Computational Biology and Chemistry 82 (2019) 65–73

Contents lists available at ScienceDirect

Computational Biology and Chemistry

journal homepage: www.elsevier.com/locate/cbac

Research Article

Structural insights of a cellobiose dehydrogenase enzyme from the basidiomycetes fungus *Termitomyces clypeatus*

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ARTICLE INFO

Keywords:
Cellobiose dehydrogenase
Termitomyces clypeatus
Carbohydrate binding module
Cellulose binding residue
Homology model
Phylogenetic relationship

ABSTRACT

Filamentous fungi secrete various oxidative enzymes to degrade the glycosidic bonds of polysaccharides. Cellobiose dehydrogenase (CDH) (E.C.1.1.99.18) is one of the important lignocellulose degrading enzymes produced by various filamentous fungi. It contains two stereo specific ligand binding domains, cytochrome and dehydrogenase - one for heme and the other for flavin adenine dinucleotide (FAD) respectively. The enzyme is of commercial importance for its use in amperometric biosensor, biofuel production, lactose determination in food, bioremediation etc. *Termitomyces clypeatus*, an edible fungus belonging to the basidiomycetes group, is a good producer of CDH. In this paper we have analyzed the structural properties of this enzyme from *T. clypeatus* and identified a distinct carbohydrate binding module (CBM) which is not present in most fungi belonging to the basidiomycetes group. In addition, the dehydrogenase domain of *T. clypeatus* CDH exhibited the absence of cellulose binding residues which is in contrast to the dehydrogenase domains of CDH of other basidiomycetes. Sequence analysis of cytochrome domain showed that the important residues of this domain were conserved like in other fungal CDHs. Phylogenetic tree, constructed using basidiomycetes and ascomycetes CDH sequences, has shown that very surprisingly the CDH from *T. clypeatus,* which is classified as a basidiomycetes fungus, is clustered with the ascomycetes group. A homology model of this protein has been constructed using the CDH enzyme of ascomycetes fungus *Myricoccum thermophilum* as a template since it has been found to be the best match sequence with *T. clypeatus* CDH. We also have modelled the protein with its substrate, cellobiose, which has helped us to identify the substrate interacting residues (L354, P606, T629, R631, Y649, N732, H733 and N781) localized within its dehydrogenase domain. Our computational investigation revealed for the first time the presence of all three domains - cytochrome, dehydrogenase and CBM - in the CDH of *T. clypeatus,* a basidiomycetes fungus. In addition to discovering the unique structural attributes of this enzyme from *T. clypeatus*, our study also discusses the possible phylogenetic status of this fungus.

1. Introduction

Cellobiose dehydrogenase (CDH) (E.C.1.1.99.18) is an extracellular enzyme secreted by various wood degrading fungi. It was first discovered in basidiomycetes fungi, *Trametes versicolor* (Westermark and Eriksson, 1974) and *Phanerochaete chrysosporium* (Westermark and Eriksson, 1975). CDH belongs to glucose-methanol-choline (GMC) family of enzymes and is a monomeric protein (Zamocky et al., 2006). The enzyme is of commercial importance for its use in biosensors and biofuel cells since it can transfer electrons from the reduced isoalloxazine ring of FAD to electrochemical cell via heme *b* (Zamocky et al., 2006; Henriksson et al., 2000). It also has the potential for use in bioremediation by demethoxylations and hydroxylations of non phenolic and poisonous aromatic waste products (Zamocky et al., 2006; Henriksson et al., 2000). It is also used in bleaching of pulp (Zamocky et al., 2006), production of lactobionic acids (Henriksson et al., 2000) etc. The enzyme consists of two domains, cytochrome (CYT) and dehydrogenase (DH), each containing a prosthetic group, heme type *b* and flavin adenine dinucleotide (FAD) respectively (Hallberg et al., 2000) and is connected by a flexible linker (Zamocky et al., 2006). Its catalytic sequence consists of both oxidation and reduction (Henriksson et al., 2000). During the oxidative half of reaction, the C1 position of saccharide is oxidized to δ-lactone and further hydrolyzed to corresponding acid in aqueous medium (Hallberg et al., 2002; Tan et al., 2015). Electrons produced by carbohydrate oxidation, are transferred by $FADH_2$ of DH domain to heme in CYT domain by inter domain

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https://doi.org/10.1016/j.compbiolchem.2019.05.013
Received 10 August 2018; Received in revised form 18 February 2019; Accepted 28 May 2019
Available online 30 May 2019
1476-9271/

electron transfer (Zamocky et al., 2006). CYT domain acts as mediator and electrons are re-transferred to an electron acceptor (Zamocky et al., 2006). CDHs found in fungi belonging to a major group, known as ascomycetes, possess an additional third domain, a carbohydrate binding module (CBM), which is absent in most of the CDH of fungi belonging to the basidiomycetes group (Henriksson et al., 2000; Harreither et al., 2011). However, a CDH protein from the basidiomycete fungus, *P. chrysosporium* has been known to own a CBM connected to cytochrome domain and the DH domain is not present (Yoshida et al., 2005) in it. The CBM, in general, plays an important role by bringing the enzyme to close association with the substrate for increasing its rate of catalysis (Lakhundi et al., 2015). In spite of absence of CBM, most basidiomycetes CDHs binds cellulose with specific cellulose binding residues located within dehydrogenase domain which is not observed in any of the ascomycetes CDHs (Harreither et al., 2011). Phylogenetic analysis of CDH showed its separation into two classes - Class I and Class II produced by basidiomycetes and ascomycetes fungi respectively with the Class II type sometimes containing the CBM (Harreither et al., 2011). If the ascomycetes CDH contain the CBM, it is classified as Class IIA type while the absence of CBM classifies it as a Class IIB enzyme (Tan et al., 2015). A third class of CDHs has been found in ascomycetes but no CDH from class III has been characterized (Harreither et al., 2011). CDH is also known to be an important player in cellulose depolymerisation by oxidative process, together with copper-dependent lytic polysaccharide monooxygenases (LPMO) (Kracher and Ludwig, 2016). Electrons that are generated from cellobiose oxidation are shuttled via CDH domains to LPMO. The reduced LPMO activates oxygen at its copper centre followed by insertion of the oxygen at the C1 or C4 atom adjacent to a glycosidic bond of cellulose molecule causing cellulosic strand break (Kracher and Ludwig, 2016).

Termitomyces clypeatus is an edible filamentous fungus belonging to the basidiomycetes group (Heim, 1951) and produces wide variety of polysaccharide hydrolysing enzymes like cellulase (Chatterjee et al., 2010), sucrase (Chowdhury et al., 2009), cellobiase (Sengupta et al., 1991), endo-1,4-β-D-xylanase (Mukherjee and Sengupta, 1985), 1,4-β-D-xylosidase (Bhattacharyya et al., 1997), α-L-arabinofuranosidase (Sinha and Sengupta, 1995), α-amylase (Ghosh and Sengupta, 1987), amyloglucosidase (Ghosh et al., 1995), including CDH (Saha et al., 2008). It produces highest amount of CDH in cellulose medium among all the fungal species (Saha et al., 2008). There is no study describing the structural and molecular features of this industrially important CDH enzyme from this source. Purpose of this study has been to fill some of these lacunae through computational works. This paper describes, for the first time, novel structural features of a CDH enzyme (*Tc*CDH) found in the fungus *T. clypeatus*. In addition our simulation studies may guide structural biologists and biochemists to investigate the geometrical and electronic positions of each domain and their specific interaction with substrate or heme or FAD in this CDH enzyme.

2. Methods and materials

2.1. Analysis of TcCDH sequence and phylogenetic tree

Nucleotide BLAST (Altschul et al., 1990) search was performed with the published CDH sequence of three basidiomycetes fungi - *Cerrena unicolor* (accession no: KC862284), *Grifola frondosa* (accession number: AB083245) and *Trametes versicolor* (accession no: AF029668) using Transcriptome shotgun assembly (TSA) database. One of the obtained sequences (accession no. GAFV01008428.1 (Mukherjee et al., 2013)) was translated (Gasteiger et al., 2003) and examined using Basic local alignment search tool for protein (BLASTP) (Altschul et al., 1990) to look for different CDH domains. The translated sequence of accession number: GAFV01008428.1 of *T. clypeatus* (*Tc*CDH) was further analyzed using Pfam (a database of protein families) (Finn et al., 2016) and multiple sequence alignment to study the domain organization and conservation of residues respectively.

Phylogenetic analysis was performed using 87 CDH protein sequences (32 from basidiomycetes, 55 from ascomycetes, Table S1) which were collected from NCBI database. The phylogenetic tree was constructed using the multiple sequence alignment program MUSCLE (Edgar, 2004) and then maximum likelihood (Felsenstein, 1981) method included in the computer program MEGA7.0.21 (Molecular Evolutionary Genetics Analysis) (Kumar et al., 2016).

2.2. Homology modelling of TcCDH

Protein BLAST (Altschul et al., 1990) was done with *Tc*CDH amino acid sequence (Mukherjee et al., 2013) to identify homologues of the enzyme along with their known conserved domains. The CDH sequences from ascomycetes and basidiomycetes groups of fungi that were homologous to the *Tc*CDH sequence were considered and aligned to identify the conserved domains from these organisms.

Homology modelling of *Tc*CDH was performed based on the crystal structure of homologous CDH from *Myriococcum thermophilum* (Tan et al., 2015) as template (PDB id 4QI6), using the modelling tool, MODELLER9.17 (Sali and Blundell, 1993). The ligands, heme and flavin adenine dinucleotide (FAD), were modelled and placed in specific geometrical orientation at cytochrome and dehydrogenase domains respectively using the same modelling program.

2.3. Modelling the substrate at active site pocket

The *Tc*CDH substrate, cellobiose was modelled in two steps; a) transforming the coordinates of cellobiose analog (ABL) from *Tc*CDH homologue (PDB id: 1NAA (Hallberg et al., 2003)) by structural superimposition and b) replacing the cellobiose analog with cellobiose (CBI) by structural superimposition of ligands. Structural alignment of 1NAA and *Tc*CDH was performed using CLICK (Nguyen et al., 2011) with CA as representative atoms. Transformed coordinates of the cellobiose analog, ABL (5-amino-5-deoxy-cellobiono-1,5-lactam), was added to *Tc*CDH. CBI coordinates were obtained from an unrelated protein structure (PDB id: 5CVY (Brunecky et al., 2017) and superimposed to ABL using CLICK (Nguyen et al., 2011) with all carbon and oxygen atoms as representatives. Transformed CBI coordinates were used to replace ABL.

The modelled protein (along with ligands) was validated next by model assessment tools such as, MODELLER's Discrete Optimized Potential Energy (DOPE) method (Shen and Sali, 2006), ProSA (Sippl, 1993) VERIFY3D (Bowie et al., 1991; Lüthy et al., 1992) and RAMPAGE (Lovell et al., 2002). DOPE is an atomic distance dependent statistical potential calculated from a sample of native protein structures (Shen and Sali, 2006). ProSA is a statistical potential that assesses the modelled protein using energy profile and compares it with the potential mean force from a set of known protein structures (Sippl, 1993). Verify3D program from Protein Structure Validation Software suit (PSVS) 1.5 (Bhattacharya et al., 2007) was used for model validation. This program analyzes the reliability of the modelled protein and characterizes the position of each amino acid with an environmental score by constructing a 3D profile (Bowie et al., 1991; Lüthy et al., 1992). Overall, stereochemistry of the model was analysed by RAMPAGE (Lovell et al., 2002). Further, CLICK (Nguyen et al., 2011) was used to obtain root mean square deviation (RMSD) for CA atoms by superimposing modelled *Tc*CDH and other PDB structures. The PDB structures used for validation study of modelled protein in terms of Root Mean Square Deviation (RMSD) and structure overlap percentage are listed in Table S2.

2.4. Molecular dynamics simulation study of TcCDH enzyme

To investigate the dynamic behavior of cytochrome and dehydrogenase domains and their recognition with substrate or heme or FAD, the classical molecular dynamic simulation study was done. The

initial atomic coordinates of this structure were taken and the missing hydrogen atoms were added to the structure using AutoPSF module of Visual Molecular Dynamics (VMD v.1.9.2.) program (Humphrey et al., 1996). In addition the substrate, heme and FAD ligands were parameterized using Swissparam program (Zoete et al., 2011). Then, the entire enzyme structure was embedded in a water box (cubic) of TIP3P water molecules of 10 Å from any edge of the protein surface. The Na^+ ions were included to neutralize the overall charge of solvated system. The molecular dynamics of solvated structure was done using Nanoscale Molecular Dynamics (NAMD v.2.9) program (Kale et al., 1999; Leroux et al., 2007) and CHARMM27 force field (Brooks et al., 1983). Water molecules and ions of the system were initially energy minimized by 500 cycles of steepest descent, followed by 1000 cycles of conjugate-gradient method. The whole system was energy minimized by 1000 cycles of steepest descent and 1000 cycles of conjugate gradient method. The system was then gradually heated from 273 K to 310 K over 100 ps under NvT conditions. The system was finally equilibrated in NpT conditions for 300 ps with an integration time step of 2 fs. The final production run was carried out for all atom simulation in the NpT ensemble for a time period of 5 ns using a time step of 1 fs. To mimic physiological conditions, the temperature was kept at 310 K by using Langevin dynamics with a damping coefficient of 5 ps^{-1}. The pressure was maintained at 1 atom using the Langevin piston Nosé–Hoover method (Gullingsrud et al., 2001), with a piston period of 100 fs and a decay time of 50 fs. Periodic boundary conditions and a cut-off distance of 12 Å, switch distance of 10 Å, and pairlist distance of 14 Å for vander Waals interactions were applied. The particle–mesh Ewald method was used to compute the long-range electrostatic interactions by specifying proper PME grid size. The SHAKE algorithm was used to constrain all bond lengths involving hydrogen atoms and the value of step per cycle (time steps per cycle) was assigned as 10. The atomic coordinates of MD structures were recorded at every 2 ps for analysis.

3. Results

3.1. Study of TcCDH domains and phylogenetic analysis

Preliminary BLAST (Altschul et al., 1990) analyses using CDH cDNA sequences of three basidiomycetes fungi - *Cerrena unicolor* (accession no: KC862284), *Grifola frondosa* (accession number: AB083245) and *Trametes versicolor* (accession no: AF029668) as query sequences, have detected good similarities with several sequences (data not shown) found in the transcriptome database of *T. clypeatus* (Mukherjee et al., 2013) published in NCBI. But one particular gene sequence (accession no. GAFV01008428.1) was consistently picked by the BLAST tool from the transcriptome database of *T. clypeatus* for all the three query sequences (respective E values are 1e-04, 0.75 and 1e-04). Since we were interested in finding the CDH gene of *T. clypeatus* and it represented a full length sequence (not a partial sequence), we focussed our attention on GAFV01008428.1 for further computational studies. This gene sequence GAFV01008428.1 of unknown function consists of 2523 nucleotides which is similar to the other fungal CDH sequences. Protein BLAST (Altschul et al., 1990) study using translated sequence of accession number: GAFV01008428.1 (Mukherjee et al., 2013) of *T. clypeatus* (named as *Tc*CDH) reveals the presence of a carbohydrate binding module (CBM) in its structure (data not shown), in addition to the cytochrome and dehydrogenase domains; the latter two domains are known to be present customarily in most CDH enzymes. Our Pfam analysis reveals that residue numbers 808–836 (amino acid number with signal sequence not removed from N-terminal end of pre-protein) consists of CBM domain of *Tc*CDH (Fig. 1(a), lower panel). It is to be clearly noted here that the CBM (Carbohydrate Binding Module) is known to be present in enzymes other than CDH also, that are involved in various types of carbohydrate degradation. Alignment of 758 CBM motifs from various carbohydrate active enzymes archived in Pfam database (Table S3(a)) with *Tc*CDH CBM has been done using ClustalX2.1 (Larkin et al., 2007) program and subsequently analysed in Jalview2.10.3b1 for multiple sequence alignment editing and visualization (Waterhouse et al., 2009). The results indicate that the key residues responsible for cellulose binding i.e. three aromatic amino acids (Y809, Y835 and Y836, brown colour in Fig. 1(a) upper panel) and three cysteine residues (C812, C823, C829, blue colour in Fig. 1(a) upper panel) are in the expected conserved positions (partial alignment data shown in Fig. 1(a), upper panel) in the CBM of *Tc*CDH. Additionally, the Pfam study also indicates that the CBM of *Tc*CDH belongs to CBM 1 family by virtue of its possessing four cysteine residues (C812, C823, C829, C839 – blue colour in Fig. 1(a) upper panel) which are involved in disulphide bond formation. To confirm the position of CBM within *Tc*CDH, BLASTP was done again using CDH sequence of *T. clypeatus* with other CBM motifs (data not shown). These results showed that the C-terminal end of the *Tc*CDH sequence contain the CBM, like other CBM containing CDHs.

BLASTP analysis reveals that the dehydrogenase domain belongs to Glucose-Methanol-Choline (GMC) family of enzymes similar to other fungal CDHs (Zamocky et al., 2006). Pfam analysis shows that residue number 260–563 consists of N-terminal part of this domain and its C-terminal part consists of residue number 654–793 (amino acid number with signal sequence not removed from N-terminal end of pre-protein) (Fig. 1(a), lower panel). Most of the class I CDHs, which do not possesses a CBM, bind to cellulose by specific cellulose-binding residues present within dehydrogenase domain (Harreither et al., 2011). This cellulose binding residues are attributed to nine aromatic amino acids in class I CDHs which is not present in any of the class II CDHs (Harreither et al., 2011). When compared by aligning dehydrogenase domain sequence of *T. clypeatus* CDH with class I and class II CDHs (13 sequences from class I and 15 sequences from class II, Table S3(b)) using Clustal Omega1.2.4 (Sievers et al., 2011) program, we observe the absence of those aromatic residues in *Tc*CDH like class II CDHs (Fig. 1(b)).

The cytochrome domain of *Tc*CDH consists of residue number 31–215 (Pfam analysis of *Tc*CDH with signal sequence not removed from N-terminal end of pre-protein, Fig. 1(a) lower panel). The cytochrome domain sequence of *Tc*CDH was aligned with other 158 cytochrome CDH motifs documented in Pfam database (Table S3(c)) using ClustalX2.1 (Larkin et al., 2007) program followed by analysis in Jalview2.10.3b1 (Waterhouse et al., 2009) program. From the alignment result (partial alignment shown in Fig. S2) the significant residues of this domain could be identified. Methionine and histidine residues (M92, Fig. S2(a); H194, Fig. S2(b) of *Tc*CDH) are conserved in all cytochrome domain motifs archived in Pfam and are predicted to be the axial ligands of heme iron as discussed for the basidiomycete, *P. chrysosporium* CDH (M65, H163 (Hallberg et al., 2000; Rotsaert et al., 2001)). A pair of conserved cysteine residues is present in the cytochrome domain (C147 and C150 of *Tc*CDH, Fig. S2(b)) that form a disulphide bond and are conserved in other cytochrome CDH motifs as well.

In order to investigate the similarity of CDH of *T. clypeatus* with those of other fungal CDHs, phylogenetic tree was constructed with 32 and 55 amino acid sequences from basidiomycetes and ascomycetes CDHs (Table S1) respectively using MEGA7.0.21 (Kumar et al., 2016). Phylogenetic tree revealed that the enzyme protein is divided into two major groups for ascomycetes and basidiomycetes (Fig. 2). *Tc*CDH is found to get clustured with the ascomycetes CDH.

3.2. Modelling the 3D structure of TcCDH

In spite of its high industrial relevance, three dimensional structure of the CDH enzyme from *T. clypeatus* is not available in the Protein Data Bank. This is because in spite of being the highest producer of CDH in cellulose medium among all the fungi that are reported till date, this fungus has not been studied much (Saha et al., 2008). Our findings of three distinct domains (cytochrome, dehydrogenase and CBM) in the

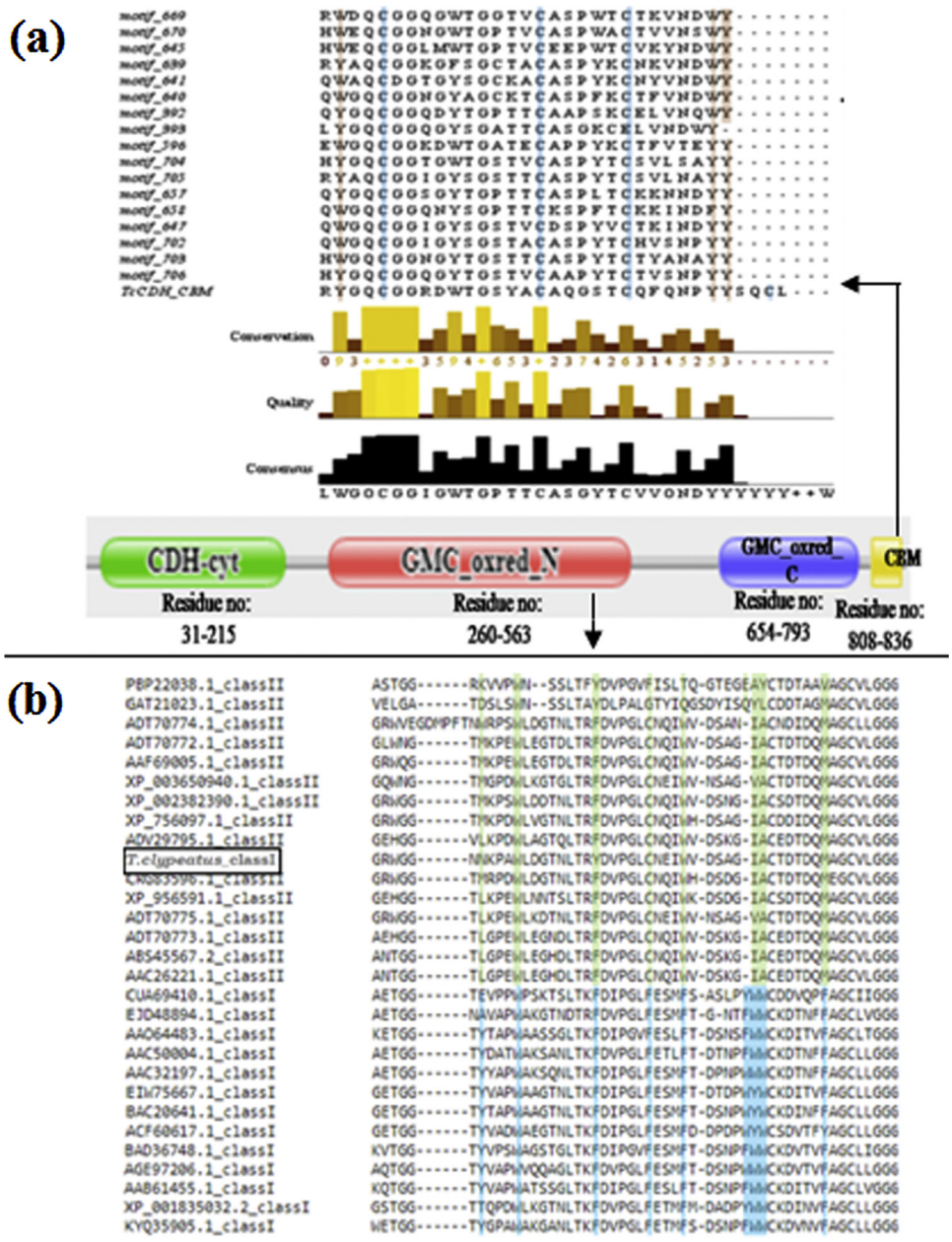

Fig. 1. (a) Domain organization of *Tc*CDH (lower panel) and CBM residues alignment (upper panel). Lower panel shows *Tc*CDH domain organization as exhibited by Pfam. Upper panel shows the Jalview2.10.3b1 image of alignment of the CBM residues of *Tc*CDH (TcCDH_CBM) with some other representative CBM motifs (of the 758 sequences analysed; data no shown) archived in Pfam. Three cysteine (C812, C823, C829 in blue colour) and aromatic amino acid residues (Y809, Y835 and Y836, brown colour) are well conserved. (b) Alignment of cellulose binding sequences of dehydrogenase domain in basidiomycetes (class I) and ascomycetes (class II) CDHs and *Tc*CDH using Clustal Omega1.2.4. Cellulose binding aromatic residues of class I basidiomycetes CDHs are marked in sky blue and the corresponding residues of class II ascomycetes CDHs in olive green. *Tc*CDH (*T. clypeatus*_class I, marked in black box) is devoid of cellulose binding aromatic residues.

CDH of this fungus, like class IIA CDHs, merits further investigation. Homology modelling of CDH from *T. clypeatus* has been carried out to partially fill this lacuna. The CDH from *M. thermophilum*, an ascomycete, has been selected as the template for this homology modelling study because it has been found to be the best hit sequence by the BLASTP program using the *Tc*CDH as query sequence and has 63% sequence identity to the query sequence, 96% query coverage and E-value 0.0. The first 24 residues in the N-terminal part of the protein sequence did not align well to the template (Fig. S1) and were predicted to be part of the signal sequence. Hence, this part of the sequence was not considered for this computational study.

Homology model using MODELLER9.17 produces the 3D structure of the protein (Fig. 3). Analysis and visualization of protein model has been done in UCSF Chimera1.11.2 (Pettersen et al., 2004). It displays the three distinct domains- cytochrome, dehydrogenase and carbohydrate binding module of *Tc*CDH. The cytochrome domain of *Tc*CDH is located in the N-terminus region and folds like a β-sandwich similar to Fab VH domain of antibody (Henriksson et al., 2000). The dehydrogenase domain is larger, and belongs to Glucose-Methanol-Choline (GMC) family of enzymes similar to other fungal CDH (Zamocky et al., 2006). These two domains are connected with linker peptide. The CBM domain is located at the C-terminal end of the protein. Pfam displayed four cysteine residues which are involved in disulfide bond formation and three of them are well conserved among other CBM motifs (Fig. 1(a) upper panel). In addition, three aromatic amino acid residues attributed for cellulose binding are well conserved in the same CBM motifs (Fig. 1(a) upper panel). Modelling with ligands has been done using the same modelling tool. The cytochrome domain binds heme iron at the exterior of the protein. The dehydrogenase domain, like other fungal CDH (Henriksson et al., 2000) binds FAD. The modelled protein is presented in Fig. 3 and a PDB file (TcCDH_Homology model.pdb) for this model has been included in the supplementary section.

Residues that interact with the heme, FAD and cellobiose, through H-bonding (Fig. S3(a), S3(b) and 4(a), respectively) and those that are within 5 Å of the respective ligands are analysed from Chimera (Pettersen et al., 2004) and listed in Table 1. Conservation of residues interacting with these ligands (heme and FAD) using respective crystal structures are presented in Fig. S4(a) and S4(b) respectively. M92 and

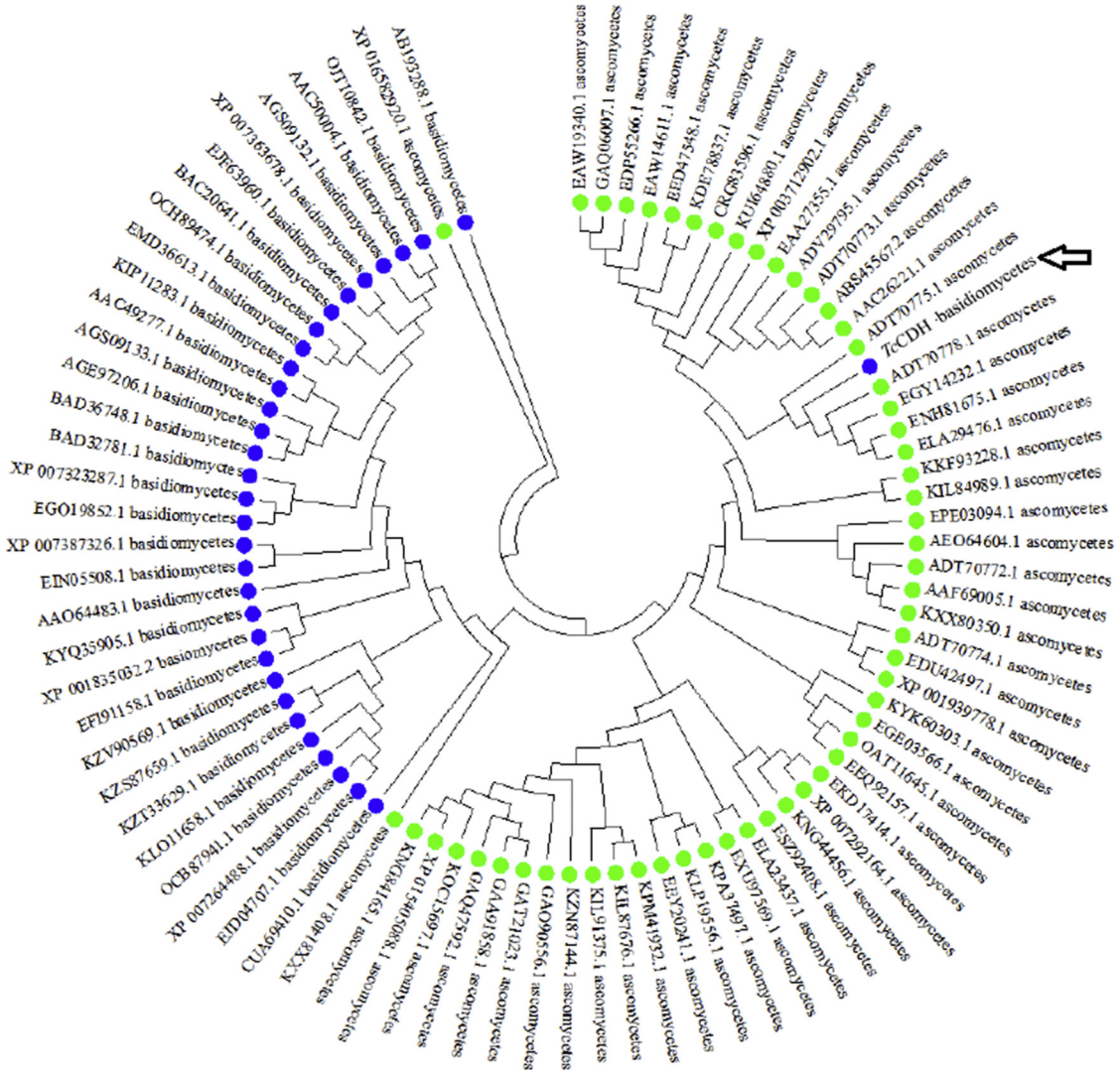

Fig. 2. Phylogenetic tree was created using MEGA7.0.21 program with 55 ascomycetes and 32 basidiomycetes CDH sequences along with *Tc*CDH sequence. Green and blue colour codes indicate ascomycetes and basidiomycetes group members respectively. *Tc*CDH (indicated by arrow) is the only basidiomycete to possess the all the three domains - cytochrome, dehydrogenase, carbohydrate-binding module and its dehydrogenase domain is devoid of the cellulose binding residues like ascomycetes CDHs; hence, it is clustered with ascomycetes group members.

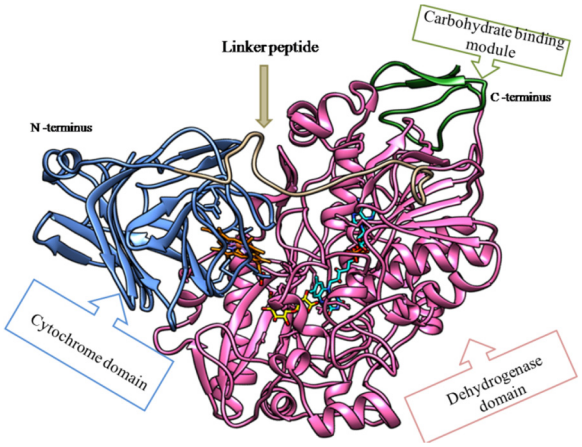

Fig. 3. Model structure of *Tc*CDH. Cytochrome domain in blue colour, dehydrogenase domain in pink, carbohydrate binding module in green, linker in tan, heme in orange, FAD in cyan and cellobiose in yellow colour. The model was visualized and analyzed in Chimera1.11.2.

H194 (pink colour in Fig. S4(a)) of *Tc*CDH cytochrome domain are determined to be the axial ligands of heme iron (Henriksson et al., 2000) on the basis of H-bonding interaction study and are within 5 Å distance of heme. These residues are also conserved among the mentioned crystal structures of heme (1D7B, 4QI3, 4QI7). Interaction study of the *Tc*CDH residues with substrate, cellobiose (CBI) have been done by comparing the modelled structure with three relevant crystal structures (at resolutions 3.2 Å or better) available in the PDB database – these are 1NAA from *Phanerochaete chyrosporium* (basidiomycetes), 4QI6 from *Myriococcum thermophilum* (ascomycetes) and 4QI7 from *Neurospora crassa* (ascomycetes). The substrate interacting residues reside within the dehydrogenase domain like other CDHs. It is found that Y649, N732 and H733 are well conserved in the PDB structures 1NAA, 4QI6 and 4QI7 (pink colour in Fig. 4(b)) that interacts with CBI through H-bonding. Furthermore, L354, P606, T629, R631, Y649, N732, H733 and N781 are well conserved among these same PDB structures (blue and pink colour in Fig. 4(b)) that are within 5 Å from substrate. Therefore, L354, P606, T629, R631, Y649, N732, H733 and N781 are predicted to be the residues which interact with the substrate. Multiple sequence alignment of the protein sequences has been done using Clustal Omega1.2.4 (Sievers et al., 2011).

Different servers of validation mentioned in materials and methods

Table 1
Interaction of *Tc*CDH residues with ligands (heme, FAD and CBI).

Ligand	Interacting residues	
Heme	< 5 Å W83, G85, V86, S87, G90, P91, M92, T93, L97, L98, M99, L112, Y117, D118, Q119, P120, G172, W173, A174, M191, K192, Q193, H194, Q197, G198, I199, W325, S328, M339, S727, R730	H-bonding M92, H194
Flavin adenine dinucleotide (FAD)	V265, G266, A267, G268, A269, G270, G271, I288, E289, K290, G291, I324, W325, C333, M339, A340, G341, C342, V343, L344, G345, G346, G347, T348, I350, N351, A352, G353, L354, T468, S469, V470, S509, A510, G511, T512, G514, L518, N732, H733, W734, D770, A771, G772, N781, P782, S783, A784, I786	G270, C342, V343, G347, L354, V470, A771, S783
Cellobiose (CBI)	N322, W325, A352, L354, A605, P606, T629, R631, E633, Y649, R730, A731, N732, H733, N781	E633, Y649, A731, N732, H733

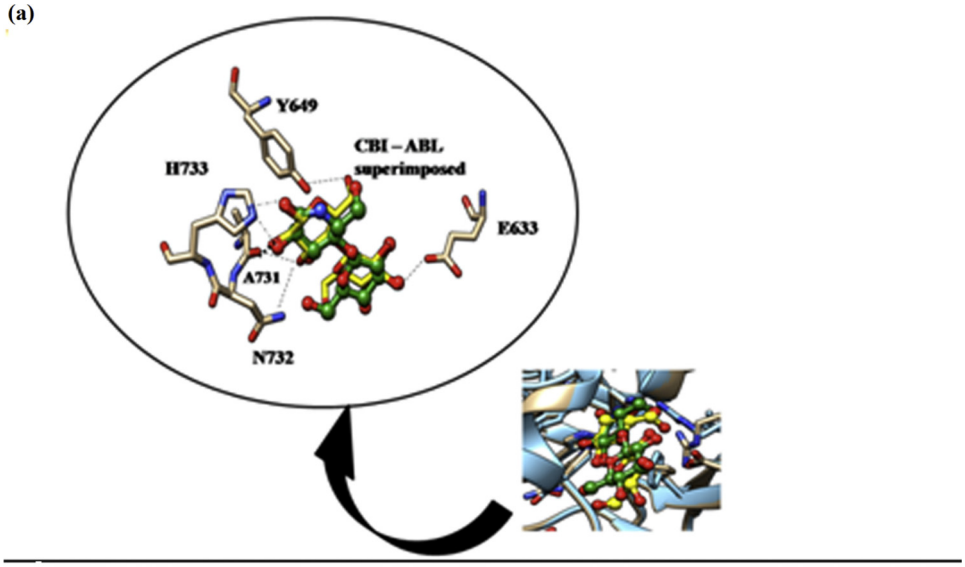

Fig. 4. (a) H-bonding interaction (black dashed lines) of cellobiose in *Tc*CDH. Cellobiose (CBI) superimposed on analog (ABL) interacts with E633, Y649, A731, N732, H733. Colour codes: red - oxygen, blue - nitrogen, green - ABL, yellow - CBI, tan and sky blue - residues. The results were rendered in Chimera1.11.2. (b) Clustal Omega1.2.4 program showing the conservation of amino acid residues which interacts with the cellobiose. L354, P606, T629, R631, Y649, N732, H733 and N781 are within 5 Å of the substrate binding site (blue, pink). Y649, N732 and H733 interact with substrate by H-bonding (pink).

section have been used to check the overall model quality for further application. MODELLER's DOPE calculates the DOPE energy of the model (Shen and Sali, 2006) which is displayed in Fig. S5. Despite a region with a high energy (around residue 200) all other regions show good DOPE energy when compared to the template. ProSAII energy plot was also used to evaluate the overall accuracy of the protein structure. The ProSAII profile confirms that the residues are all in energetically well-optimised environments (negative energy values; Fig. S6), with very few exceptions (positive energy values) (Sippl, 1993). Verify3D program provides a score to each residue for determining their packing quality and majority of residues should have 3D-1D score above 0.2 (Lüthy et al., 1992). The overall score of the model is 0.48 which is in

the acceptable range according to Verify3D rules as shown in Fig. S7. Ramachandran plot using RAMPAGE indicate that 96.2% residues are in the favoured region, 3.2% residues are in allowed regions, 0.6% residues are in the outlier region (Fig. S8) (Lovell et al., 2002). Furthermore, the cytochrome and dehydrogenase domains of *Tc*CDH are superimposed on respective domains of *M. thermophilum* (4QI3 and 4QI4). The RMSD value (CA atoms) between those superimposed complex structures are 0.65 Å and 0.62 Å for cytochrome and dehydrogenase domain respectively (Fig. S9). The value of RMSD less than 1 indicates that the model protein is reasonably similar to the template and of good quality.

3.3. Analysis of MD structure of TcCDH enzyme

In the MD simulated structures, substrate (cellobiose), FAD and heme are observed to retain proper geometrical positions at their respective binding pocket which are similar in the model structure determined earlier. The cellobiose is occupied at substrate binding pocket (residue id: 802) of dehydrogenase domain and FAD is observed at cofactor binding region of the same domain (residue id: 842) whereas the position of heme is found at the cytochrome domain (residue id: 841) (Table S4(a) and S4(b)). During MD simulation, the loop region between cytochrome and dehydrogenase domain has been found to be more flexible and may possibly help the dynamic motion of the two domains (Fig. S10). The three domains of CDH that were found in the model structure have been found to be retained in the simulated structure as well and the MD simulated protein is presented in supplementary section (Fig. S10). A PDB file (TcCDH_MD.pdb) for this structure has been also included in the supplementary section.

4. Discussion

Traditionally, organisms were classified in to different groups based on their morphological properties. But with the advent of Molecular Biology, plentiful molecular features of various biomolecules became available in the form of DNA and protein sequences. This helped researchers to categorize organisms more precisely on the basis of their molecular properties. The fungus *T. clypeatus* was classified into basidiomycetes group according to its morphological characteristics (Heim, 1951) way back in 1951 at which time all these molecular data about various organisms were virtually non-existent. Our own analysis of *T. clypeatus* mycellial cells under microscope reveals the presence of basidium (data not shown), which is the main feature of all basidiomycetes fungi and supports this classification from the morphological viewpoints. But investigations of CDH enzyme sequence and structure from *T. clypeatus* using computational tools reveal a number of similarities with ascomycetes CDHs, such as, i) CDH from this source *T. clypeatus* possesses a discrete carbohydrate binding module (CBM) (Fig. 1) which is mostly found in ascomycetes class IIA CDHs and is generally absent in basidiomycetes CDHs. The CBM domain, when present in the CDH enzyme, enhances the rate of cellulose hydrolysis by increasing the enzyme concentration on the substrate surface (Lakhundi et al., 2015). Where present within a carbohydrate degrading enzyme, a CBM involves amino acid sequence with discrete fold having carbohydrate binding activity (Lakhundi et al., 2015). It consists of 30–200 amino acids, may be located either in C or N terminal ends or occasionally centrally positioned within the polypeptide chain (Lakhundi et al., 2015). Based on amino acid sequence similarity, the CBMs from various carbohydrate degrading enzymes have been classified into at least 71 different families of which CBMs that are found in the CDHs are positioned within Family 1 (Tan et al., 2015; Lombard et al., 2013). The 3D structures of 71 representative families of CBM have been interpreted so far (http://www.cazy.org) (Lombard et al., 2013). Characterization of CBM domain of various groups described in the Pfam database shows that the important residues for carbohydrate binding - three aromatic amino acid residues and three cysteine residues - are well conserved in the carbohydrate binding motifs (CBM) of 758 carbohydrate active proteins including the ascomycetes IIA CDHs (Fig. 1(a), upper panel and Table S3(a)). These central residues of CBM which contribute to the binding on surface of cellulose are already reported for the Class IIA CBMs of ascomycetes CDH enzymes (Harreither et al., 2011) and a class I CDH variant from *P. chrysosporium* (Yoshida et al., 2005). *Tc*CDH also contains these residues and this confirms the CBM nature of this particular domain in *Tc*CDH. CDH of all other basidiomycetes (except *P. chrysosporium* (Yoshida et al., 2005)) and Class IIB CDH from ascomycetes, are devoid of this particular CBM domain. Our finding of a distinct carbohydrate binding module (CBM) in *Tc*CDH, as unravelled by our computational studies, is supported by experimental findings of Saha et al. (2008) who, with the help of binding studies (Saha et al., 2008), have shown that *T. clypeatus* CDH possesses high affinity for both crystalline cellulose and amorphous cellulose. This high affinity of CDH towards cellulose binding probably is possible when a CBM is present as because studies by Duan et al. (2016) and Nishijima et al. (2015) have shown that mutating the aromatic amino acids (in other systems) within the CBM domain lead to decreased affinity of the enzyme towards cellulose. ii) Dehydrogenase domain of *Tc*CDH does not contain cellulose binding residues; similarly class IIA and IIB ascomycetes CDHs (Fig. 1(b)) are also devoid of these residues within their dehydrogenase domains. Basidiomycetes CDHs (except *P. chrysosporium* (Yoshida et al., 2005)) are also known to have the dehydrogenase domain which, on the other hand, contains these conserved cellulose binding residues (Harreither et al., 2011). This is yet another major similarity of the *Tc*CDH with the CDHs belonging to the ascomycetes group. The cytochrome domain of *Tc*CDH, which is present in both ascomycetes and basidiomycetes groups, possesses the specific conserved residues which interact with heme and a pair of cysteines (Fig. S2) and this finding is in agreement with other CDH cytochrome motifs archived in Pfam indicating the importance of this domain in catalytic process of CDH activity.

The phylogenetic analysis presented here provides a new perception of classification of *T. clypeatus* CDH and categorizes it with ascomycetes group (Fig. 2). *Tc*CDH is the only basidiomycete to possess all the three domains – it possesses both the cytochrome and dehydrogenase domains found in the ascomycetes and basidiomycetes in addition to the CBM domain found in the class IIA CDHs of ascomycetes. In addition, its dehydrogenase domain has been found to be devoid of the cellulose binding residues as found in case of the ascomycetes; this is in contrast, on the other hand, with the dehydrogenase domain found in basidiomycetes where this domain contains specific cellulose binding residues discussed earlier. From these findings it is clear that the *Tc*CDH is more similar to the ascomycetes CDH than that of the basidiomycetes CDH (Fig. 2) in spite of itself being classified as belonging to the basidiomycetes group. Thus, the phylogenetic tree signifies a close relation of *Tc*CDH with class II CDH of ascomycetes. This is inspite of *T. clypeatus* sharing many important morphological features because of which it was classified as basidiomycetes in the first place by Heim (1951) in 1951. It is to be noted here that this is not the only enzyme of *T. clypeatus* which showed ascomycetes like features. Another carbohydrate metabolizing enzyme, phosphoketolase (Pk), which was cloned from *T. clypeatus* (accession no: KF690709) and expressed in *Escherichia coli*, showed noticeable similarity with ascomycetes phosphoketolases as well (Sarkar and Roy, 2014). Phylogenetic analysis of Pk sequence from *T. clypeatus* with other bacterial and fungal Pk sequences revealed that *Metarhizium anisopliae*, an ascomycete fungus, is its closest relative (Sarkar and Roy, 2014).

Homology model of CDH from *T. clypeatus* has been constructed in order to evaluate its detail structure (Fig. 3). The model has displayed the two distinct domains, cytochrome and dehydrogenase along with the ligands, heme and FAD respectively (Fig. 3). In addition, the model also has displayed the CBM domain very clearly (Fig. 3). Hence, the presence of all three domains in *Tc*CDH as determined by the Pfam analysis earlier is fully corroborated by the homology model of the

enzyme. We have also rebuilt the protein along with its substrate, cellobiose, which helped us to investigate the substrate interacting residues. These residues are localized in the dehydrogenase domain and compared by aligning with other PDB structures from both basidiomycete and ascomycetes fungi. Eight residues (L354, P606, T629, R631, Y649, N732, H733, N781) and three residues (Y649, N732, H733) are found to be conserved in all of them on the basis of 5 Å resolution and H-bonding interactions respectively (Fig. 4(b)). The simulation studies described for the model structure also confirm the presence of three usual domains of the CDH and the ligands have been found to be present in the binding pockets of their respective domains (Fig. S10).

In conclusion, the results of our computational investigations reveal true identity of a gene (accession no. GAFV01008428.1 (Mukherjee et al., 2013)) from *T. clypeatus* as a CDH enzyme. Four reasons in support of this conclusion and as discussed above are: i) presence of all three domains that are also found in the CDH of ascomycetes, i.e. cytochrome domain, dehydrogenase domain and the CBM; ii) the CBM of the *Tc*CDH, with its three cysteine and three aromatic amino acid residues, has been found to be highly conserved structurally when compared with the CBM domains of CDH from the ascomycetes group of fungus; iii) its cytochrome domain contains the conserved histidine and methionine (that bind with heme) as well as two cysteine residues (that help in the formation of disulphide linkages) that are universally present within the cytochrome domains of CDH of both the ascomycetes and basidiomycetes fungi and iv) presence of cellobiose specific eight substrate binding residues that are found within the dehydrogenase domain of the CDH of both the groups.

From an examination of its CDH sequence, phylogenetic placement and homology model, it is evident that *T. clypeatus* is close to ascomycetes group in spite of being morphologically similar to basidiomycetes group. The discordance in morphological (Heim, 1951) and molecular properties may perhaps be due to *T. clypeatus* being an intermediate fungus between ascomycetes and basidiomycetes since it bears mixed properties of both the groups. The work accomplished here reports the lack of cellulose binding residues within the dehydrogenase domain of *Tc*CDH similar to the ascomycetes CDHs but in deference to that of the basidiomycetes CDH. It seems that lack of those residues in *Tc*CDH and other ascomycetes made them to retain CBM to perform cellulose binding function. By combining the morphological features (Heim, 1951) and molecular properties of *T. clypeatus*, we can presume that it is a new group of fungus having properties of both ascomycetes and basidiomycetes and it is on its way to be fully differentiated as a basidiomycete in course of evolution in distant future. The CBM and dehydrogenase domains of *Tc*CDH may fuse during evolutionary process in coming days to perform both cellulose binding and catalytic function in a single domain like in the dehydrogenase domain of basidiomycetes CDHs. This scheme of thinking is in line with the general idea that basidiomycetes evolved from the ascomycetes during evolution and are more advanced in nature (Zhao et al., 2014; Liu et al., 2017). Also, our findings appear to be in synchrony with the observation of Zamocky et al. (2004) who predicted that with the availability of additional CDH sequences, examples of class-I CDHs with a CBM, or class-II CDHs devoid of CBMs may be discovered (Zámocký et al., 2004). In fact this is one of the unique and novel finding of our report that *T. clypeatus*, in spite of having a class-I CDH, harbours a CBM, in line with the prediction of Zamocky et al. (2004). This may have happened in course of the evolution during which the TcCDH may have acquired the CBM domain in it, as predicted by Zamocky et al. (2004).

The results of our computational studies of this enzyme described in this paper has logically opened up additional avenues that can be explored in future, for example, i) the roles of conserved and non-conserved substrate interacting residues found in the active site of *Tc*CDH can be investigated by mutagenesis experiments as described in (Ramachandran et al., 2016), ii) homology model of *Tc*CDH can be used for substrate docking studies so that the interactions of the protein with the ligands can be assessed using scoring and energy parameters as described (Selvaraj et al., 2016; Kim et al., 2016). In addition to this, experiments like NMR spectroscopy and isothermal titration calorimetry (ITC) studies (Courtade et al., 2016) can be performed with *Tc*CDH to study its interaction with lytic polymonooxygenase (LPMO) to mediate cellulose depolymerisation (Kracher and Ludwig, 2016). No less important will be the cloning and characterisation of the *Tc*CDH gene for the purpose of engineering and expressing it in suitable hosts for various industrial applications.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

This work has been supported in part by a grant from UGC, India to AR[a*] and other departmental funds by DBT, GoI. SB[a] is thankful to UGC and Visva-Bharati University for fellowship support. MSM[b] and AR[b] would like to acknowledge a Wellcome trust-DBT, India alliance for a senior fellowship to MSM[b]. We also thank members of the MSM[b] lab for useful discussions and criticisms.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.compbiolchem.2019.05.013.

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