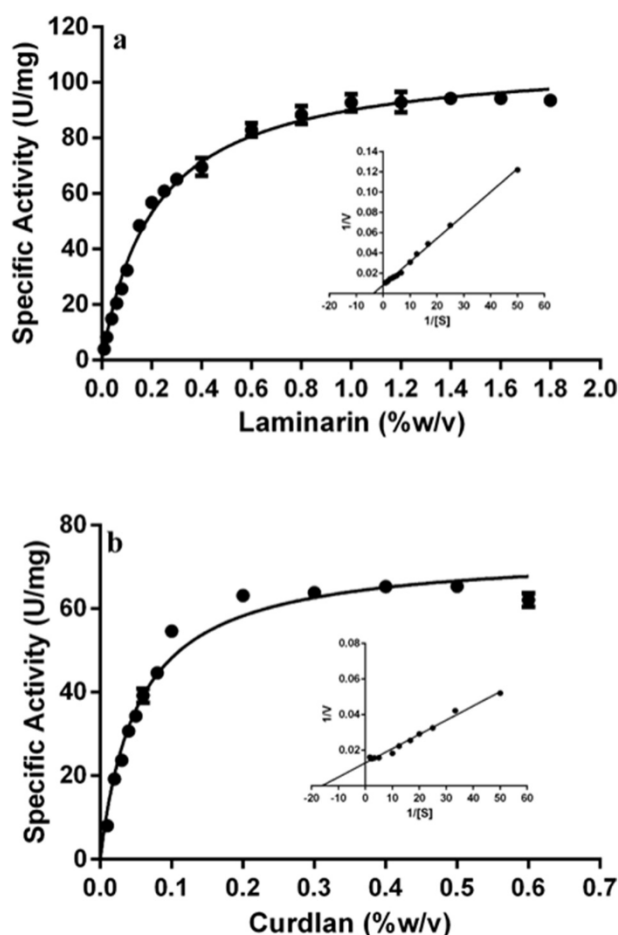


Fig. 3. Michaelis-Menten and Lineweaver-Burk (inset) plots for determining the kinetic parameters of CtLam81A for laminarin (a) and curdian (b). The assay was performed in 100 μ l reaction mixture containing different concentrations of substrates at pH 7.0 and incubated at 75 $^{\circ}$ C for 10 min as described in **Material and methods**. The reactions were carried out in triplicate sets.

2.7. Mode of action of CtLam81A

The CtLam81A hydrolyzed products of curdian were analysed by thin layer chromatography (TLC) on silica gel-coated aluminium plate (TLC Silica gel 60 F254, 20 $\times$ 20 cm, Merck). A 100 μ l reaction was set up by adding 10 μ l of CtLam81A (0.02 mg/ml) to 90 μ l of 0.55% (w/v) curdian, dissolved in 50 mM HEPES buffer (pH 7.0) and incubated at 75 $^{\circ}$ C for different time intervals. The resulting reaction mixture (100 μ l) was treated with an equal volume of absolute ethanol and centrifuged at 13,000g for 10 min. The supernatant containing the released oligosaccharides was transferred to a clean micro-centrifuge tube and concentrated to 20 μ l (for time interval 1 min–5 min) and to 40 μ l (for

time interval 10 min–24 h) by evaporating in the hot-air oven at 80 $^{\circ}$ C. After this, the amount of reducing sugar of curdian oligosaccharides was measured by the method as described earlier [28, 29]. Precisely 0.8 μ l of concentrated samples and of glucose (2.0 mg/ml) were loaded on the TLC plate. The TLC plate was dried for 5 min and kept in the developing chamber. The mobile phase consisted of ethyl acetate/acetic acid/water in the ratio 2:2:1 [30]. The plate was dried and oligosaccharides were visualized by spraying the visualizing solution (sulphuric acid: methanol 5:95, v/v and α -naphthol 0.5%, w/v). The degree of polymerization of oligosaccharides formed in the 5 min reaction was analysed by performing Matrix-Assisted Laser Desorption Ionization–Tandem time of Flight mass spectrometry (MALDI–TOF/TOF MS) in the positive ion reflectron mode by using an ultrafleXtreme system (Bruker Daltonics). The sample for MALDI–TOF analysis was prepared by mixing 1 μ l of concentrated 5 min sample with 0.3 μ l of 0.01 M NaCl and 0.5 μ l of 50 mg/ml 2,5-dihydroxybenzoic acid in 50% (v/v) acetonitrile and spotted onto a stainless steel target plate [31].

2.8. Generation of mutant CtLam81A-E515A by site-directed mutagenesis

To study the mode of binding of CtLam81A with the substrate, the catalytic residue, Glu515 was mutated to alanine. The catalytic base, Glu515 of CtLam81A was identified by multiple sequence alignment with the closest characterized homologues of family 81 glycoside hydrolase from *Bacillus halodurans* GH81 (BhGH81; Q9KG76) and *Thermobifida fusca* Lam81A (TfLam81A, AAZ56163) was created by Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and visualized in ESPript 3.0 (<http://esprict.ibcp.fr/ESPript/ESPript/>). The catalytic base and catalytic acid residues of BhGH81 were recently determined by Pluvinage et al., 2017, which are conserved as E515 and D438 respectively, in CtLam81A. The mutant (CtLam81A-E515A) was generated by site-directed mutagenesis by using the NZYmutagenesis Kit (NZYTech, Lisbon, Portugal) using the plasmid CtLam81A-pET28a as template. The primers used were as follows: “sense 5'-GCCGACGGCAACAATC AAGCGTCATCATCGGAGGCCATCAAC-3'” and “antisense – 5'-GTTGAT GGCCTCCGATGATGACGCTTGATTGTTGCCGTCGGC-3'”. The generated plasmid was sequenced to ensure that only the appropriate mutation had been incorporated into the amplified DNA. The recombinant pET28a derivative encoding CtLam81A-E515A was used to transform *E. coli* BL-21 cells, expressed and purified as described earlier. The activity of mutant (CtLam81A-E515A) was determined in 100 μ l reaction mixtures containing 1% (w/v) laminarin in 50 mM HEPES buffer (pH 7.0) and 10 μ l purified mutant (CtLam81A-E515A) (1 mg/ml). The reaction was incubated at 75 $^{\circ}$ C for 10 min.

2.9. Crystallization of CtLam81A-E515A, data collection and processing

The mutant CtLam81A-E515A (30 mg/ml) was co-crystallized in the presence of laminaritetraose (30 mM) and laminarihexaose (30 mM) supplemented with 0.5 mM CaCl_2 , using the sitting drop vapor-diffusion method and drops were set at 277 K. The drops were prepared by adding an equal volume (1 μ l) of protein-ligand mixture and reservoir solution on 96-well crystallization plates (SWISSCI 'MRC' 2-Drop Crystallization Plates, Douglas Instruments, Berkshire, UK) using a nanodrop robotic dispensing system (Oryx8, Douglas Instruments). Several commercial screens, namely the PEG/Ion HT and CS1/CS2 (Hampton Research, Aliso Viejo, CA, USA), the JCSG screen (Jena Bioscience, Jena, Germany) were used. CtLam81A-E515A crystallized only in one of the conditions of the JCSG screen containing 30% (w/v) 2-methyl-2,4-pentanediol, 0.1 M sodium acetate pH 4.6 and 0.02 M calcium chloride. CtLam81A-E515A crystals were harvested from the crystallization drop and transferred into a cryo-protectant solution mimicking the mother liquor supplemented with 30% (w/v) glycerol. A complete dataset was collected at beamline ID23-1 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and the crystal diffracted up to a resolution of 1.4 $\text{\AA}$. The dataset was processed with

Table 2
Effect of metal ions on the activity of CtLam81A.

Metal ions	Specific activity (U/mg)	Residual enzyme activity (%)
Control	100	100
Ca^{2+} (10 mM)	130.9 $\pm$ 2.7	130.9 $\pm$ 2.7
Mg^{2+} (10 mM)	133 $\pm$ 1.2	133 $\pm$ 1.2
Co^{2+} (10 mM)	93.6 $\pm$ 0.9	93.6 $\pm$ 0.9
Ni^{2+} (10 mM)	94.4 $\pm$ 1	94.4 $\pm$ 1
Zn^{2+} (10 mM)	26.2 $\pm$ 0.5	26.2 $\pm$ 0.5
Mn^{2+} (10 mM)	26.1 $\pm$ 1.2	26.1 $\pm$ 1.2
Cu^{2+} (10 mM)	14.2 $\pm$ 0.5	14.2 $\pm$ 0.5
EDTA (10 mM)	67.7 $\pm$ 1	67.7 $\pm$ 1

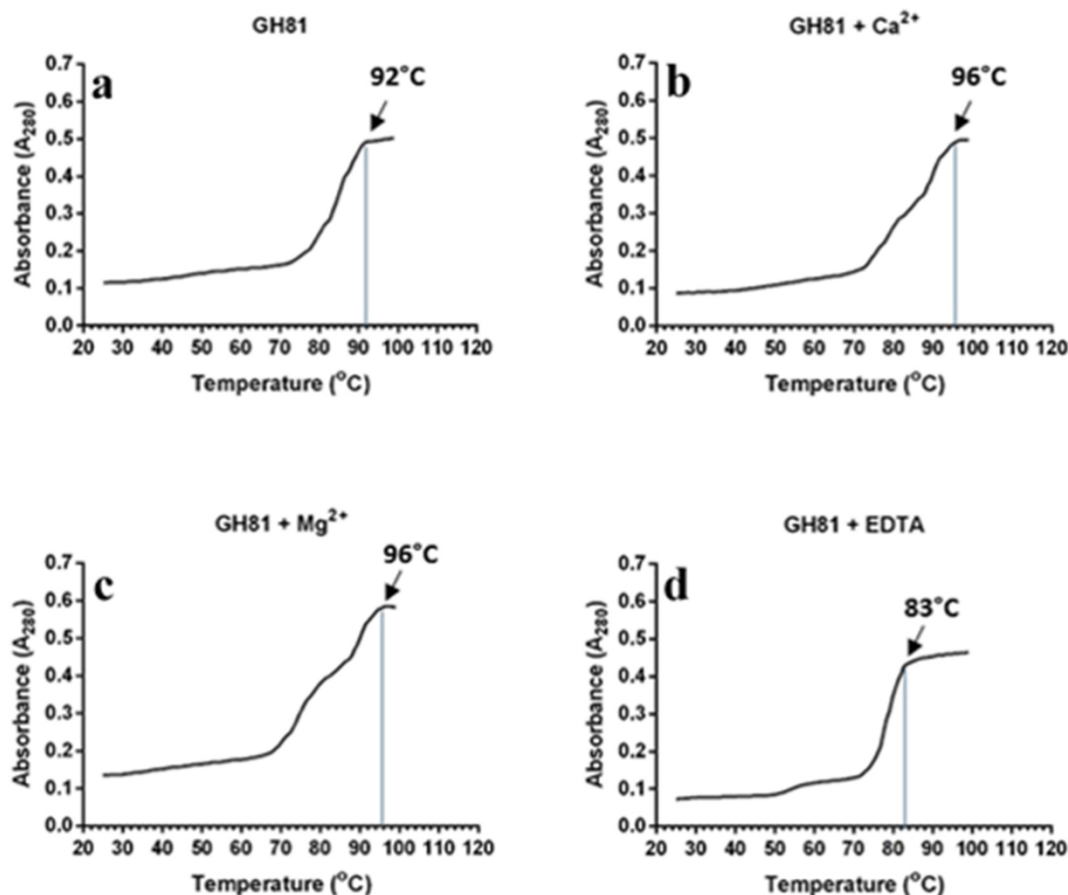


Fig. 4. Temperature melting curve of CtLam81A (a), CtLam81A + 10 mM Ca^{2+} ions (b), CtLam81A + 10 mM Mg^{2+} (c) and CtLam81A + 10 mM EDTA (d). 50 μg of CtLam81A protein was suspended in 1 ml 50 mM HEPES buffer (pH 7.0) and subjected to different temperatures on a UV-Visible spectrophotometer.

XDS [32] and scaled with AIMLESS [33]. The crystal indexed in the body-centered orthorhombic space group $I2_12_12_1$ with unit-cell parameters $a = 61.92 \text{ \AA}$, $b = 139.03 \text{ \AA}$ and $c = 197.91 \text{ \AA}$. The Matthews coefficient calculations suggested the presence of one molecule per asymmetric unit (Matthews coefficient of $2.61 \text{ \AA}^3/\text{Da}$) with a solvent content of 53%. The data-collection and processing statistics are presented in Table 3.

2.10. Structure solution and refinement

At the time of the structure determination process, the only structural model available for phasing was the family 81 glycoside hydrolase from *Rhizomucor miehei* (PDB: 4K3A) with a sequence identity of 27%. The phasing model was optimized by pruning non-conserved residues and domains by using Chainsaw [34] prior to Phaser-MR [35] from the PHENIX package [36]. AutoBuild [37] from PHENIX [36] was used for automated model building producing a model for CtLam81A-E515A with 684 residues with R_{work} and R_{free} of 0.198 and 0.213, respectively. Restrained refinement to structure completion was performed with PHENIX.REFINE [38], with iterative inspection of the electron density maps and manual rebuilding with COOT [39]. In the final stages of refinement, the R_{work} and R_{free} converged to 10.8% and 13.3%. The geometrical validation and model improvement was carried out by using PDBREDO [40, 41] and other validation programs such as PROCHECK [42] and MOLPROBITY [43]. The analysis of the model showed that 97.6% of the protein residues are in the most favored or additionally allowed regions of the Ramachandran plot, while only 0.14% are in disallowed regions (Residue H79). Refinement statistics are summarized in Table 3.

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2.11. Accession codes

Coordinates and observed structure factor amplitudes for CtLam81A-E515A have been deposited in the Protein Data Bank under the accession code 6FOP.

3. Results and discussion

3.1. Cloning, expression and purification of CtLam81A

The gene encoding CtLam81A was amplified by PCR and cloned in to pET28a. The resulting recombinant plasmid, identified by *NheI*-*XhoI* restriction digestion and sequenced to verify the integrity of the nucleic acid encoding CtLam81A, was used to transform into *E. coli* BL21 cells. Recombinant CtLam81A was purified by immobilized metal ion affinity chromatography (IMAC) and analysed by SDS-PAGE (Fig. 1). Purified CtLam81A revealed a molecular size of approximately, 82 kDa, which is in agreement with the expected size deduced from the protein primary sequence.

3.2. Substrate specificity of CtLam81A and CtLam81A-E515A

The hydrolytic capacity of CtLam81A against a diverse range of plant cell wall polysaccharides was tested (Table 1). CtLam81A displayed the highest activity against laminarin (100 U/mg) followed by curdlan (65 U/mg), indicating that CtLam81A displayed activity against both soluble and partial soluble substrates. There is no report available on the activity of family 81 GH against curdlan till date. The enzyme displayed a residual activity against lichenan (1 U/mg) and β -D-glucan (0.5 U/mg)

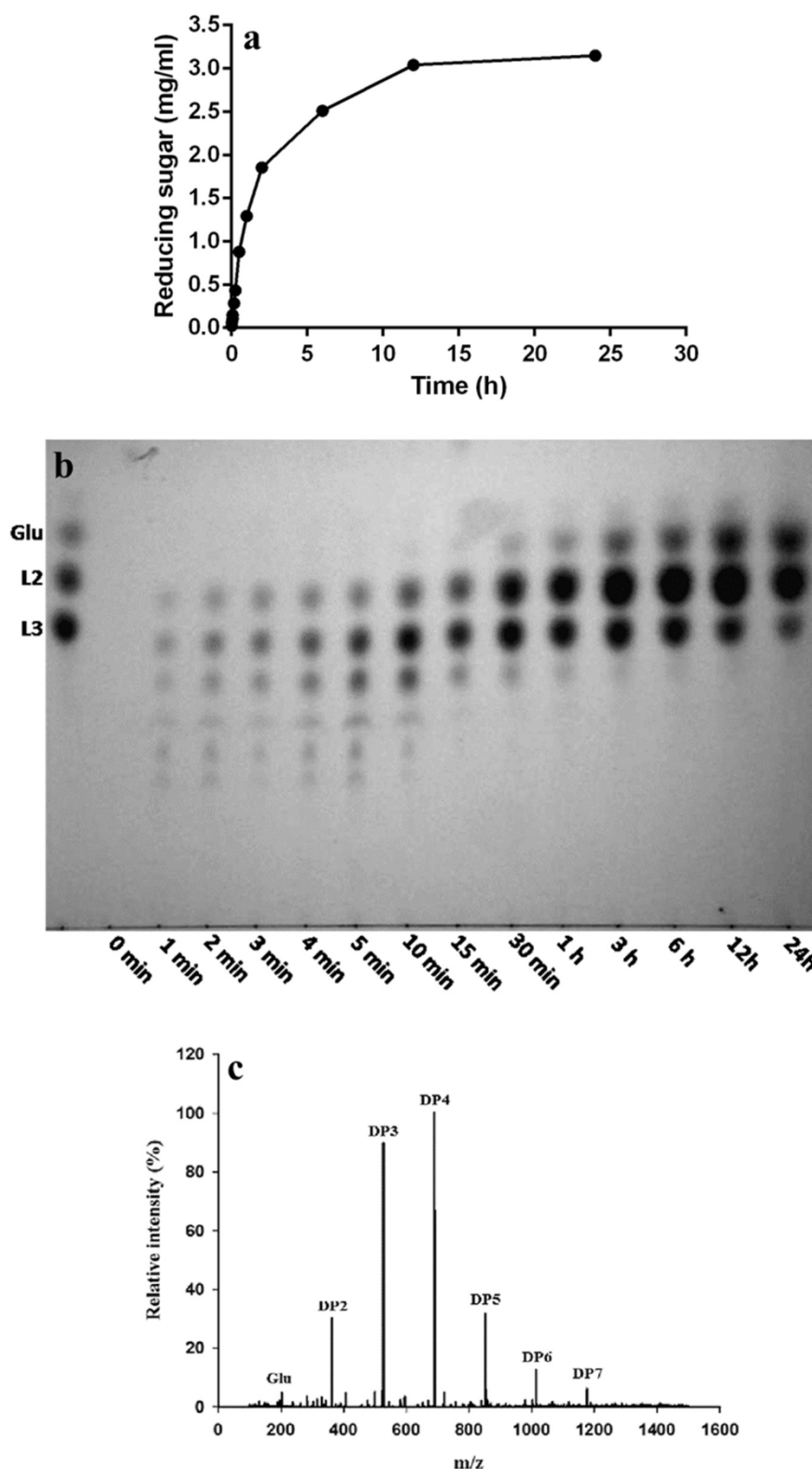


Fig. 5. Reducing sugar released by CtLam81A hydrolysis of curdlan at different time intervals (a), TLC analysis, Glu, L2 and L3 are glucose, laminaribiose and laminaritriose as standard (b) and MALDI-TOF analysis for mass confirmation (c). The assay was performed in 100 μ l reaction mixture containing curdlan (0.5%, w/v) at 75 $^{\circ}$ C and pH 7.0 (HEPES) for different time intervals as described in [Material and methods](#).

and was unable to depolymerize birchwood xylan, beechwood xylan, carboxy methylcellulose (CMC), hydroxyethyl cellulose, mannan and carob galactomannan. These results indicated that CtLam81A efficiently hydrolyses β -1,3-glucan polymer but is unable to recognize other type of carbohydrate linkages. The weak activity observed against mixed

glucan (β -1,3; 1,4-linkage) lichenan and β -D-glucan was probably due to the presence of β -1,3 linkages in low abundance. Thus, the substrate specificity of CtLam81A is similar to other GH81 enzymes [21–23]. The mutant CtLam81A-E515A showed no activity against any of the enzyme substrates (Table 1).

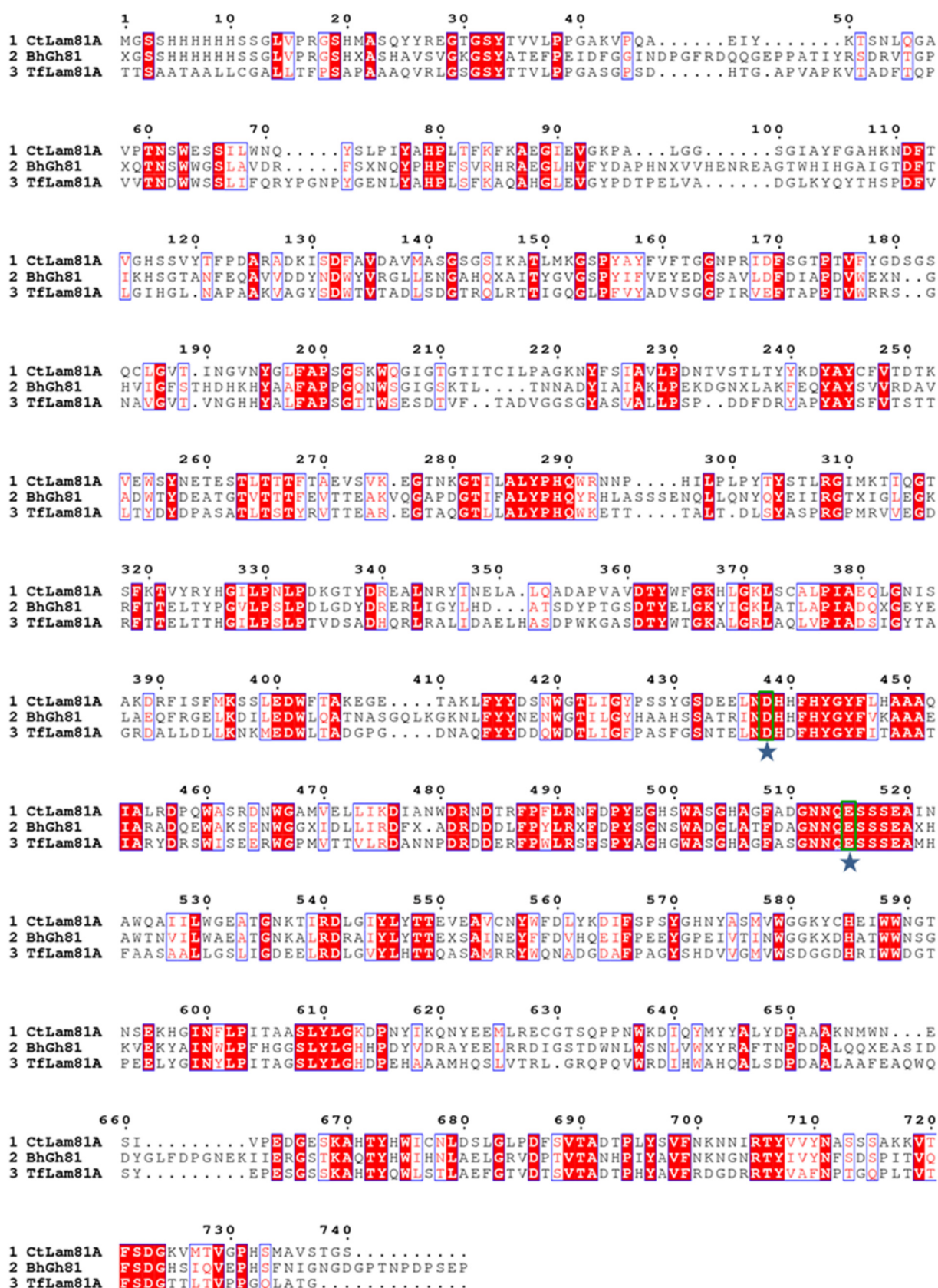


Fig. 6. Multiple sequence alignment (MSA) of CtLam81A with the homologous protein of the family 81 glycoside hydrolase viz. BhGh81 from *Bacillus halodurans* and TfLam81A from *Thermobifida fusca*. MSA was performed with the help of Clustal Omega program and the conserved amino acid residues were viewed by ESPrnt 3.0.

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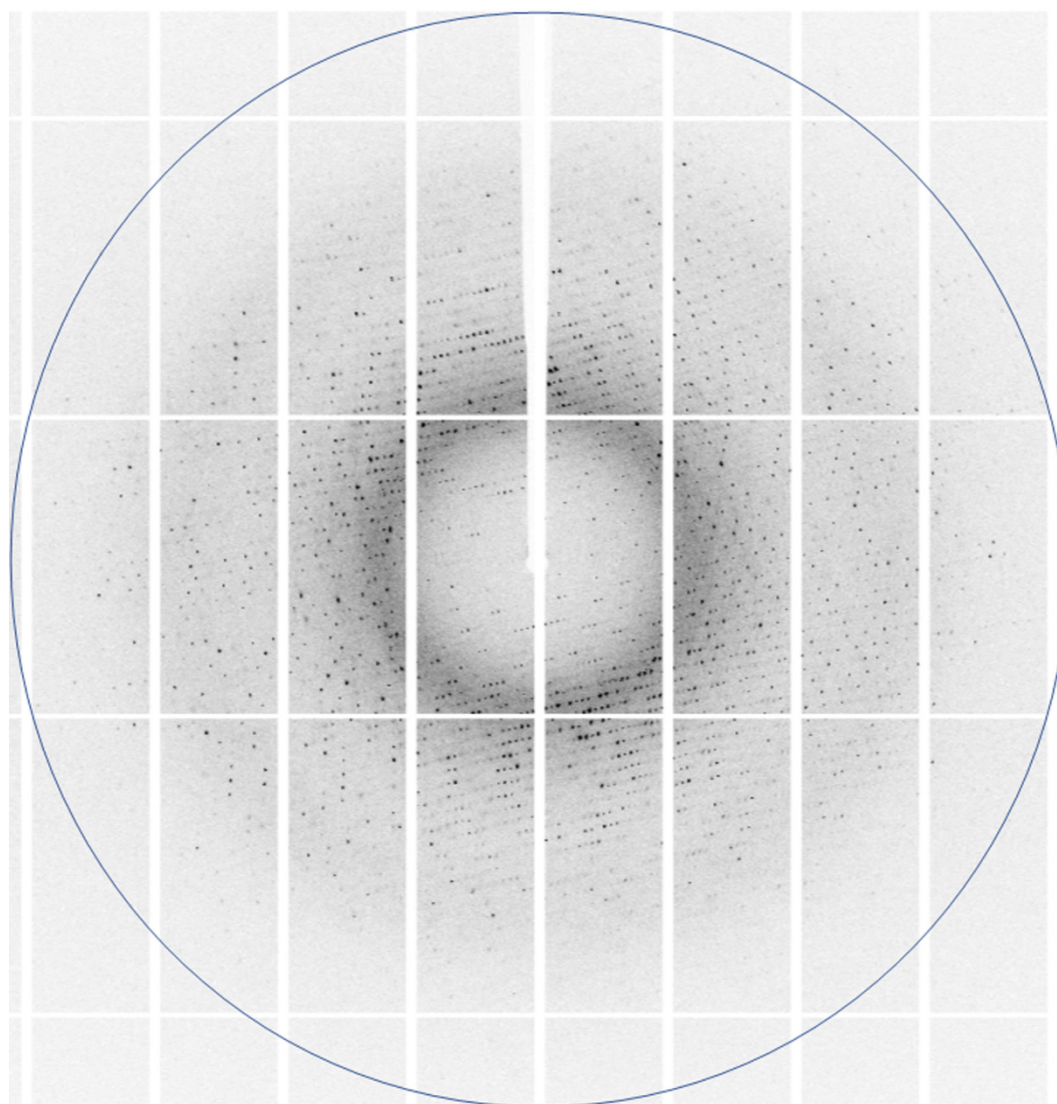


Fig. 7. Representative diffraction pattern of a CtLam81A-E515A crystal at 100 K (the outer circle corresponds to 1.4 Å resolution), collected on a PILATUS 6M-F detector with a crystal-to-detector distance of 202.2 mm. The X radiation wavelength was 0.9918 Å. The crystal indexed in space group $I2_12_12_1$ with the unit-cell parameters: $a = 61.92$ Å, $b = 139.03$ Å, and $c = 197.91$ Å.

3.3. Biochemical properties of CtLam81A

CtLam81A displayed an optimum pH of 7.0 and retained more than 80% activity at pH 5.5 and 90% of activity at pH 8.0 (Fig. 2a). Similar pH optimum was reported for Lam81 from *Thermobifida fusca* [23] whereas, SpEng2 from *S. pombe* and ScEng2 from *S. cerevisiae* showed optimum pH 6.0 and 6.5, respectively [22]. CtLam81A displayed stability in a wide range of pHs (6.5–9.0) (Fig. 2b). However, CtLam81A rapidly lost stability at pHs below 6.5 and above 9.0 (Fig. 2b). The optimum temperature for CtLam81A activity was 75 °C (Fig. 2c). In addition, CtLam81A retained 100% of its activity at 70 °C and 93% of its activity at 75 °C after 1 h incubation (Fig. 2d), indicating the thermophilic nature of the enzyme. This is the first report on an enzyme from family 81 GH that shows the stability up to 75 °C. The thermal tolerance revealed by CtLam81A makes it suitable for industrial application as several industrial processes require thermostable enzymes for easy mixing, better substrate solubility, high mass transfer rate and reduced risk of contamination [44].

The kinetic parameters of CtLam81A were determined by analyzing the Michaelis-Menten plot and Lineweaver-Burk plot. CtLam81A displays a $V_{\max}$ of 109.0 ± 1.8 U/mg and a K_m of 2.1 ± 0.12 mg/ml for laminarin and a $V_{\max}$ of 74.2 ± 2.5 U/mg and a K_m of 0.54 ± 0.06 mg/ml for

curdlan (Fig. 3). The effect of various cations and EDTA on the activity of CtLam81A was also investigated. The result indicated that the enzyme activity of CtLam81A increased in the presence of Ca^{2+} and Mg^{2+} ions, while activity was inhibited in the presence of Mn^{2+} , Zn^{2+} and Cu^{2+} ions and EDTA and there was no effect of Co^{2+} and Ni^{2+} ions on enzyme activity (Table 2). However, this result differs from previous studies, which showed no effects of Ca^{2+} and Mg^{2+} ions on the activity of SpEng2 from *S. pombe* and ScEng2 from *S. cerevisiae* [22]. The observed decrease in activity resulting from incubation with EDTA indicated that CtLam81A requires Ca^{2+} ions for structural stability and efficient catalysis. To study the effect of Ca^{2+} and Mg^{2+} ions in deep, CaCl_2 and MgCl_2 concentration was varied between 0.1 and 10 mM in the reaction mixture. The result revealed that enzyme activity of CtLam81A increased by 31% and 33% in the presence only 1 mM of Ca^{2+} and Mg^{2+} ions, respectively.

3.4. Protein melting curve of CtLam81A

The melting temperature of CtLam81A was found to be 92 °C (Fig. 4a). This melting temperature was shifted towards a higher temperature, 96 °C in the presence of 10 mM Ca^{2+} (Fig. 4b) or 10 mM

Table 3

Data collection and processing statistics for the CtLam81A-E515A crystal. Values in parentheses correspond to the highest resolution shell.

Data set	CtLam81A-E515A
Source	ID23-1, ESRF
Detector	PILATUS 6M-F
Wavelength (Å)	0.9918
a (Å)	61.92
b (Å)	139.03
c (Å)	197.91
Space group	I2 ₁ 2 ₁ 2 ₁
Molecules per ASU	1
Matthews coefficient (Å ³ ·Dalton ^{−1})	2.68
Mosaicity (°)	0.10
Resolution range (Å)	45.15–1.40 (1.42–1.40)
<I/σI>	14.7 (2.8)
Wilson B-factor	15.8
R _{merge} (%) [*]	6.6 (60.8)
R _{pim} (%) ⁺	2.8 (24.8)
Half-dataset correlation CC1/2	0.998 (0.904)
Multiplicity	6.6 (6.9)
No. of observed reflections	1,103,933 (55838)
No. of unique reflections	166,379 (8147)
Completeness (%)	99.5 (99.0)
Anomalous completeness (%)	97.9 (97.4)
Anomalous multiplicity	3.3 (3.5)
Solution method	MR
Refinement statistics	
R _{work} ^{**}	0.108
R _{free}	0.133
Rmsd bonds (Å)	0.007
Rmsd angles (°)	0.937
Average B (Å)	20.0
Main chain	18.2
Ligands	33.7
Water molecules	33.8
Residues per ASU	717
Ligands per ASU	12 MPD, 2 ACT, 2 Na ⁺ , 4 Cl [−] , 3 Ca ²⁺ , 4 Mg ²⁺ , 2 Ni ²⁺
No. of water molecules	714
Total no of atoms	6652
Ramachandran favored (%)	97.5
Ramachandran outliers (%)	0.14
PDB accession code	6FOP

^{*} Where *I* is the observed intensity, and $\bar{I}$ is the statistically weighted average intensity of multiple observations.

⁺ A redundancy-independent version of *R*_{merge}.

^{**} Where *I*_{calc} and *I*_{obs} are the calculated and observed structure factor amplitudes, respectively (*R*_{free} is calculated for a randomly chosen 5% of the reflections).

Mg²⁺ ions (Fig. 4c). However, 10 mM EDTA shifted the melting temperature to a much lower temperature of 83 °C (Fig. 4d). This observation suggests that Ca²⁺ and Mg²⁺ ions thermally stabilize CtLam81A and provide resistance to unfolding and denaturation at higher temperatures, whereas, EDTA removes native metal ions and induces instability. These results are also corroborated by the CtLam81A activity profile in the presence of Ca²⁺, Mg²⁺ ions and EDTA as described in the previous section. Taken together, data relating to protein melting and enzyme activity suggested that Ca²⁺ and Mg²⁺ ions are involved in maintaining enzyme structural integrity.

3.5. Mode of action of CtLam81A

The amount of reducing sugar released during the hydrolysis of curdlan is displayed in Fig. 5a. This indicated that levels of reducing sugar saturated after 12 h. The hydrolysed products generated by CtLam81A during the degradation of curdlan were analysed by TLC (Fig. 5b). The chromatogram showed the presence of β-1,3-oligosaccharides of various degrees of polymerization (DP). Initially, after first 5 min of the reaction, a wide range of oligosaccharides were formed, whereas towards the end of 24 h, only glucose and laminaribiose were the major products. Thus, the data suggest that CtLam81A displays a typical endoglucanase activity. Further MALDI-TOF analysis of hydrolysed products of curdlan produced by CtLam81A enzyme in 5 min displayed peaks at *m/z* 203.7, 363.5, 526.7, 689.5, 852, 1014 and 1176.3 (as sodium TH-2226_146106035

adduct, [M + Na⁺]) which corresponded to glucose (Glu), Laminaribiose (DP2), Laminaritriose (DP3), Laminaritetraose (DP4), Laminaripentaose (DP5), Laminarihexaose (DP6) and Laminariheptaose (DP7) (Fig. 5c). These oligosaccharides have been used as prebiotics [9] and also anticancer agents [11, 12].

3.6. 3D structure analysis of CtLam81A-E515A

Multiple sequence alignment of CtLam81A with other family 81 glycoside hydrolases from *Bacillus halodurans* GH81 (BhGH81; Q9KG76) and *Thermobifida fusca* Lam81A (TfLam81A, AAZ56163) suggested that E515 and D438 may act as base and acid catalyst, respectively (Fig. 6). In order to study the mode of interaction of CtLam81A with its ligand, its catalytically inactive mutant (CtLam81A-E515A) was constructed by site-directed mutagenesis. The results showed that the mutant, CtLam81A-E515A gave no activity against β-1,3-glucan substrates (Table 1), supporting a catalytic role for E515. Tetragonal crystals containing one molecule of CtLam81A-E515A per asymmetric unit were obtained using a solution of 0.1 M sodium acetate pH 4.6, 0.02 M calcium chloride and 30% (w/v) 2-methyl-2,4-pentanediol as the precipitating agent. The crystals diffracted up to 1.4 Å resolution (Fig. 7) and the data collection statistics are presented in Table 3. To solve the structure of CtLam81A-E515A, sequence alignments were performed to find the highest similarity models that could lead to good initial phases obtained by molecular replacement (MR). Structure determination was

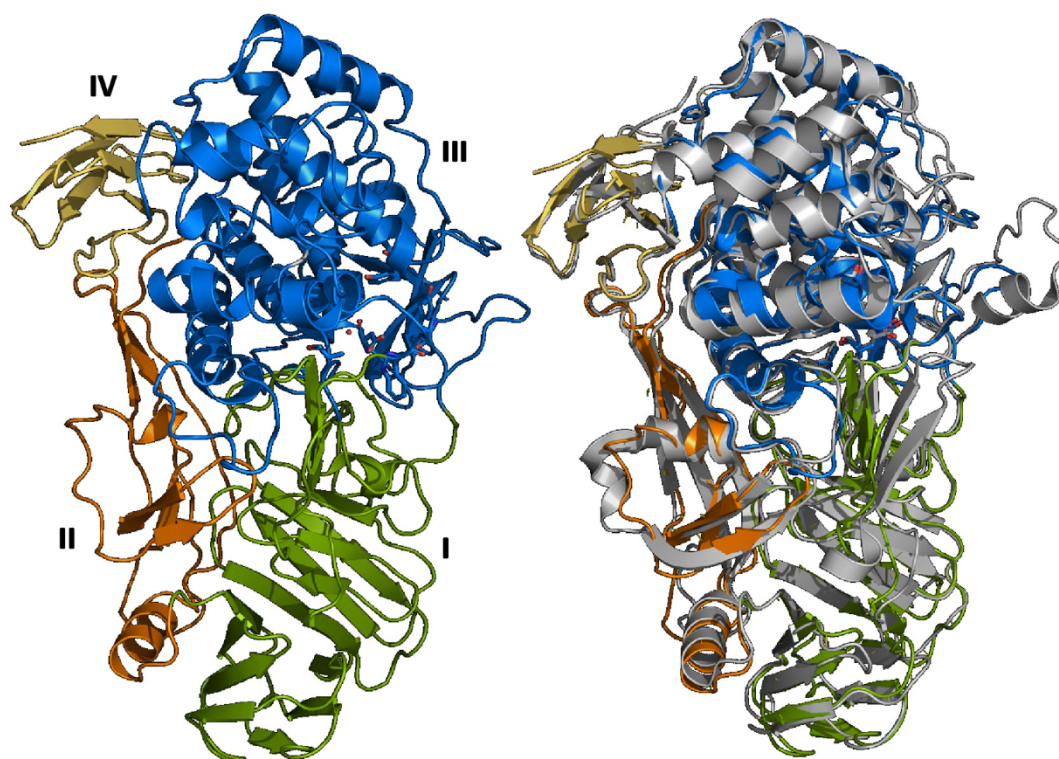


Fig. 8. The overall structure of CtLam81-E515A. The composite domains I–IV are labelled and colored green, orange, blue and yellow, respectively. In the right panel, the superposition of the BhGH81 is shown as a grey cartoon. All figures were produced using PyMOL v.2.0 (<http://www.pymol.org/>; Schrodinger LLC).

performed by MR using as search model, the family 81 glycoside hydrolase from *Rhizomucor miehei* (PDB code 4K3A), the highest similarity structure available at the time of structure solution that showed 27% sequence identity to CtLam81A-E515A. In the initial attempts to solve the phase problem, the non-conserved amino acids were pruned to facilitate the rotational and translational searches. Nevertheless, an MR solution could only be obtained while searching for small sections of the protein separately: Domain I, from residues 1 to 310; Domain II from residues 311 to 654. This procedure is normally used for large, multi-domain or oligomeric proteins, where a high degree of flexibility can be expected between the different domains/subunits. A solution was obtained for domain II. Automated model building was used, producing a preliminary model with 684 residues and a R_{work} and R_{free} of 0.198 and 0.213. The preliminary inspection of the electron density map, calculated after structure solution by MR, showed no evidence of the presence of the ligand bound at the active site of CtLam81A-E515A. The electron density for the mutated residue 515 was confirmed. In the final stages of refinement, the R_{work} and R_{free} converged to 10.8% and 13.3%. The structure solution details and refinement statistics are listed in Table 3. Fig. 8 shows the overall structure obtained and its superposition with the recently solved structure of family 81 GH from *Bacillus halodurans* [24], that shows 41% sequence similarity with CtLam81A, revealing their structural conservation (rmsd of 1.19 Å for 697 C_{α} atoms). The structure of CtLam81A-E515A, presented in Fig. 8 (PDB code 6FOP), comprises one polypeptide chain (residues 38 to 740), 714 water molecules and has approximate dimensions of $45.2 \times 41.2 \times 83.7$ Å³. The accessible surface area of the structure to solvent is about 23,748 Å². The CtLam8-E515A structure consists of 15 α -helices and 38 β -strands, comprising a four-domain organization starting from the N-terminus of a β -sandwich domain (I, green color), an α/β -domain (II, orange), an $(\alpha/\alpha)_6$ barrel domain (III, blue), and finally a small 5-stranded β -sandwich domain (IV, yellow) (Fig. 8). With the exception of a small defined secondary structure in the N-terminal and a C-terminal domain, this overall structure has been described in detail by Zhou et al.

(2013) [25] for the RmGH81 structure from *Rhizomucor miehei* and more recently by Pluvinage et al. [24] for BhGH81 from *Bacillus halodurans*. Similar to CtLam81, BhGH81 also has the C-terminal domain that appears to be unique for these two enzymes from *C. thermocellum* and *B. halodurans* organisms. This domain appears to separate the $(\alpha/\alpha)_6$ barrel domain from the following domain, which is a dockerin module in CtLam81 and a CBM6 module in BhGH81. In RmGH81, these 50 amino acids from the C terminus of the crystallized protein are not present and may comprise another uncharacterized domain similar to that seen in CtLam81 and BhGH81. Several solvent ions and molecules could be identified in the electron density, including three calcium ions, four chloride ions, two sodium ions, four magnesium ions, two nickel ions and twelve MPD (methyl pentanediol) molecules. The Ca (3) atom is coordinated in an octahedral geometry by residues D401, T404, E409 and K412 (Fig. 9A) and the Mg (1) coordinated by residues I67, N70, Q71, Y72 and A125 (Fig. 9B). This high number of cations has not been reported for other members of GH81 family and may be related to the higher thermal stability and higher catalytic activity observed for CtLam81A in the presence of Ca^{2+} and Mg^{2+} , reported above. These structural ions have a probable protective role, conferring structural stability to the enzyme.

3.7. The substrate binding site

As revealed by the crystal structure and its superposition with BhGH81, the carbohydrate-binding pocket of CtLam81A is very similar to BhGH81 (Fig. 9E). In RmGH81 and BhGH81 the residues E553, E557, D475 and E542, E546, D466, respectively, were proposed to be the potential acid and base catalytic residues. The corresponding conserved residues in CtLam81 are E515, E519, and D438, which are located in the base of a cleft present near the center of the $(\alpha/\alpha)_6$ barrel. These residues provide strong evidence for holding and the retention of the substrate. The residues Y363 and Y445 are involved in the formation of hydrogen bonds with E519 and are highly conserved in all GH family

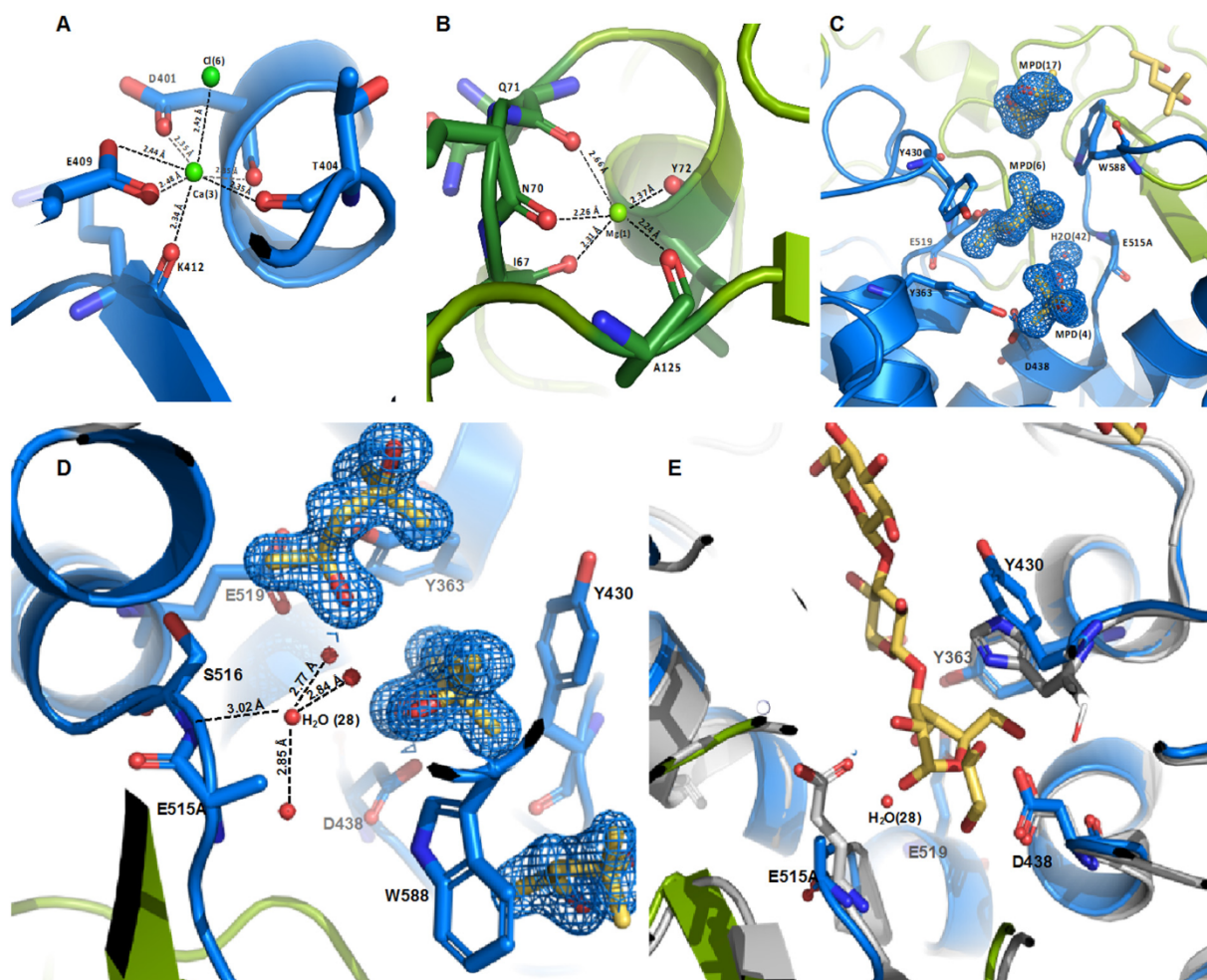


Fig. 9. Ligands interaction with CtLam81-E515A. A) Calcium interaction with domain I of CtLam81-E515A. B) Magnesium interaction with domain III of CtLam81-E515A. C) MPD interaction in CtLam81-E515A active site. The $2mF_o - DF_c$ electron density map is contoured at 1σ . D) Active site of CtLam81-E515A with the $2mF_o - DF_c$ electron density map contoured at 1σ . E) Active site of CtLam81-E515A superposed with the active site of BtGH81 complex with products resulting from L6 cleavage. CtLam81-E515A (pdb: 6FOP) is shown in green cartoon and sticks for domain I and blue for domain III, water from CtLam81-E515A as red spheres and magnesium and calcium as green spheres. In the superposition, BtGH81 (pdb: 5T4G) is shown in grey cartoon and sticks and L3 from BtGH81 as yellow sticks. The figure was produced by using PyMOL v.2.0 (<http://www.pymol.org>; Schrodinger LLC).

81 members. There are twelve MPD molecules found in the CtLam81E515A crystal structure. These were probably imported during the crystallization and cooling since MPD was present in high concentrations in the crystallization and cryoprotectant solutions. The aromatic side chain of W588, that provides the classic CH- π interactions to the sugar unit, is making the π interactions with an MPD molecule. A similar interaction with MPD is observed for residue W589. Other two molecules of MPD are present in the vicinity of catalytic residues E515A (proton donor), E519 (basic catalyst) and D438 (acid catalyst) (Fig. 9D). One of the MPD molecules contributes to a network of two hydrogen bonds with two neighboring water molecules and interacts with residues Y363 and K367. The second MPD molecule makes a network of three hydrogen bonds with residue D438 and two neighboring water molecules. The N atom of S516 hydrogen bonds to one water molecule, itself bound by three other water molecules (Fig. 9D.) As reported by Pluvinau et al. [24], if A515 was a glutamate, as in the wild type, it would not be well positioned, to directly attack the substrate. Therefore, this arrangement suggests that this water molecule is activated by E515, becoming a catalytic water, since it is reasonably well positioned to attack the substrate (Fig. 9E).

4. Conclusions

Here we described the biochemical properties of recombinant CtLam81A from *Clostridium thermocellum*, an enzyme that is a major TH-2226_146106035

component of the cellulosome, a high molecular mass multi-enzyme complex that orchestrates the deconstruction of structural carbohydrates. CtLam81A displayed maximum activity against laminarin and therefore was named Laminarinase 81A (CtLam81A). CtLam81A displayed maximum activity at neutral pH and 75 °C. The enzyme was stable in the pH range, 7.0 to 9.0 and was thermally stable up to 75 °C for 1 h. Ca^{2+} or Mg^{2+} ions enhanced the activity of CtLam81A and also shifted the melting temperature by 5 °C confirming their involvement in catalysis and also to provide protein stability. During the initial stages of hydrolysis of β -1,3-glucans, as analysed by TLC and MALDI-TOF, CtLam81A releases predominantly a range of oligosaccharides with different DP confirming an *endo*- β -1,3-glucanase activity. The mutant CtLam81A-E515A was co-crystallized and tetragonal crystals diffracted up to 1.4 Å resolution and structure was solved by Molecular Replacement. The structure revealed the presence of three Ca^{2+} and four Mg^{2+} ions and this high number of metal ions has not been reported so far in any other member of family GH81. These structural ions have a probable protective role, conferring structural stability to CtLam81A. The conserved catalytic residues in CtLam81 are E515, E519, and D438 located at the base of the cleft present near the $(\alpha/\alpha)_6$ barrel. Like in *Rhizomucor miehei* and *Bacillus halodurans* GH81, the predicted catalytic base is E515 and D438 might be the acid catalyst. CtLam81A may display a large array of biotechnological applications including its use in agriculture to control fungal diseases. It can also be used in combination with

exo- β -1,3-glucanase to produce fermentable sugar in bioethanol industry. The thermostability of CtLam81A makes it suitable for industrial applications as several industrial processes require thermostable enzymes for easy mixing, better substrate solubility, high mass transfer rate and lowering the risk of contamination. Finally, the capacity of CtLam81A to produce β -1,3-glucan oligosaccharides of different DP may be applied to the production of prebiotics, immune-activation precursors or anti-cancer agents.

Acknowledgements

This research work was carried out under bilateral joint DST (Ministry of Science and Technology, India) project (No. DST/INT/Portugal/P-14/2013) in collaboration with FCT Lisbon, Portugal (grant references PTDC/BIA-MIC/5947/2014, PTDC/BBB-BEP/0869/2014, IF/01621/2013/CP1183/CT0002, PEst-C/EQB/LA0006/2013 and UID/Multi/04378/2013 to Research Unit UCIBIO also co-financed by the ERDF under the PT2020 Partnership Agreement (POCI-01-0145-FEDER-007728)) and in part supported by funding from Indian Institute of Technology Guwahati (IIT), Guwahati, Assam, India. The authors are also grateful to Professor Maria João Romão, for usage of the Macromolecular Crystallography Lab facilities (FCT-NOVA, Caparica), to Central Instrument Facility, IIT Guwahati for providing the MALDI-TOF facility and to the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and the Diamond Light Source (DLS, Oxfordshire, UK) for access to synchrotron facilities.

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Structural characterization of an endo β -1,3-glucanase of family 81 glycoside hydrolase (CtLam81A) from *Clostridium thermocellum*

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Running title: Structural characterization of CtLam81A

Received: February 13, 2018

Accepted: August 15, 2018

Published: August 20, 2018

Abstract

The gene sequence of a family 81 glycoside hydrolase from *Clostridium thermocellum* Cthe_0660, (GenBank accession number: ABN51485.1) was retrieved from CAZy (Carbohydrate-Active Enzyme) database. The gene (2151 bp) encoding the catalytic module of Cthe_0660, an endo β -1,3-glucanase (CtLam81A) cloned, expressed earlier was used in this study. The secondary structure analysis of CtLam81A by PsiPred showed the presence of 23% α -helices and 23% β -strands whereas, CD analysis showed 24% of α -helices and 24% of β -strands. The secondary structure analysis by Circular Dichroism corroborated the PsiPred results. The 3D modeled structure of CtLam81 generated by Swiss-Model server and energy minimized by Swiss-PDB viewer displayed an N-terminal β -sandwich domain, a $(\alpha/\alpha)_6$ domain and a short β -sandwich domain at C-terminal. The quality assessment of modelled structure by Ramachandran plot displayed 94% residues in favoured region, 4.6% in allowed region and 1.4% in outlier region. The superposition of CtLam81 modeled structure with its closest homologue Bh0236 (PDB ID: 5t49) from *Bacillus halodurans* indicated that Glu515 acts as a catalytic base, while Asp438 acts as a catalytic acid. Multiple sequence alignment showed that these catalytic residues are conserved within the family 81 glycoside hydrolase. CtLam81 showed lowest binding energy against laminaripentaose and the amino acid residues, Tyr363, Trp364, Tyr430, Trp588, Trp589 and Trp637 create the binding pocket at active site to hold the ligand.

Key words: Family 81 glycoside hydrolase; β -1,3-glucanase; Laminari-oligosaccharides; Circular Dichroism; Homology modeling; Docking; *Clostridium thermocellum*

Note - Coloured Figures are available on the Journal Website in "Archives" Section

Introduction

β -1,3-glucan is a major carbohydrate found in bacteria (*Alcaligenes faecalis*, *Agrobacterium rhizogenes*, *Agrobacterium radiobacter*), as a linear chain of β -1,3-linked glucose (McIntosh et al., 2005) while in fungi (*Lentinus edode*, *Sclerotium rolfsii*, *schizophyllum commune*) and algae (*Laminaria digitata*) a chain of β -1,3-glucan branched either with β -1,6-linked glucose or with short chain of β -1,3-glucan (McIntosh et al., 2005; Volman et al., 2008). β -1,3-glucan is hydrolysed by β -1,3-glucanase enzyme, which are classified into two types, exo- and endo- acting. Exo- β -1,3-glucanase (EC 3.2.1.58), acts on the terminal of chain and releases glucose oligomers (Mouyna et al., 2013). These exo-acting enzymes fall into the glycoside hydrolase families 3, 5, 17 and 55. Endo- β -1,3-glucanase (EC 3.2.1.39) acts randomly on the β -1,3-glucans and releases mixture of oligosaccharides. Endo- β -1,3-glucanases fall into families 16, 17, 55, 64, 81 and 128 of glycoside hydrolase (GH). Thus, both GH17 and GH55 families contain β -1,3-glucan specific enzymes that have both, exo- or endo-activities. Family 81 glycoside hydrolases are known for their endo- β -1,3-glucanase activity and are widely distributed in plants, yeast, fungi, bacteria, archaea and viruses. Presently, family 81 GH contains 674 proteins distributed over bacteria, archaea, eukaryotes and viruses (<http://www.cazy.org/GH81.html>). All of the proteins in family 81 GHs share a common size of around 650 amino acids. Some of its members are biochemically characterized like β -glucan-binding protein (GBP) from *Glycine max* (Fliegmann et al., 2005), *TfLam81* from *Thermobifida fusca* (McGrath & Wilson, 2006) and Eng2 from *Saccharomyces cerevisiae* (Martin-Cuadrado et al., 2008). ^1H NMR spectroscopic analysis of GBP hydrolysed product revealed the retaining hydrolytic mechanism of this family (Fliegmann et al., 2005). So far only two enzymes, *RmLam81A* from *Rhizomucor miehei* (Zhou et al., 2013) from *Bacillus halodurans* (Pluvinaige et al., 2017) provide information about crystal structure solved from family GH81. Crystal Structure analysis of

RmLam81A depicted the presence of β -sandwich domain at N-terminal and $(\alpha/\alpha)_6$ domain at C-terminal connected *via* additional domain between them. Similar type of domain was also found in *BhGH81* protein. Interaction study of laminarin with *BhGH81* revealed that the active site of this protein can accommodate maximum five glucose residues of β -1,3-glucan (Pluvinaige et al., 2017). *Clostridium thermocellum* contains multienzyme complex, known as cellulosome. Cellulosome complex is made by a high affinity interaction between dockerin modules of the cellulosomal catalytic proteins and cohesin modules on the scaffoldins (Fontes and Gilbert, 2010). One such cellulosomal enzyme of *Clostridium thermocellum* is β -1,3-glucanase (*Cthe_0660*) belongs to family 81 GH. The catalytic component, *CtLam81A* of *Cthe_0660* was cloned and purified earlier (unpublished results) was used in the present study. In this study, the secondary structure elements of *CtLam81A* were determined by Circular Dichroism and also confirmed by PsiPred. The 3D structure of *CtLam81A* was modeled by homology modeling and ligand docking study was performed to identify the amino acid residues involved in the catalysis.

Material and Methods

Retrieval of amino acids sequence of CtLam81A - The gene sequence of family 81 glycoside hydrolase protein of *Clostridium thermocellum* ATCC27405 having gene accession number ABN51895.1 and uniprot ID A3DD6 was retrieved from CAZy database (<http://www.cazy.org/>). The boundaries of the family 81 GH catalytic domain (*CtLam81A*) in the amino acid sequence of *Cthe_0660* protein was identified by using the conserved domain database (<http://www.ncbi.nlm.nih.gov/cdd/>). The 2151 bp gene encoding *CtLam81A* protein was cloned, expressed and purified earlier in our laboratory (Unpublished results).

Secondary structure analysis of CtLam81A - Secondary structure of *CtLam81A* protein sequence for alpha helices, beta strands, turns and loops were predicted with the help of

PsiPred v3.3 method (<http://bioinf.cs.ucl.ac.uk/psipred/>). The composition of secondary structure of CtLam81A was also determined by Circular dichroism (CD). The purified CtLam81 at a concentration 0.7 μ M in 50mM phosphate buffer, pH 7.5 was used for CD analysis. The CD spectrum was recorded on a spectro-polarimeter (JascoJ-815, Japan) at 25°C using 1 nm bandwidth over far UV region between 190 to 250 nm at scanning rate of 50 nm/min. The CD data were presented by difference in molar extinction coefficient ($\Delta\epsilon$, deciliter mol⁻¹ cm⁻¹) as a function of wavelength (Kelly et al., 2005). Percentage of α -helix and β -sheet were measured by web based K2D3 software package (<http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/>) (Andrade et al., 1993).

Homology modelling CtLam81A-The three dimensional structure of CtLam81A was modelled with the help of SWISS-MODEL server. SWISS-MODEL is a web based server, which automatically identify structural templates based on BLAST analysis and generate 3-D protein structure (<https://swissmodel.expasy.org/>). The best modelled structure was selected based on the alignment score with templates.

Model refinement and quality assessment - The best modeled structure was energy minimized by Swiss-PDB viewer. Swiss-PDB viewer comprises a version of GROMOS 43B1 force field (Van Gunsteren et al., 1996). This force field allows the evaluation of the energy of structure as well as it repairs the distorted geometry through energy minimization. Then the quality of energy minimized structure was checked by plotting the Ramachandran plot using RAMPAGE server (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>). This modeled structure was also validated by using the Verify-3D program (http://services.mbi.ucla.edu/Verify_3D/), which analyzes the compatibility of atomic model with the amino acid sequence (Liithy et al., 1992).

Prediction of active site and mechanism of action - The amino acid residues in the active site are generally conserved in the proteins of the same family. To identify the amino acid

residues at active sites, *CtLam81A* was structurally aligned with the structure of *BhGH81* (PDB ID- 5T49) using Pymol. To confirm whether these residues are conserved within family 81 GHs, multiple sequence alignment (MSA) was performed with the already characterized homologous proteins of the family81 GHs viz. *Bacillus halodurans* GH81 (*BhGH81*; Q9KG76), *Thermobifida fusca* Lam81A (*TfLam81A*, AAZ56163), *Glycine max* Gbp (*GmGbp*; BAA11407), *Aspergillus fumigatus* EngA (*AfEngA*; AAF13033), *Saccharomyces cerevisiae* Eng2 (*ScEng2*; AAB82378) and *Rhizomucor miehei* Lam81 (*RmLam81A*; KC847083). The percentage similarity between amino acid sequences of *CtLam81A* with its homologous proteins from family 81 GH was analysed by BLAST tool (Altschul et al., 1990). MSA was performed with the help of CLUSTALW program (<http://www.genome.jp/tools-bin/clustalw>) and the conserved amino acid residues were viewed by ESPript 3.0 (<http://esprict.ibcp.fr/ESPript/ESPript/>). To study the mechanism of hydrolysis the distance between the two catalytic amino acids (Asp438 and Glu515; from C₈toC₈) of *CtLam81A* was measured in the molecular visualization system PyMol 2.0.

Molecular dynamic simulation of CtLam81A modeled structure - Molecular dynamic (MD) simulation of *CtLam81A* was performed by using GROMACS v 5.14 (Berendsen et al, 1995). GROMOS96 53a6 force field was used to calculate the Protein forces, where the protein *CtLam81A* was placed within in a cubic box of single point charge (SPC) with water molecules. The *CtLam81A* protein charges were neutralized by adding the 15 numbers of counter ions (Na⁺). Then the whole system was equilibrated for 500 ps in NVT ensemble (constant number of particles, volume and temperature) was carried out for restraining the solute atoms. This system was again equilibrated for 500 ps by NPT ensemble (constant number of particles, pressure and temperature) twice, once with restraints and then without restraints. Production run was performed for 30 ns with NPT ensemble adopting a 2 fs integration time. The linear constraint solver (LINCS) algorithm (Hess et al, 1997) was

employed to constrain the bonds associated with hydrogen atoms and radius of gyration. Throughout the production run the modeled CtLam81A structure was analyzed as a time dependent function to ascertain its stability in the solvent system. The variation in the CtLam81A protein backbone (root mean square deviation, RMSD) was estimated by the least square fitting method.

Biding interaction analysis of CtLam81A with the ligands - Molecular docking of CtLam81A with the laminari oligosaccharides was performed by using SwisDock, the web based server (<http://www.swissdock.ch/docking>). Laminaribiose, laminaritriose and laminaritetraose were downloaded from PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Laminaripentaose and laminarihexaose were generated using the GLYCAM server (Kirschner et al., 2008). The modelled CtLam81A was saved in PDB format and the ligands were saved in Mol2 file format for docking analysis in the Swiss Dock tool. Swiss Dock generates large number of ligand binding results. The enzyme-ligand docked complex showing the strongest binding with maximum of negative binding energy were selected. This ligand bound structure was downloaded and visualized in PyMol 2.0. The depiction of ligand interaction with the amino acid residues of the protein was generated using the PDBsum Generate tool (<https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html>).

Results and Discussion

Molecular architecture of CtLam81A

CtLam81A from *Clostridium thermocellum* contains an N-terminal catalytic domain and a type I dockerin domain at the C-terminal connected via linker. The dockerin domain binds to cohesion domain, located on the scaffoldin protein in cellulosome complex (Ding et al, 2001). The 2151 bp gene encoding family 81 glycoside hydrolase (CtLam81) and linker was cloned in pET-28a(+) vector, expressed in *E. coli* BL21 cells and purified (Unpublished

results). Total length of purified protein(CtLam81A) is 740 amino acids consisting of 23 amino acids of pET28a(+) vector, 660 amino acids of catalytic domain and 64 amino acids of linker at the C-terminal. The theoretical molecular size of CtLam81A is 82.4 kDa.

Secondary structure analysis of CtLam81A

Secondary structure of CtLam81A predicted by PsiPred tool showed the presence of 14 α -helices (23%), 31 β -strands (23%) and 45 random coils (54%) (Fig.1). CtLam81A showed almost an equal amount of β -strands and α -helices. The N-terminal of CtLam81A showed the predominance of β -strands and the C-terminal showed the abundance of α -helices. The predicted secondary structure was also confirmed by circular dichroism analysis of purified CtLam81A. The CD spectrum of CtLam81A was analysed with the help of K2D3 server, which gives the percentage of α helix and β strand by comparing the available secondary structures of known proteins (Louis-Jeune et al., 2012). This analysis showed that CtLam81A contains 24% α -helices and 24% β -strands (Fig. 2). The results of CD analysis corroborated with the secondary structure predicted by PsiPred method. This distribution of secondary structure elements is common in family 81 GH (Zhou et al., 2013).

Homology modeling and structure validation

The web based server SWISS-MODEL generated the 3-D model structure of CtLam81A by using the structural template of protein BhGH81(PDB ID- 5T49) having 42% sequence identity with query coverage 100%. The overall structure of the modelled CtLam81A (Fig. 3A) consisted of three domains, N- terminal β -sandwich domain I (red colour), an $(\alpha/\alpha)_6$ barrel domain II(green colour) and a short β -sandwich domain III (yellow colour) at C-terminal. This modeled structure was energy minimized and validated before docking analysis. Ramachandran plot of CtLam81A modeled protein showed that 94% of the residues found in the favourable region and 4.6 % of residues found in allowed region and only 1.4% residues (Phe177, Thr189, Lys221, Asn465, Asp481, Thr592, Ile661 and Pro663)

was found in outlier region (Fig. 3B). This indicated that the amino acid residues in the modelled CtLam81A occupied favourable phi (ϕ) and psi (ψ) backbone dihedral angles. Verify_3D result showed that 95.25% of the residues in modeled structure had an averaged 3D-1D score ≥ 0.2 , which indicated the compatibility of amino acids with the modelled structure (Fig. 3C). ProSA result of modelled CtLam81A indicated that the protein is error free and reside in the x-ray zone with Z-scores of -9.88 (Fig. 3D).

Catalytic cleft and mechanism of catalysis of CtLam81A

The amino acids residues involved in the catalysis and in substrate binding were explored by superimposing the CtLam81A modelled structure with crystal structure of BhGH81 (PDB ID – 5T49) having RMSD, $0.495 \text{ \AA}$ as shown in Fig. 4A. The catalytic core of CtLam81A existed within $(\alpha/\alpha)_6$ barrel domain of the protein. The superposition of modelled structure of CtLam81A (shown in green colour) with BhGH81 (shown in cyan colour) showed that β -sheet and α -helix of CtLam81A aligned well with the β -sheet and α -helix of BhGH81 (Fig. 4A). However, some loop regions (43-65 amino acids, 96-105 amino acids, 214-222 amino acids and 296–302 amino acids) of the CtLam81A modelled structure do not align with the BhGH81 structure. The analysis of superimposition showed that Glu515 acts as catalytic base and Asp438 acts as acid catalyst while Trp588 and Trp589 helps in binding the ligand (Fig. 4B). CtLam81A shared amino acid sequence similarity with homologous proteins from *Bacillus halodurans* GH81 (BhGH81; Q9KG76), *Thermobifida fusca* Lam81A (TfLam81A, AAZ56163), *Glycine max* Gbp (GmGbp; BAA11407), *Aspergillus fumigatus* EngA (AfEngA; AAF13033), *Saccharomyces cerevisiae* Eng2 (ScEng2; AAB82378) and *Rhizomucor miehei* Lam81 (RmLam81A; KC847083) as shown in Table 1. The multiple sequence alignment of CtLam81A showed that the catalytic amino acids are conserved in all family 81 glycoside hydrolases. Trp588 is conserved in both bacterial and plant proteins, while Trp589 is conserved in bacteria only (Fig. 5). Glycoside

hydrolases catalyse the glycosidic bond, either by inverting or retaining hydrolytic mechanism. It has been found that, in the inverting mechanism, the average distance between two carboxyl groups of acid/base catalytic residues is 10.5 Å and in the retaining mechanism the average distance is only 5.5 Å (Zechel & Withers, 1999). The superimposition of *CtLam81A* structure with crystal structure of *BhGH81* indicated that Glu515 is catalytic base and Asp438 is catalytic acid. The distance between these two catalytic residues was found to be 8.3 Å (Fig. 6), indicating the inverting hydrolytic mechanism for *CtLam81A*. The same inverting hydrolytic mechanism was also reported for β -glucan-binding protein (GBP) a member of family 81 glycoside hydrolases from soybean (Fliegman et al., 2005).

Molecular dynamics simulation of CtLam81A modeled structure

Molecular dynamic simulation of modeled *CtLam81A* was performed in order to analyse the structure stability and compactness of the structure over a 30 ns duration. The MD simulation results showed the fluctuation in RMSD value of *CtLam81A* up to 5 ns and thereafter the structure was completely stable till 30 ns. The overall deflection was 0.32 nm RMSD (Fig. 7A). The radius of gyration (*R_g*) of *CtLam81A* structure remained between 2.31 and 2.39 nm till the end of the run (Fig. 7B). Based on the MD simulation result of *CtLam81A*, it was concluded that the modelled structure of *CtLam81A* structure has a stable conformation.

Docking study of CtLam81A with ligands

The docking study of *CtLam81A* was performed in order to analyse the interaction of ligands with its active site. The binding energies of laminari-oligosaccharides with *CtLam81A* are shown in Table 2. Laminaripentaose showed the strongest binding with maximum of negative binding energy of -11.47 kcal/mol. The docking of laminaripentaose on surface of *CtLam81A* is shown in Fig. 8A. The non-reducing end of laminaripentaose is expressed as $-n$ subsite and the reducing end as $+n$ subsite. This result revealed that the

catalytic cavity of CtLam81A can occupy maximum of five glucose residues of β -1,3 glucan. Similar results were reported for β -1,3-glucanase (*BhGH81*) from *Bacillus halodurans* (Pluvinage et al., 2017). The aromatic amino acid residues viz. Tyr363, Trp364, Tyr430, Trp588, Trp589 and Trp637 in the catalytic pocket are involved in the holding of laminaripentaose. Trp 589 of CtLam81A lies near the -2 subsite of the laminaripentaose and Trp 588 lies near the +1 subsite, which helps in holding the ligand, as also reported for *BhGH81* from *Bacillus halodurans* (Pluvinage et al., 2017). The catalytic core residues, Tyr363, Lys367, Tyr430, Asp438, Asp513 and Glu519 of CtLam81A are making polar interactions with laminaripentaose (Fig. 8B), while, the residues Trp364, Gly431, Phe441, Ala502, Glu515, Trp588, Ile599, Leu602, Trp637 and Asp665 present at the catalytic core are involved in hydrophobic interactions with the ligand.

Conclusion

The amino acid sequence of CtLam81A was analysed for secondary structure elements by PsiPred tool and CD. CD analysis showed 24% of α -helices and 24% of β -strands. Modelled structure of CtLam81A showed the presence of an N-terminal β -sandwich domain, a $(\alpha/\alpha)_6$ domain and a short β -sandwich domain at C-terminal. The active site lies in the $(\alpha/\alpha)_6$ domain of the protein. Superimposition of CtLam81A with *BhGH81* and MSA confirmed that Glu515 is catalytic base and Asp438 is catalytic acid, which are 8.3 Å apart and involved in hydrolysing β -1,3-glucan by inverting mechanism. The docking study of CtLam81A with ligands showed the strongest binding energy against laminaripentaose, because the active site pocket of CtLam81A can occupy 5 glucose residues of β -1,3-glucan. The aromatic amino acid residues Tyr363, Trp364, Tyr430, Trp588, Trp589 and Trp637, Trp 589 and Trp 588 create a binding pocket for holding the ligand at the catalytic site.

Acknowledgements

This research work was carried out under bilateral joint DST (Ministry of Science and Technology, India) project (No. DST/INT/Portugal/P-14/2013) in collaboration with FCT Lisbon, Portugal. The authors thank Prof. T. Punniyamurthy, Head, Department of Chemistry, Indian Institute of Technology Guwahati for providing CD facility.

Abbreviations

GH, glycoside hydrolase; CD, Circular Dichroism

Conflict of Interest

The authors declare no conflict of interest.

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Table 1. Blast analysis of CtLam81A with its homologues from family 81.

Organism	PDB ID	Query coverage (%)	Identity (%)	e-Value	Total Score
<i>Bacillus halodurans</i> C-125	5T4A	100	42	0	553
<i>Thermobifidafusca</i> YX-ER1	-	96	43	0	570
<i>Glycine max</i>	-	83	24	4e-35	130
<i>Aspergillus fumigatus</i> Af293	-	71	26	5e-35	130
<i>Saccharomyces cerevisiae</i> YPA84	-	64	27	2e-30	116
<i>Rhizomucormiehei</i>	4K3A	44	27	2e-25	112



Table 2. Binding analysis of CtLam81A with Laminari-oligosaccharides.

Ligand	Binding free energy, ΔG (kcal/mol)	Residue making polar interactions	Residue making Hydrophobic interactions
Laminaribiose	-7.29	Glu515, Asp438	Tyr430, Gly431, Glu435, Asn437, Phe 441, Trp588
Laminaritriose	-8.24	Asn513,Asn437	Phe508, Glu435,Gly431, Ser503, Tyr430, Asp438, Trp558, Trp589, Asp438, Trp588, His442, Phe441, Glu515, Glu519, His442, Ile599
Laminaritetraose	-7.01	Tyr430, Trp588, Trp637	Trp589, Tyr353, Asp665, Trp364
Laminaripentaose	-11.47	Tyr363,Lys367, Tyr430,Asp438, Asp513,Glu519	Trp637,Asp665,Ile599,Leu602,Trp364,Glu515,Trp588,Gly431,Ala502,Phe441
Laminarihexaose	-10.18	Tyr363,Glu519, Asp438,Asn513, Tyr430	Trp588,Phe508,Asn437,Ala502,Glu515,Phe441,Leu602,Lys367,Trp589,Asn590,Asn636,Trp637

Figures Legends

Figure 1: Secondary structure determination of CtLam81A by PsiPred server showing the amino acid residues involved in forming α -helix (cylinders), β -sheet (arrow) and random coil (continuous line).

Figure 2: Circular dichroism (CD) spectrum of CtLam81A for determining the percentage of secondary structure elements. The CD data were presented by difference in molar extinction coefficients ($\Delta\epsilon$, decilitre $\text{mol}^{-1} \text{cm}^{-1}$) as a function of wavelength. The purified CtLam81 at a concentration 0.7 μM in 50 mM phosphate buffer, pH 7.5 was used for CD analysis. The CD spectrum was recorded at 25°C using 1 nm bandwidth over far UV region between 190 to 250 nm at scanning rate of 50 nm/min.

Figure 3: (A) Cartoon representation of modeled structure of CtLam81A displaying N-terminal β -sandwich domain I (red colour), an $(\alpha/\alpha)_6$ barrel domain II (green colour) and a short β -sandwich domain III (yellow colour) at C-terminal. Quality assessment of modelled CtLam81A by (B) Ramachandran plot of modelled CtLam81A, (C) Verify-3D and (D) Prosa web server.

Figure 4: (A) Superimposition of CtLam81A modeled structure (green) with BhGH81 crystal structure (cyan) and (B) Superimposition of active site residues of CtLam81A (red colour) with active site residues of BhGH81 (yellow colour) by molecular visualization system PyMol 2.

Figure 5: Multiple sequence alignment of CtLam81A with the homologous protein of the family 81 glycoside hydrolase viz. *Bacillus halodurans* GH81 (BhGH81; Q9KG76), *R. miehei* Lam81 (RmLam81A; KC847083), *T. fusca* Lam81A (TfLam81A, AAZ56163), *A. fumigatus* EngA (AfEngA; AAF13033), *S. cerevisiae* Eng2 (ScEng2; AAB82378) and *G. max*Gbp (GmGbp; BAA11407). The

conserved amino acid residues are shown in red background and semi conserved residues are shown in box. This figure was generated by EsPript3.0 (<http://espript.ibcp.fr/>).

Figure 6: Determination of hydrolytic mechanism (Retaining or Inverting) of CtLam81A enzyme by measuring the distance (from C_{δ} to C_{δ}) between the acid catalyst (Asp438) and catalytic base (Glu515) of CtLam81A by molecular visualization system PyMol 2.

Figure7: Molecule dynamic (MD) simulation of modeled CtLam81A showing (A) RMSD plot and (B) Radius of gyration plot

Figure8: (A) Surface view of CtLam81A showing docking of Laminaripentaose with the amino acid residues of the catalytic cleft and (B) 2D Schematic presentation of Laminaripentaose interaction with the amino acid residues at active site of CtLam81A. Dashed lines show the hydrogen bonds and the amino acid residues shown in arc with spokes are making hydrophobic interactions with the ligand.

Fig. 1

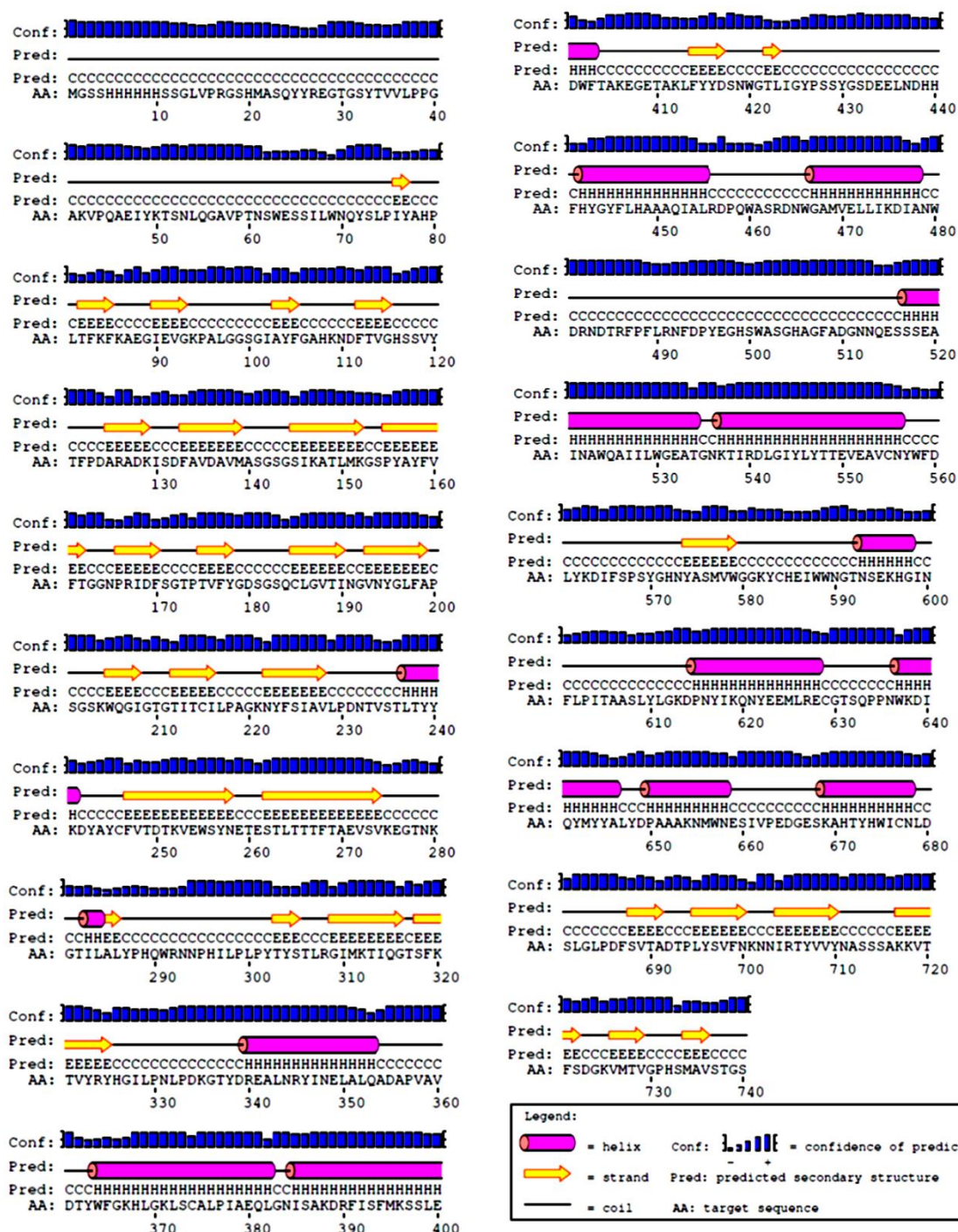


Fig. 2

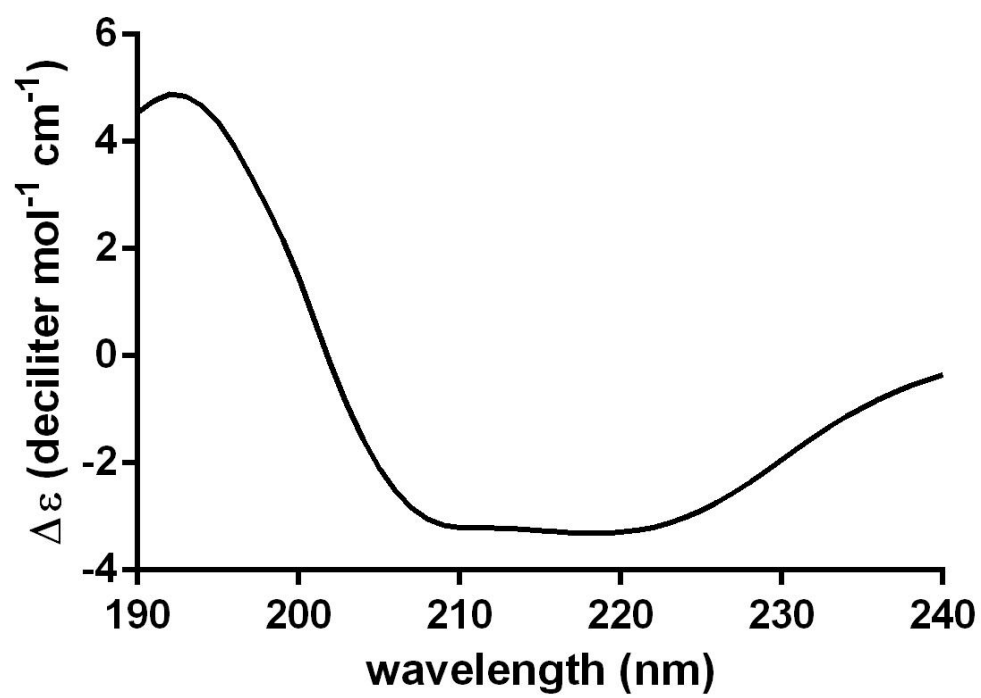
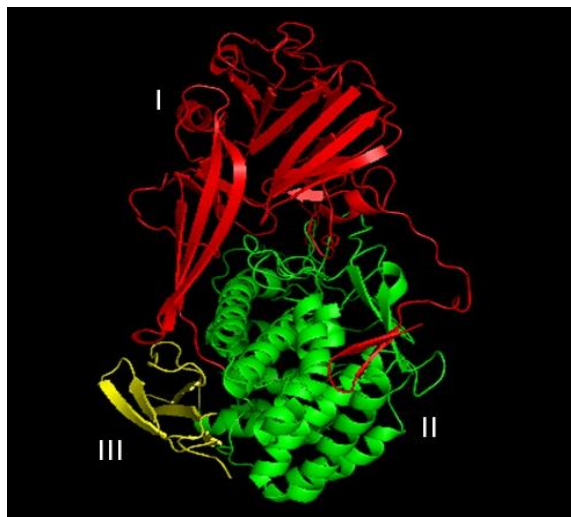
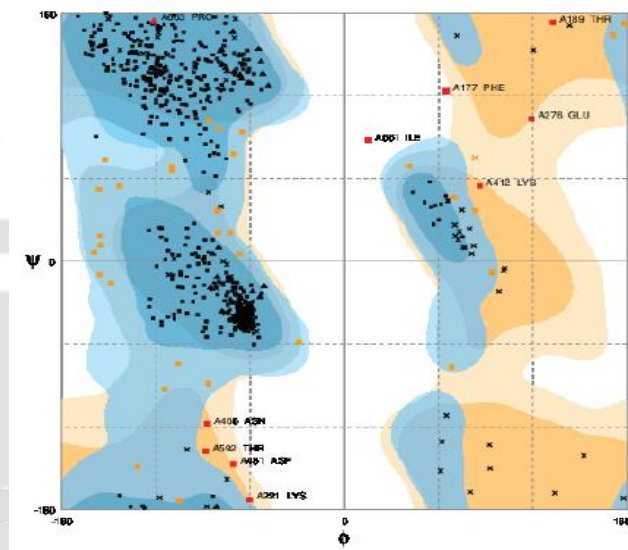


Fig. 3

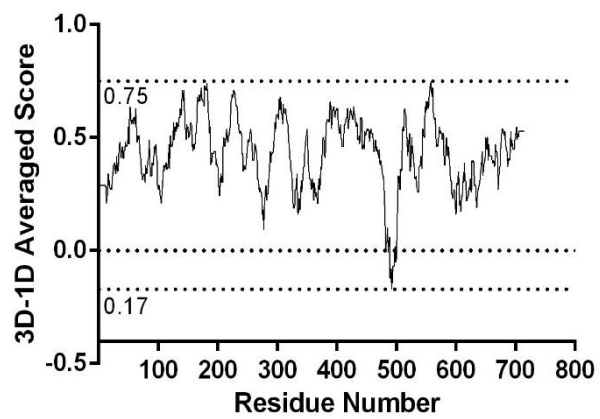
A



B



C



D

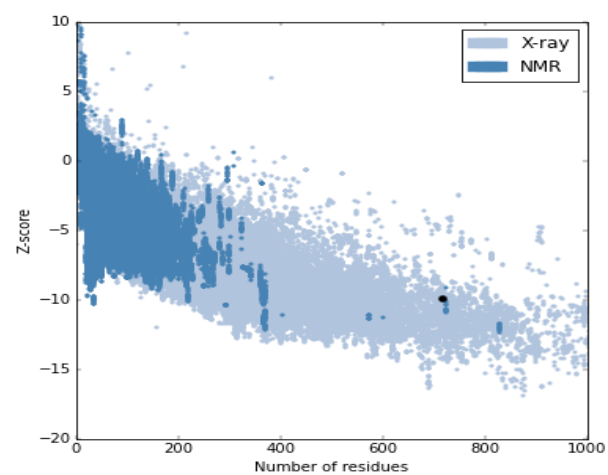


Fig. 4

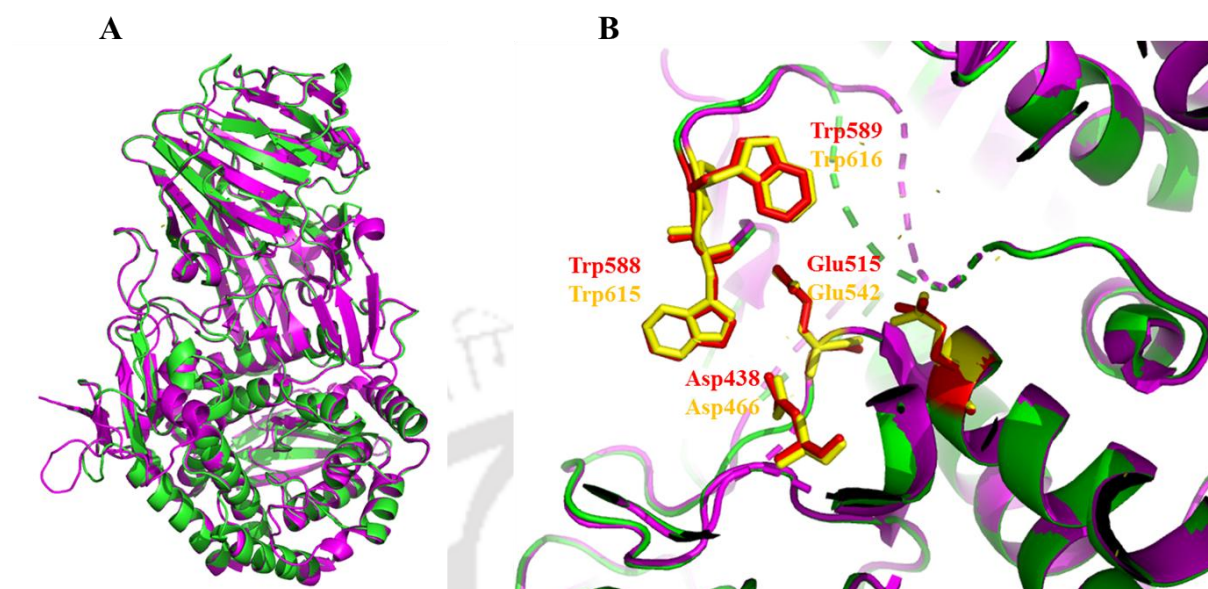


Fig. 5

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1	CtLam81A		MGSSHHHHHHSS...	GLVPRGSHMASQYYREG.																																																								
2	BhGh81		XGSSHHHHHHSS...	GLVPRGSHXASHAVSVG.																																																								
3	RmLam81			MRFQV																																																								
4	TfLam81A	MS.....	HASRRRWRRR....	TTSAAATAALLCG...ALLTFPSAPAAAQVRLG.																																																								
5	AfEngA																																																											
6	ScEng2	MCYSRQAIPPPVFNRP	GGTTNRGPPPLP	PRANVQPPVCSSSENSKPRENRVAGESLRTPS																																																								
7	GmGbp																																																											
		30	40	50																																																								
1	CtLam81A		TGSYTVVLP.....	GAKPQ.....A.....EIY.....K																																																								
2	BhGh81		KGSYATEFPE.....	IDFGGI.....NDPGFRDQQGEPPATIYR																																																								
3	RmLam81	...IVAAATITMIT	SYIPGVASQSTSDGDD	LFVVSNFDPKSIFPEIKHPFEP...MYA																																																								
4	TfLam81A		SGSYTTVLPP.....	GASGPS.....D.....HTG.APVAPKV																																																								
5	AfEngA		MGI.VSFLKPVV.L..	ANGQDVFPVS.....TGPNSQDDWLE...ERS																																																								
6	ScEng2	SSNPLADSQVNSD	NIFQSPVL.SNLKAPPS	VFNKV.....QHPVPK....PNI																																																								
7	GmGbp		MVNIQNTSY.....	IFPQTQSTVLPDPSKFFS																																																								
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2	BhGh81	S	D	R	V	T	G	P	X	Q	T	N	S	W	G	S	L	A	V	D	R		F	S	X	N	Q	Y	P	H	P	F	S	V	R	H	R	A	E	G	L	H	...	V	F	Y	D	A	P	H	N	X	V	V						
3	RmLam81	N	T	E	N	G	K	I	V	P	T	N	S	W	I	S	N	L	F	Y	P	S	A	...	D	N	L	A	P	I	T	P	D	P	Y	T	L	R	L	L	D	G	Y	G	G	N	...	P	G	L	T	I	R	Q	P	S	A	K		
4	TfLam81A	T	A	D	F	T	Q	P	V	V	T	N	D	W	S	S	L	I	F	Q	R	Y	P	G	N	P	Y	G	E	N	L	Y	A	H	P	L	S	F	K	A	Q	A	H	G	L	E	...	V	G	Y	P	D	T	P	E	L	V	A		
5	AfEngA	S	V	N	T	D	T	P	V	E	T	N	K	F	Y	C	G	L	F	L	G	...	T	Q	T	N	N	T	F	T	H	P	Y	S	V	A	W	V	K	G	G	T	S	Q	S	Y	G	M	A	I	S	H	V	E	S	N				
6	ScEng2	D	D	Q	S	V	D	P	L	E	T	N	K	F	Y	T	N	M	L	D	...	D	N	T	Q	P	I	W	T	H	P	Y	S	I	W	F	S	R	D	P	...	E	L	F	G	L	A	N	H	T	L	A	S							
7	GmGbp	S	N	L	L	S	S	P	L	P	T	N	S	E	F	F	Q	N	F	V	L	K	N	...	G	D	Q	Q	E	Y	I	H	P	Y	L	I	K	S	S	N	S	L	...	S	L	S	Y	P	S	R	Q	A	S	S	A					
		100	110	120	130																																																							
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2	BhGh81	H	...	E	N	R	E	A	...	G	T	W	...	H	I	H	G	A	I	G	T	D	F	T	...	I	K	H	S	G	...	T	A	N	F	E	Q	A	V	V	D	D	Y	N	D	W	Y	V	R	G	L	L	...							
3	RmLam81	V	L	G	S	Y	P	P	T	N	D	V	P	Y	T	D	A	G	Y	M	I	N	S	V	V	D	L	R	...	L	T	S	S	E	W	S	D	V	P	D	R	...	Q	V	T	D	W	D	H	L	S	A	N	L	R	L	S	T		
4	TfLam81A		D	G	L	...	K	Y	Q	Y	T	H	S	P	D	F	V	...	L	G	I	H	G	...	L	N	A	P	A	K	V	A	G	S	D	W	T	V	T	A	D	L	...																	
5	AfEngA	I	V	A	H	G	P	V	N	T	A	I	P	G	S	P	I	S	Y	V	N	P	I	G	H	S	V	L	...	L	S	A	S	E	...	L	G	P	S	T	V	L	T	T	E	N	P	L	P	F	S	A	N	A	V	L	R	P		
6	ScEng2	Q	...	R	V	F	D	T	T	T	N	P	P	R	F	Y	F	N	P	T	N	I	K	S	F	V	...	F	K	A	R	E	...	F	V	S	S	N	D	I	K	L	E	F	R	D	M	K	H	M	S	M	C	L	M					
7	GmGbp	V	I	...	F	Q	...	V	F	N	P	D	...	L	T	...	I	S	A	P	Q	G	P	K	Q	G	P	P	G	K	H	L	I	S	S	Y	S	D	L	S	V	...	T	L	...															
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1	CtLam81A	.	A	S	G	S	I	K	A	T	Y	G	V	G	S	P	Y	A	F	V	F	T	G	G	N	P	R	I	D	F	S	G	T	P	T	V	F	Y	G	D	S	G	...	S	Q	C	L	G	V	T	...	I	N	G	V	N				
2	BhGh81	.	E	N	G	A	H	Q	X	A	I	T	Y	G	V	G	S	P	Y	I	F	V	E	Y	E	D	G	S	A	V	L	D	F	D	I	A	P	D	V	W	E	X	N	...	G	H	V	I	G	F	S	T	H	D	H	K	H			
3	RmLam81	P	Q	D	S	N	S	I	D	F	P	I	V	R	G	M	A	Y	I	T	A	N	Y	N	N	L	T	P	Q	F	L	S	Q	H	A	I	S	V	E	A	D	E	K	K	S	D	D	N	T	S	T	F	S	...	G	R	K	F	K	
4	TfLam81A	.	S	D	G	T	R	Q	L	R	T	I	G	Q	L	P	F	V	Y	A	D	V	S	G	G	P	I	R	V	E	F	T	A	P	P	T	V	W	R	R	S	...	G	N	A	V	G	V	T	...	V	N	G	H						
5	AfEngA	S	A	S	S	S	Q	S	I	T	I	P	V	V	Q	G	M	G	F	V	T	G	I	Y	S	N	L	Q	P	K	I	Q	S	G	V	F	F	T	K	M	V	T	A	G	S	...	P	R	...	T	G	I	F	K						
6	ScEng2	S	L	S	S	S	Q	F	I	E	F	P	L	V	Q	G	M	G	F	V	T	A	I	Y	H	D	L	G	F	E	L	R	S	A	V	G	F	R	S	L	E	R	I	...	S	...	V	N	...	E	R	Y	G	K						
7	GmGbp	.	D	F	P	S	S	N	L	S	F	F	L	V	R	G	S	P	Y	L	T	V	S	V	T	Q	P	T	P	L	...	S	I	T	T	H	S	I	L	S	F	S	...	S	N	...	D	S	N	T	K									
		200	210	220	230																																																							
1	CtLam81A	Y	G	L	F	A	P	S	G	S	K	W	Q	G	I	G	T	G	T	I		T	C	I	L	P	A	G	K	N	Y	F	S	I	A	V	L	P	D	N	T	V	S	T	L	T														
2	BhGh81	Y	A	A	F	A	P	P	G	Q	N	W	S	G	I	G	S	K	T	L		T	N	N	A	D	Y	I	A	I	A	K	L	P	E	K	D	G	N	X	L	A																		
3	RmLam81	I	T	M	N	D	D	P	T	S	T	F	I	I	Y	S	L	G	D	K	...	P	L	E	L	R	K	Q	D	N	S	N	L	V	A	S	K	P	Y	T	G	V	I	R	V	A	K	L	P	A	P	...	E	F	E	T				
4	TfLam81A	Y	A	L	F	A	P	S	G	T	T	W	S	E	S	D	T	V	F		T	A	D	V	G	G	S	G	Y	A	S	V	A	L	L	P	S	P	...	D	D	F	D																	
5	AfEngA	Y	S	L	S	L	E	D	G	T	S	W	L	L	Y	A	T	P	D	D	G	S	D	P	Q	L	...	Q	L	A	S	N	S	E	I	I	G	P	A	G	W	S	G	T	I	Q	V	A	K	N	P	A	G	A	...	S	G	E	K	
6	ScEng2	Y	N	I	Q	L	E	N	N	R	N	W	I	L	Y	L	T	S	P	D	Y	S	F	P	Q	D	F	Q	I	S	L	L	D	S	N	T	I	I	S	S	H	K	I	N	G	L	I	C	Q	L	S	...	A	...	D	S	V	P		
7	GmGbp	Y	T	F	Q	E	N	N	G	Q	T	W	L	L	Y	A	T	S	P	I		K	L	N	H	T	L	S	E	I	T	S	N	A	F	S	G	I	I	R	I	A	L	L	P	D	S	D	S	K	H	E	A							
		240	250	260	270	280	290																																																					
1	CtLam81A	Y	Y	K	D	Y	A	Y	C	F	V	T	D	T	K	V	E	W	S	...	Y	N	E	T	E	S	T	L	T	T	F	T	A	E	V	S	V	K	...	E	G	T	N	K	G	...	T	I	L	A	L	Y	P	H	Q	W	R	N	N	P
2	BhGh81	K	F	E	Q	Y	A	Y	S	V	V	R	D	A	V	A	D	W	T	...	Y	D	E	A	T	G	T	V	T	T	F	E	V	T	T	E	A	K	V	Q	G	A	P	D	G	...	T	I	F	A	L	Y	P	H	Q	Y	R	H	L	A
3	RmLam81	L	L	D	A	S	R	A	V	W	P	T	G	G	D	I	S	A	R	S	D	D	N	N	G	A	S	Y	T	I	K	W	K	T	...	N	...	S	N	E	A	P	L	T	Y	A	A	H	L	T	S	I	D							
4	TfLam81A	R	Y	A	P	Y	A	Y	S	F	V	T	S	T	T	L	T	Y	D	...	Y	D	P	A	S	A	T	L	T	S	T	Y	R	V	T	T	E	A	R	...	E	G	T	A	Q	G	...	T	L	L	A	L	Y	P	H	Q	W	K	E	T
5	AfEngA	F	Y	D	N	S	S	G	V	Y	A	V	E	G	A	V	M	G	S	...	V	S	E	S	T	G	T	Y	S	L	M	W	A	K	...	A	G	K	D	A	Q	N	T	P	L	M	F	A	L	P	H	M	E	S	F	D				
6	ScEng2	S	I	D	M	A	A	G	C	Y	P	V	Y	C	D	L	S	G	Q	T	V	D	E	H	F	T	N	Y	R	F	N	Y	T	V	...	A	G	Y	...	S	Q	S	G	T	T	L	M	Y	A	L	P	H	K	A	A	F	T			
7	GmGbp	V	L	D	K	Y	S	S	C	Y	P	V	S	G	K	A	V	F	R	...	E	P	F	C	V	E	Y	N	W	E	K	...	K	...	D	S	G	D	L	L	L	A	H	L	P	L	H	V	Q	L	L	R								
		300	310	320	330	340																																																						
1	CtLam81A	...	H	I	L	P	L	P	Y	T	S	T	L	R	G	I	M	K	T	...	I	Q	G	T	S	F	K	T	V	Y	R	H	...	G	I	L	P	...	N	L	P	D	K	G	T	Y	D	R	E	A	L									
2	BhGh81	S	S	S	...	E	N	Q	L	L	Q	N	Y	Q	Y	E	I	I	R	G	T	...	X	I	G	L	E	G	K	R	F	T	T	E	L	T	Y	P	...	G	V	L	P	...	S	L	P	D	L	G	D	Y	D	R	E	R	L			
3	RmLam81	D	S	N	V	K	...	R	T	D	M	...	T	L	Q	S	A	T	K	G	P	...	M	T	A	L	V	G	N	E	W	T	L	R	E	T	E	L	S	P	...	E	W	L	P	...	L	Q	A	A	P	...	N	P	T	T	I			
4	TfLam81A	...	T	A	L	T	...	D	L	S	Y	A	S	P	R	C	P	M	R	V																																								

Fig. 6

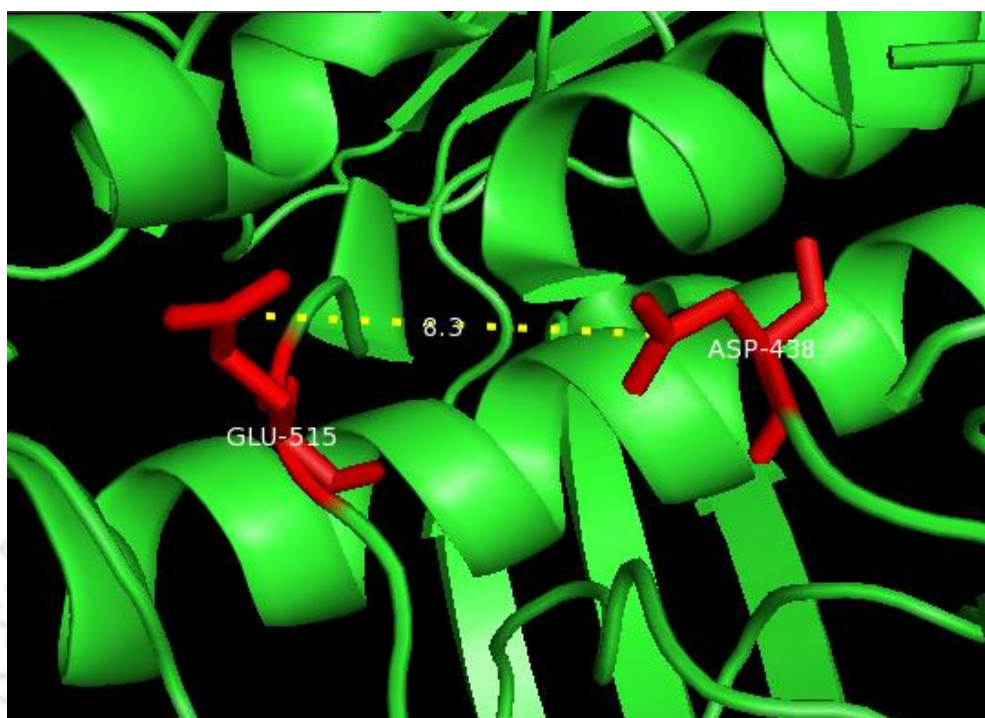


Fig. 7

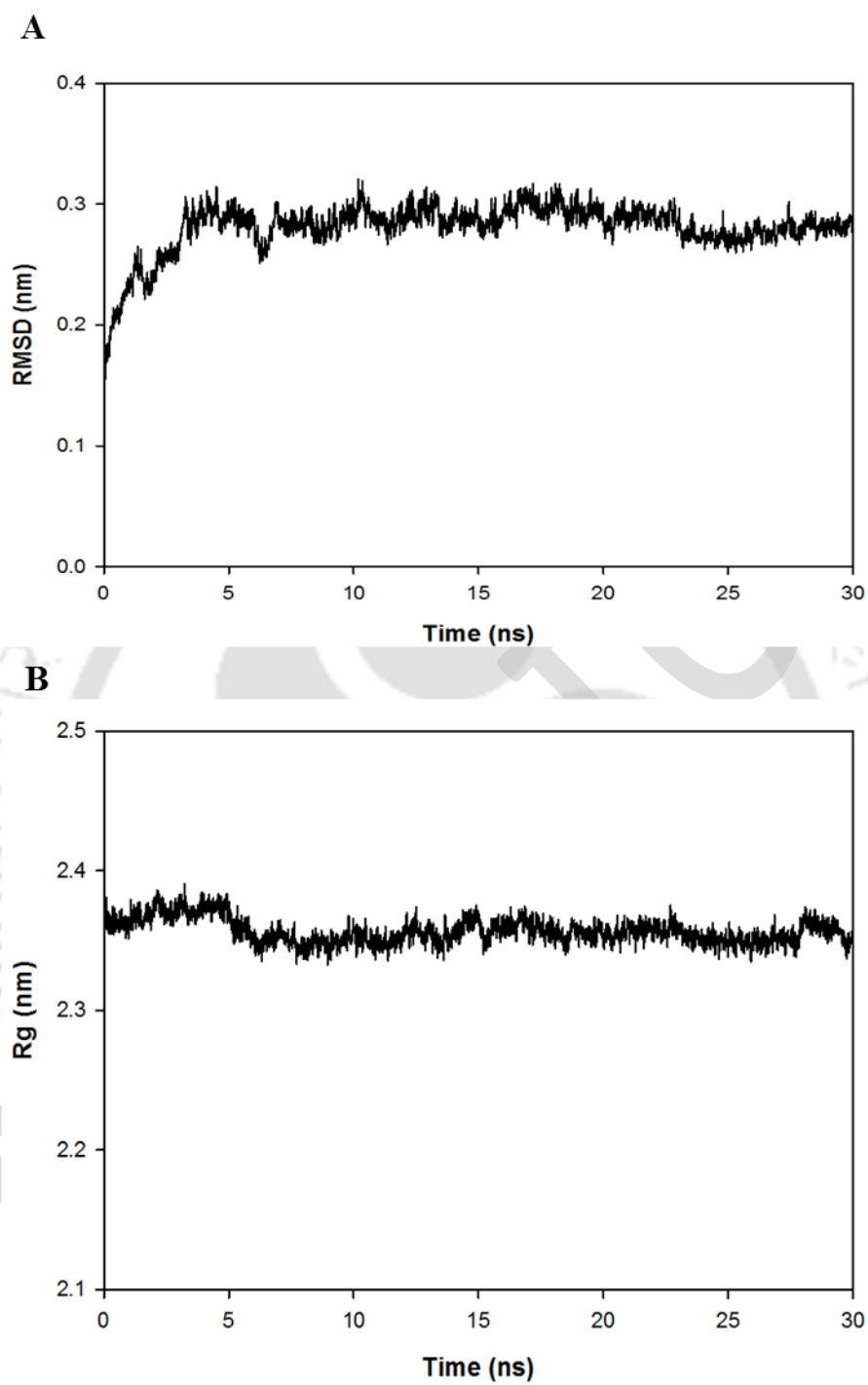
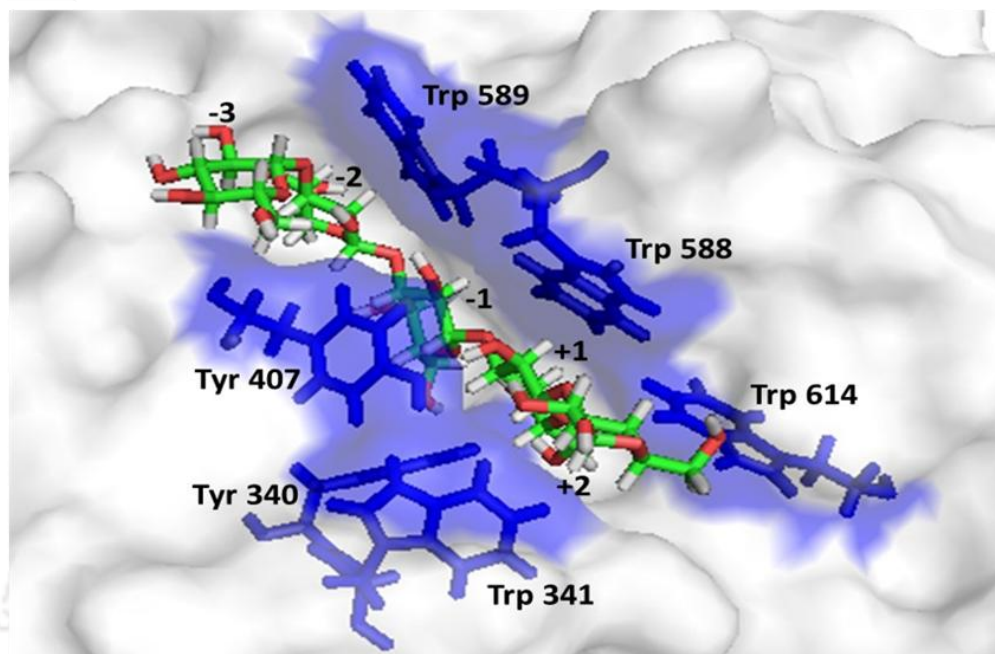


Fig. 8

A



B

