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Protein engineering and yeast surface display of cellulases for improved bioethanol production

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Abstract:

Bioethanol production is important because of its positive impact on the environment. Cellulose is the most abundant raw material available. The enzymatic breakdown of cellulose by the cellulase enzyme leads to the formation of glucose, aiding in the production of bioethanol with commercial potential and lower production costs. Three cellulase enzymes endocellulase, cellobiohydrolase and β-glucosidase, were protein-engineered at variable regions via rational design. The protein-engineered genes were modelled, and molecular docking was performed to assess the binding affinity of the enzymes for the cellulose substrate. Compared with the wild-type enzymes, the engineered enzymes displayed greater stability, with a significant increase in amino acid interactions. Codon optimization, and synthetic gene synthesis, followed by vector preparation and subcloning into yeast for surface display, were carried out. The transformed OC21 yeast cells were analyzed for enzyme activity, and expression studies revealed that the membrane fractions had enzyme activity of endocellulase (28±0.0024 IU/ml), cellobiohydrolase (16±0.04 IU/ml) and β-glucosidase (11±0.09 IU/ml), confirming the surface anchorage of the enzymes. The protein-engineered enzymes resulted in higher hydrolysis efficiencies of 81.9±2.2% and 78.1±1.2% for swollen cellulose and pretreated rice straw, respectively, at the 10-gram level. Separate and simultaneous saccharification studies of swollen cellulose and pretreated rice straw for ethanol production by thermotolerant *Saccharomyces cerevisiae*OC21 surface displaying yeast strains at 42°C to asses fermentation efficiency. Separate saccharification and fermentation yielded 93.6±0.58% and 91.4±0.32%, respectively, for swollen cellulose fermentation and pretreated rice straw fermentation. A significant improvement in the production of bioethanol was observed in simultaneous saccharification and fermentation for swollen cellulose and pretreated rice straw, with 95.1±0.45% and 92.3±0.29% ethanol fermentation efficiency, respectively, in fermentation by surface-displaying OC21 yeast. The ethanol was further purified by distillation and molecular sieve absorption to obtain fuel grade ethanol with purity of 99.6 ± 0.2% (v/v). This result demonstrates that engineered cellulase enzymes, surface displayed in yeast strains can be an effective approach for improved bioethanol production.

Keywords: Protein engineering, Molecular docking, Cellulases, Yeast surface display, Bioethanol production.

1. Introduction:

Over the past few decades, the excessive use of fossil fuels has led to a significant rise in pollution levels and many adverse health effects [1]. Bioethanol plays a leading role as a sustainable alternative to fossil fuels. [2]. Bioethanol is derived from biomass and proven to be eco-friendly, with a low carbon footprint [3]. Cellulose is the most abundant natural raw material, with a production of 10^{11}-10^{12} tons per year [4]. Cellulases are a group of enzymes that play important roles in the breakdown of cellulose a complex polymer of glucose [5]. The global economy today relies heavily on a range of fossil energy sources, including oil, coal, and natural gas. These resources are widely used to produce fuel, generate electricity, and manufacture various goods [6]. The exponential growth of the population demands enormous fuel needs for sustainability and development; therefore, ethanol production from cellulose can be a potential and safer alternative to fossil fuels [7].

However, bioethanol production depends heavily on the availability of inexpensive feedstocks, efficient conversion technologies and improved fermentation processes. In this context, lignocellulosic biomass can be an important resource for bioethanol production as it is obtained from agricultural residues [8]. Pretreatment can improve enzyme accessibility for efficient biomass conversion, enzymatic cellulose degradation is mediated by endocellulase, cellobiohydrolase and β-glucosidase [9]. Insufficient activity of any component, including enzyme activity, substrate accessibility, abundance and synergistic action, can significantly affect bioethanol production. Therefore, improving cellulase performance is an optimal strategy for increased bioethanol production [10].

Protein engineering refers to the process of modifying the structure of an existing protein to customize its properties. This powerful technology not only deepens our fundamental understanding of enzyme function and evolution but also plays a crucial role in optimizing enzymes for use in pharmaceuticals, green chemistry, and biofuel production [11,12]. The protein engineering approach enhances enzyme activity under various conditions, such as elevated temperatures and the presence of organic solvents, salts, and other physiological conditions [13]. Yeast surface display is widely used to study protein expression on the surface of yeast; yeast surface display also plays a vital role in protein engineering [14]. YSD allows the study of protein affinity, expression, specificity, and stability [15]. The use of engineered enzymes in bioethanol production can increase yield and improve the overall fermentation efficiency [16]. The abundance and cost efficiency of cellulose make it a dependable raw material for most bioprocesses [17].

Hence, this study focuses on the protein engineering of cellulase enzymes, which are expressed yeast surface displays for efficient bioethanol production from lignocellulosic materials.

2. Materials and Methods:

Enzymes:

Commercial endocellulase, cellobiohydrolase, and β-glucosidase enzymes for hydrolysis studies are procured from Novozymes. Protein-engineered cellulose enzymes were surface displayed and expressed by a transformed thermotolerant yeast strain in the laboratory.

Microorganisms, Plant material, Vectors, Restriction enzymes, Genes and Ligases

Rice straw used as lignocellulosic substrate in this study was collected from Jallapally Farm, Pothangal Mandal, Nizamabad District, Telangana, India (18.5478859° N, 77.7859339° E). The plant material was collected from privately owned agricultural land after obtaining permission from the respective landowner.

The thermotolerant yeast *Saccharomyces cerevisiae* EBY100was mutated and developed earlier in our laboratory. The pCTO CP yeast surface display vector was constructed in our laboratory. NheI and BamHI restriction enzymes and T4 ligases obtained from fermenter studies. The genes of the engineered enzymes were synthesized and procured from Mr. Gene Germany.

2.1.1 Protein Engineering:

a. Sequence collection

It is aimed to engineer native endocellulases, cellobiohydrolases, and β-glucosidase enzymes. After a thorough study, 50 sequences of each endocellulases, cellobiohydrolase, and β-glucosidase sequences were retrieved via NCBI BLAST, and 3 conserved native sequences were selected as templates for protein engineering.

b. Domain conservation studies

Conservative evolution studies of for endocellulase, cellobiohydrolase, andβ-glucosidase domains were carried out via multiple sequence alignment via ClustalW. Fifty sequences obtained via BLAST were analysed via ClustalW. Conserved regions and variable regions were identified, and a matrix was prepared to develop the mutants.

c. Protein modifications by protein engineering

Rational protein engineering was performed on only the identified variable regions to obtain stable and functional proteins for 3 cellulases, as described below.

Engineering of Endocellulase

1. Replacement of N with T at 321 positions N321T.
2. Replacement of N with H at 321 positions N321H
3. Replacement of N with E at 321 position N321E.
4. Replacement of four aspartates within the active site centre of endoglucanase with alanine.
5. Replacement of four aspartates within the active site centre of endoglucanase with glutamine.

Engineering of the CBH enzyme

1. The CBH was designed with CBD.
2. CBH was designed without CBD
3. Replacement of the alanine of 224 with histidine A224H
4. Replacement of the glutamic acid 217 with aspartic acid. E217D

Engineering of Betaglucosidase

1. 294 of G was replaced with F. G294F
2. 294 of G replaced with W. G294W
3. 294 of G replaced with Y. G294Y
4. Addition of CBD of CBHII to BGL.

d. Structure building and molecular docking.

The 3D structures of the wild-type and engineered cellulase protein sequences were developed via Modeler 9.1 homology modelling studies. The template PDB was obtained from NCBI PDB BLAST. Among the Hits, 1H1N for endocellulase, 1CB2 for CBHII, and 1EXl for Bgl were selected as templates because of their high homology. 1H1N for endocellulase, 1CB2 for CBHII, and 1EXL for Bgl were used as templates for the wild-type sequence, and the developed wild-type structure was used as the template for other altered mutant sequences. Twenty model files were generated for each sequence. Among the 20 models, one model with the lowest RMS value was selected, and PROCHECK was run for stability analysis via Ramachandran plots. For these structures, docking was performed with cellulose, CMC, cellobiose, and glucose to determine the substrates and glucose binding sites.

Molecular docking studies were performed to assess the molecular interactions between the target and the ligand via FlexX software.

e. Codon optimization

The wild-type and selected mutein sequences of all three engineered enzymes were codon optimized via DNA 2.0 software for maximum expression of cellulases in *Saccharomyces cerevisiae.* Codon optimization was carried out above the 15% threshold [18] as per the codon usage frequency of the standard *Saccharomyces cerevisiae* table. After confirming that protein engineering was successful, a gene cloning strategy was used. Three genes under the same individual promoter were cloned, and cloning was performed separately.

2.1.2 Synthetic gene synthesis

After protein engineering, 2 mutants for each of the 3 cellulases synthesized at Mr Gene Germany. The synthetic genes were further used for cloning in *S. cerevisiae* for yeast surface display.

2.2.1 Yeast Surface Display Vector Preparation and Subcloning

Synthetic genes were provided in a standard plasmid, which was further subcloned and inserted into the pCTO CP surface display vector with the Gal promoter. Alpha factor signal peptides were incorporated into*S. cerevisiae* for surface display. The surface display plasmid developed in our laboratory earlier (pCTO CP) was used in these studies. The synthetic Half 2-Agglutinin gene 120bp N-terminal, (Gly 4, Ser)3 linker, and Nhe site were maintained after the GAL promoter with the EcoR I site, followed by MCS for cloning of the desired genes. The constructed vector map was constructed and illustrated in (Fig. 1).

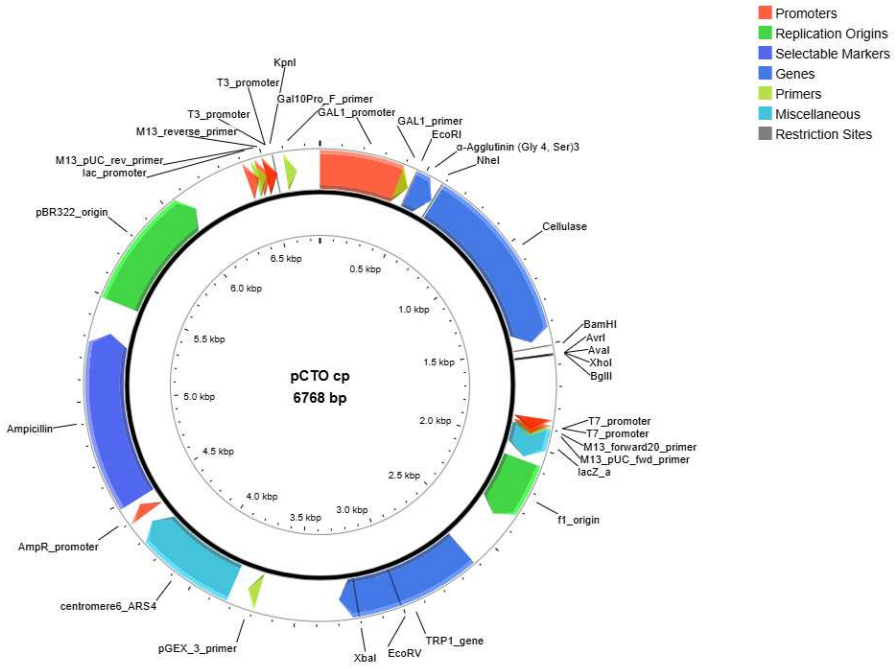


Figure 1. pCTO CP vector map

The cel, CBH II, and bgl engineered synthetic genes encoding engineered endocellulase, cellobiohydrolase II and β-glucosidase were selected for cloning into the yeast surface expression vector pCTO CP in *S. cerevisiae*. The genes were digested with NheI, BamHI and ligated via T4 ligase and transformed into *E. coli* DH5α.

Transformation and selection of *S. cerevisiae*

The plasmid used for yeast transformation was isolated from transformed *E. coli*DH5α cells via a Miniprep Qiagen kit. The EYB100 *S. cerevisiae* mutant is a tryptophan-negative auxotrophic strain that grows at 40°C.Auxotrophic *S. cerevisiae* EYB100 strain (Trp-ve) competent cells and plasmids at ratios of1:0.01, and 1:0.02, used for electrotransformation. Electroporation was carried out at 1.6 V for 9s.

The samples were plated on tryptophan negative selective medium (YNB minimal medium without tryptophan) and incubated for 3 days at 40°C. The recombinant yeasts were selected by their growth on a selective medium. The surface display of cellulase and cellulase activity were studied.

Expression and activity studies

The selected OC21 yeast recombinants were grown in YEPC selective media. The supernatant, live washed cells, heat-killed and membrane isolate yeasts were used for analysis of FPase, CMCase, and β-glucosidase activities to assess the endocellulose, cellohydrolase, and β-glucosidase enzymes.

The cells were harvested via centrifugation and subsequently washed with normal saline. Half of the washed cells were treated with 100mM β-mercaptoethanol in 0.1M phosphate buffer at 70°C for 4 hr. Half of the MTOH-treated cells, were lysed via sonication. Intactly MTOH-treated, washed cells and lysates were used for cellulase activities.

On the basis of activity, one clone each of endocellulase, cellobiohydrolase and β-glucosidase was selected from the EBY100 OC21 transformants.

Preparation of swollen cellulose and pretreatment of rice straw:

Swollen cellulose was prepared by soaking crystalline cellulose overnight in 80% ortho phosphoric acid and subsequently neutralizing with 0.2M NaOH and washing.

Rice straw (dried) was ground via a laboratory cutting mill to a particle size of 5 mm, and immersed in a flask containing water (60 mL/g solid) at 25°C for 3 days to form a solid slurry. Then, sodium chlorite (0.4 g/g solid) and glacial acetic acid (84 mg/g solid) were added to the solid slurry, and the mixture was heated to 80°C in a water bath with gentle swirling at intervals. After a 1 h reaction time, the mixture was bubbled with air to remove the yellowish-green chlorine dioxide that was generated during the reaction. The treated rice straw was collected and washed with water. The water-washed sample was sun-dried for 3 days before further use.

Lignocellulosic hydrolysis of swollen cellulose and pretreated rice straw with commercial and YSD enzymes:

Hydrolysis studies of swollen cellulose and pretreated rice straw by commercial cellulases and yeast surface display enzymes were performed at the 2- and 10-gram levels. Two and 10 grams of lignocellulosic substrate were made by suspending 2% and 10% of the substrates in 100 mM citrate buffer, respectively, followed by sterilization. Two percent (v/w dry substrate) of three individual (endoglucanase, cellobiohydrolase, and β-glucosidase) commercial enzymes were added to 2- and 10-gram-level substrates of swollen cellulose and rice straw for hydrolysis studies. Two percent (v/v) of the surface-displaying enzyme Yeast was added to swollen cellulose, and rice straw flasks at the 2- and 10-gram levels were pretreated separately. Commercial enzymes (endoglucanase, cellobiohydrolase and β-glucosidase) were obtained from Novozymes [19]. The hydrolysis efficiency was calculated by estimation of the cellulose before and after hydrolysis.

Ethanol fermentation studies of swollen cellulose and pretreated rice straw by separate and simultaneous saccharification

Separate saccharification at 42°C and fermentation

The saccharification of swollen cellulose and pretreated rice straw was carried out separately at the 10-gram level via the fed batch fermentation method. Substrates at the 5-gram level were separately suspended in 500 ml flasks. One hundred millilitres of 100 mM citrate buffer was added to the flasks, followed by the addition of 2% (v/w) three commercial enzymes. A Thermotolerant yeast VS3 pellet was added and incubated at 42°C for 24 hours. A second batch of 5 grams of substrate was added to further proceed with the reaction, and the mixture was incubated at 42°C for 48 hours.

During the incubation, samples were drawn at 6 hr time intervals ranging from 12 hours to 72 hours. The samples were analysed for reducing sugars via the DNS method and were estimated for ethanol via gas chromatography.

Simultaneous saccharification and fermentation at 42°C

Simultaneous saccharification of swollen cellulose and pretreated rice straw was carried out separately via the 10% batch fermentation method. Five grams of each substrate was separately suspended in 250 ml flasks. One hundred milliliters of 100 mM citrate buffer was added to the flasks. Five milliliters of each surface-displayed yeast pellet was added and

incubated at 42°C for 24 hours. A second batch of 5 grams of substrate was added after 24 h to further proceed with the reaction, and the mixture was incubated at 42°C for 48 h.

During the incubation, samples were drawn at 6 hr time intervals ranging from 12 hours to 72 hours. The samples were analyzed for reducing sugars by DNS and cellulose by the Apegraff method [18]. and ethanol estimation via gas chromatography.

2.7 Estimation of glucose, ethanol, and fermentation efficiency:

Estimation of glucose, cellulose, and ethanol contents

Estimation of glucose was performed via the DNS method. The cellulose content before and after hydrolysis was measured via the Apegraff method [20].

The estimation of ethanol content was carried out via gas chromatography. The method involved a SHIMADZU GC-2020 instrument with a flame ionization detector. GC was carried out according to the NREL procedure LAP # 011, using a ZB-Wax column [21].

Fermentation efficiency

The amount of ethanol obtained (0.511) per gram of consumed sugar was taken as the practical yield and divided by the theoretical yield to obtain the ethanol fermentation efficiency [22].

The formula used to calculate the ethanol fermentation efficiency.

Fermentation efficiency (%) = actual ethanol produced (per gram of sugar used)/(theoretical ethanol produced per gram of glucose), (0.511) ×100

Ethanol purification through multi stage distillation and molecular sieve dehydration

The ethanol concentrate obtained after three successive distillation cycles was dehydrated using activated 3Å molecular sieves. The sieves were activated at 250°C for 4h, collected, and packed in adsorption column. Ethanol was passed through the column at 40°C to ensure the collection of dehydrated ethanol in airtight containers. The dehydrated process was continued until molecular sieve saturation, and ethanol purity was subsequently determined by GC-FID [23].

Statistical analysis:

The experiments were repeated thrice in triplicate (n=9) and values with standard deviation are presented.One-way ANOVA followed by Tukey's test was performed to find out the significance of the data and p-values of less than 0.05 were considered statistically significant.

3.Results:

Protein engineering of 3 enzymes

Endocellulase, cellobiohydrolase and β-glucosidase enzyme sequences were collected, protein sequences were modified at variable regions, protein modifications were performed, and engineered cellulase gene sequences were codon optimized and obtained via synthetic gene synthesis from Mr. Gene, Germany. The obtained genes were subsequently subcloned and inserted into pCTO CP and transformed into the S. cerevisiae EYB100 strain (Trp-ve).

Modelling

After BLAST searches of the selected sequences (Cel (AAA34213), CBHII (AAA72922, BglI (Q9P8F4)), sequences with more than 90% similarity were selected. A homologous structure was developed for the wild-type sequences and altered sequences. 1H1N for

endocellulase, 1CB2 for CBHII and 1EXl for Bgl were used as templates for the wild-type sequence, and the developed wild-type structure was used as the template for other altered mutant sequences. Twenty model files were generated for each sequence. Among the 20 models, one model with the lowest RMS value was selected, and PROCHECK was run and analysed via Ramachandran plots.

The PDB structures of the 3 enzymes are as follows (Fig. 2).

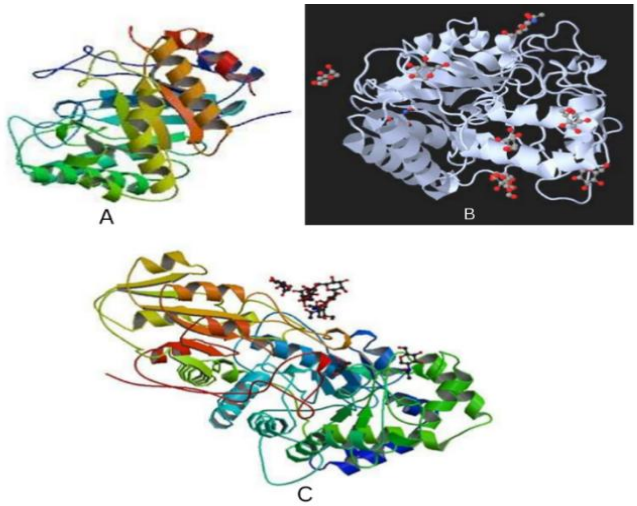


Figure 2. Structures of 1HIN (A), 1cb2 (B) and 1EX1 (C).1H1N for endocellulase, 1CB2 for CBHII and 1EXl for Bgl.

Table 1. Energy values of wild and engineered proteins.

Protein	**Energy Value (KJ/mol)**
Wild endocellulase	-15295
Endocellulase mutein 1	-16621
Endocellulase mutein 2	-15754
Wild Cellobiohydrolase	-17634
Cellobiohydrolase mutein 1	-17838
Cellobiohydrolase mutein 2	-17838
Wild β-glucosidase	-73792
β-glucosidase mutein 1	-73057
β-glucosidase mutein 2	-73057

The energy values of the enzymes were evaluated, and the results revealed that the engineered enzymes had improved stability compared with that of the wild-type enzymes, resulting in improved enzyme activity (Table 1).

Modelling studies and stability studies of wild-type and engineered enzymes followed by docking of wild-type and engineered enzymes with cellulose and cellobiose were performed to determine the cellulase activity and molecular affinity of the enzymes as illustrated in (Table 2).

Table 2. Affinity studies of wild and engineered proteins.

Proteins interactions in docking	**Affinity (kcal/mol)**
Wild endocellulase X cellulose	-5.621
Endocellulase mutein 1 X cellulose	-6.211
Endocellulase mutein 2 X cellulose	-6.424

Wild Cellobiohydrolase X cellulose	-7.598
Cellobiohydrolase mutein 1X cellulose	-7.617
Cellobiohydrolase mutein 2 X cellulose	-7.617
Wild Beta-glucosidase X cellobiose	-6.193
Beta-glucosidase mutein 1 X cellobiose	-6.199
Beta-glucosidase mutein 2 X cellobiose	-6.199

Docking studies revealed that, compared with wild-type enzymes, mutein enzymes have improved affinity for substrates.

Molecular docking.

Docking studies of wild-type and mutant endocellulases with cellulose

With substrate cellulose,7 amino acid interactions were observed for wild-type endocellulase, and 16 AA interactions were observed for mutant endocellulase (Fig. 3).

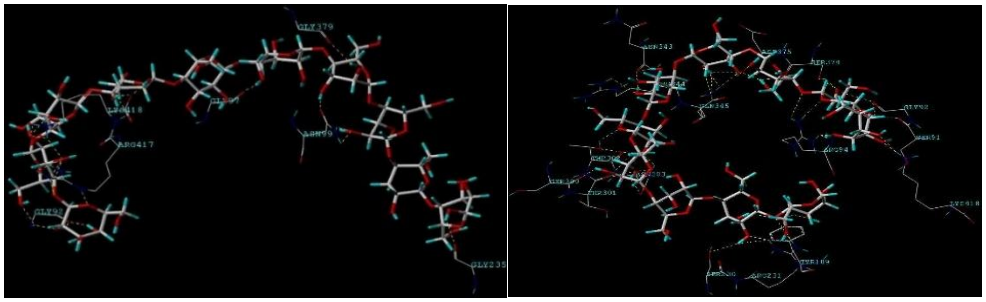


Figure 3. Docking studies of wild-type and mutant endocellulases with cellulose

Docking the wild-type and mutant cellobiohydrolase with cellulose:

With the substrate cellulose, only six AA interactions were observed for the wild-type cellobiohydrolase, whereas 15 amino acid interactions were observed for the mutant enzyme (Fig. 4).

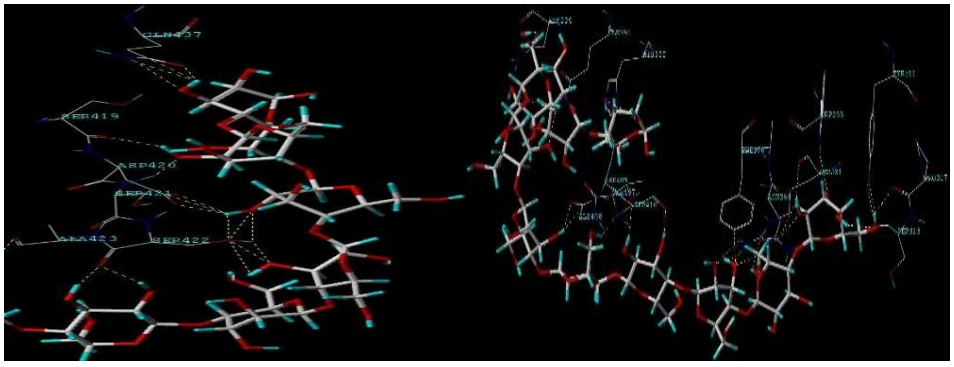


Figure 4. Docking studies of wild-type and mutant cellobiohydrolases with cellulose.

Docking studies of wild-type and mutant β-glucosidases with cellobiose

Five and twelve amino acid interactions with cellobiose were observed for wild-type and mutant beta-glucosidase, respectively (Fig. 5).

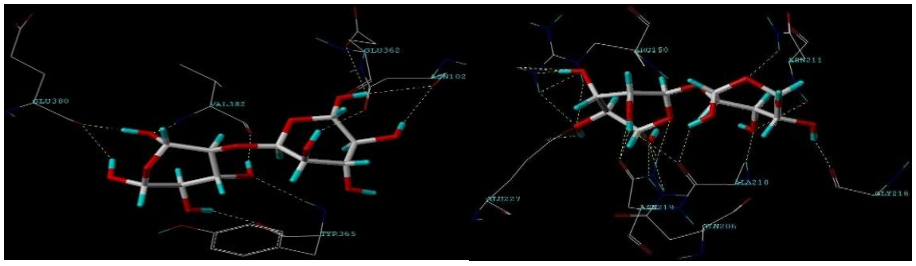


Figure 5. Docking studies of wild-type and mutant beta glucosidases with cellobiose

Compared with the native protein structures, the engineered protein structures of the 3 enzymes were found to be stable and active.

3.2 Gene cloning:

After engineering and confirmation of stability and activity, gene subcloning studies of synthetic engineered cellulases in the pCTOCP vector were carried out for yeast surface display (Fig. 6).

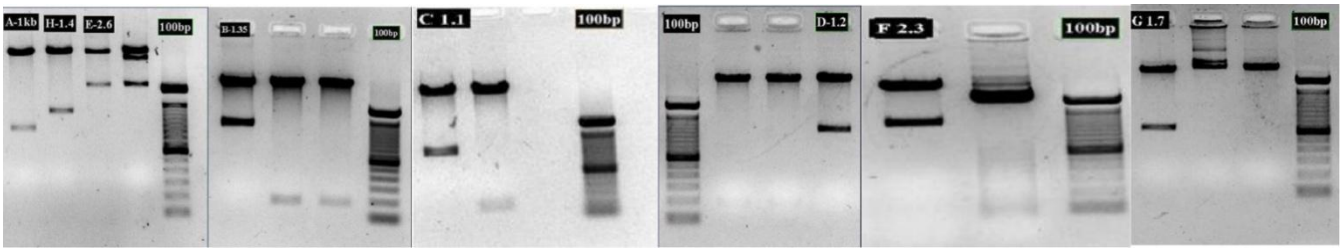


Figure 6. Yeast Surface Display sub cloning studies of synthetic genes consisting of A: Cellobiohydrolase without CBD. 1 Kbps. B. Cellobiohydrolase, 1.35 Kbps; C. Endocellulase without CBD, 1.1 Kbps; D. Endocellulase, 1.23 Kbps; E. Beta Glucosidase, 2.65 Kbps; F. Beta Glucosidase without CBH, 2.3 Kbps; G. Cellobiohydrolase with additional CBD, 1.7 Kbps. H. Endocellulase with an additional CBD of 1.4 Kbps.

Confirmation:

Positive clones were confirmed by growth of EBY strain on Trp negative medium and gene presence via PCR as illustrated in (Fig. 7).

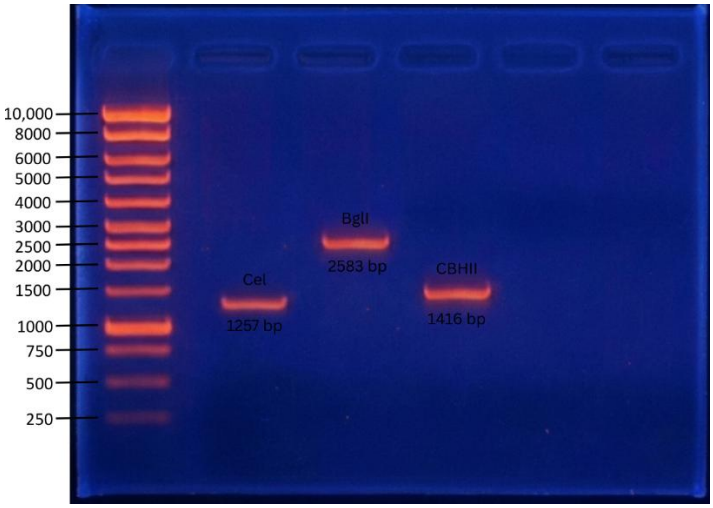


Figure7.PCR confirmation of engineered endocellulase, β-glucosidase, cellobiohydrolase.

Surface display expression studies

Expression and enzyme activity of surface displayed yeast were assessed. Various portions of surface displaying yeast were studied (Table 3).

Table 3. Expression and enzyme activity of surface-displayed OC21 yeast strains.

Sample	OC21CP1 (Endocellulase) CMCase IU/ml	OC21CP2 (Cellobiohydrolase) FPase IU/ml	OC21CP3 (β-glucosidase) β-glucosidase IU/ml
Supernatant	Nil	Nil	Nil
Intact yeast	19±0.09	10±0.2	8±0.4
Heat killed yeast	5±0.074	2.4±0.022	2.1±0.024
Membrane	28±0.024	16±0.04	11±0.09
Mercapto ethanol treated and washed yeast	Nil	Nil	Nil
Mercaptoethanol treated Washed yeast and lysed protein	Nil	Nil	Nil
Mercaptoethanol treated supernatant	2.5±0.08	1.2±0.04	0.8±0.02

Enzyme activity was significant in the intact yeast and membrane fractions, indicating that the enzymes are surface anchored and membrane bound. No activity was observed in the supernatant, indicating that the enzymes were not secreted but were membrane-bound. Further confirmation was obtained, as the mercaptoethanol-treated supernatant had the lowest enzyme activity, indicating strong anchoring of proteins.

The membrane fractions had maximum enzyme activities of 28 IU/ml, 16 IU/ml, and 11 IU/ml for endocellulase, cellobiohydrolase and β-glucosidase, respectively.

Hydrolysis studies of lignocellulosic materials

The hydrolysis efficiency of swollen cellulose was reported to be 75.4±0.4 g and 74.8±2.1 g at the 2- and 10-gram levels, respectively, by commercial enzyme hydrolysis and 82.3±0.2 g and 81.9±2.2 g at the 2- and 10-gram levels, respectively, by YSD enzyme hydrolysis.
The hydrolysis efficiency of pretreated rice straw was reported to be 72.5±0.7g and 71.8±0.9g at the 2-gram level by commercial enzyme hydrolysis and 78.6±0.9g and 78.1±1.2g at the 2-gram level by YSD enzyme hydrolysis. The hydrolysis profile of these enzymes was included in (Table 4).

Table 4. Hydrolysis of lignocellulosic materials by commercial enzymes and yeast surface display enzymes.

	Swollen cellulose (2 grams)		Swollen cellulose (10 grams)		Pretreated Rice straw (2 grams)		Pretreated Rice straw (10 grams)	
Enzymatic treatment	Commercial	YSD	Commercial	YSD	Commercial	YSD	Commercial	YSD
Cellulose	2.0±0.2 g	2.0±0.11	10±0.2 g	10±2.1 g	2.0±0.1 g	2.0±0.12	10±0.9g	10±0.24g

before Hydrolysis		g				g		
Cellulose after hydrolysis	1.508±0.3 g	1.644±0.1 g	7.48±0.1 g	8.19±0.1 g	1.45±0.21g	1.572±0.11g	7.18±0.2 g	7.81±0.21g
Hydrolysis efficiency	75.4±0.4 g	82.3±0.2 g	74.8±2.1 g	81.9±2.2 g	72.5±0.7 g	78.6±0.9 g	71.8±0.9 g	78.1±1.2 g

Ethanol fermentation studies by separate, simultaneous saccharification and fermentation.

The ethanol fermentation studies were done by separate and simultaneous saccharification followed by fermentation of swollen cellulose and pretreated rice straw. The results are described in (Table 5) and (Table 6).

Table 5. Ethanol-fed batch fermentation studies of swollen cellulose and pretreated rice straw at the 10-gram level by separate saccharification.

	Swollen cellulose (grams)	Pretreated rice straw (grams)
Level of fermentation	10 grams level	10 grams level
Total cellulose before hydrolysis (grams)	10±0.02	10±0.03
Cellulose Hydrolyzed	7.47±0.09	7.15±0.08
Hydrolysis efficiency	74.7±0.47	71.5±0.39
Glucose produced	8.27±0.14	7.92±0.17
Glucose consumed	7.74±0.17	7.24±0.18
Glucose left	0.53±0.008	0.68±0.009
Ethanol produced	3.96±0.074	3.70±0.067
Ethanol fermentation efficiency	93.6±0.58%	91.4±0.32%

Table 6. Ethanol fermentation studies of Swollen cellulose and pretreated rice straw at 2 and 10-gram level by Simultaneous Saccharification and fermentation.

	Swollen cellulose	Pretreated rice straw
Level of fermentation	10 grams level	10 grams level
Total cellulose before hydrolysis (grams)	10±0.01	10±0.05
Cellulose Hydrolyzed	8.18±0.05	7.80±0.07
Hydrolysis efficiency	81.8±0.56	78.0±0.55
Glucose produced	9.08±0.06	8.66±0.05
Glucose consumed	8.63±0.041	7.99±0.04
Glucose left	0.45±0.01	0.67±0.01
Ethanol produced	4.40±0.02	4.08±0.03
Ethanol fermentation efficiency	95.1±0.45%	92.3±0.29%

Ethanol was fermented from swollen cellulose, and rice straw was pretreated by separation and simultaneous saccharification at the 10-gram level to assess the ethanol fermentation efficiency. With engineered cellulase surfaces displaying thermotolerant yeast, more ethanol production and fermentation efficiency are achieved by simultaneous saccharification and fermentation than by commercial enzymes used for separate saccharification and fermentation with thermotolerant yeast.

Ethanol purification

The concentrated ethanol fraction with 95% (v/v) was obtained after third distillation. Molecular sieve column effectively absorbed residual water from ethanol stream, resulting in final ethanol purity of 99.6± 0.2% (v/v). The dehydrated ethanol was clear and colorless, confirming successful production of anhydrous ethanol suitable for fuel and industrial applications.

4. Discussion:

Protein engineering enhances the stability and binding affinity of a protein to its respective substrate [24]. The conserved regions of a protein cannot be protein engineered, as changes in conserved regions may result in the loss of the entire activity of the protein or in changes in the conformation of the protein on an exponential scale, altering its 3D structure and making it an unstable protein [25]. The enzyme sequences were collected from various databases and research papers, and multiple sequence alignment was performed via CLUSTAL W to obtain conserved and variable regions.

Molecular modelling and interaction studies were performed on the endocellulases, cellobiohydrolases and beta-glucosidases of wild-type and engineered variants against cellulose and cellobiose, respectively. A significant improvement in molecular interactions was observed in engineered proteins, indicating that protein engineering improves binding affinity and enzymatic functions. Vigneri et al. [26] reported similar findings on insulin, where the modification of insulin with one to three amino acid sequences resulted in greater affinity and activity than those of endogenous insulin. YSD cellulase enzymes were studied to analyse the hydrolytic properties of yeast in the present study. Poomani et al. (2024) [27] also conducted a similar study to assess the hydrolytic activity of endocellulase enzyme surfaces displayed on yeast and concluded that histidine is the key amino acid residue involved in the interaction of cellulase with cellulose. A similar importance of histidine was reported in the present study. In the engineering of the endocellulase enzyme, replacement of N with T at position 321 resulted in optimal activity at pH 5.4 [28]. The N321E mutation changed the optimal activity to pH 4.0, resulting in a large increase in specific activity. Compared with the wild-type enzyme, the N321H mutant enzyme was more active over a broader pH range [29]. Replacement of four aspartates within the active site centre of endoglucanase with alanine and glutamine resulted in an increase in substrate binding, and an increase in substrate binding affinity caused by aspartate mutation was also reported by Kim et al. (2001) [30]. In the engineering of cellobiohydrolases, the replacement of alanine 224 with histidine and glutamic acid 217 with aspartic acid was found to yield a more thermostable enzyme [31]. Histidine substitution in flexible fragments of firefly luciferase modifies its thermostability [32].

Compared with the parent strain, Betaglucosidase was engineered by the replacement of G294 with aromatic compounds such as F, W, and Y, resulting in higher activities for

substrate recognition; the hydrolytic activities also increased [33], which led to the conclusion that aromatic compounds confer greater substrate recognition activities. CBH CBD-BGL exhibited the highest rate of hydrolysis, approximately fourfold higher than that of the native enzyme; CBD-CBD-BGL was twofold higher than that of the native enzyme. These findings were also supported by the studies of Liu et al. (2018) [34] & Méndez-Líter et al. (2017) [35].

Compared with the native protein structures, the engineered protein structures of the 3 enzymes were found to be stable. Compared with docking with native molecules, the molecular docking of ligand cellulose molecules with the engineered protein resulted in greater interactions and greater stability, as confirmed by improved RMS values. Zhang et al. (2025) [36] also confirmed that protein engineering conferred stability and improved energy compared with native proteins. The molecular modelling of natural flavonoids was performed, followed by molecular docking with VEGFR, and the potential of natural flavonoids for effective lung cancer treatment was determined [37].

Compared with extracellular expression, surface display of proteins is more beneficial. In the present study, the surface activities of endocellulase (28±0.0024 IU/ml), cellobiohydrolase (16±0.04 IU/ml) and β-glucosidase (11±0.09 IU/ml) on the membrane fractions were observed, indicating that the protein anchored to the membrane. Khorsand et al. (2024) [38] reported that, compared with other expression systems, the surface display system is a more efficient system of expression for the production of recombinant proteins. Yeast surface display was also used to screen nanobodies for cytochrome P450 enzymes by Sun et al. (2024) [39].

Swollen cellulose and rice straw at concentrations of 2 and 10% were used as substrates in this study, whereas Poomani et al. (2024) [27] evaluated hydrolysis and fermentation studies in the presence of microcrystalline cellulose as a substrate.

The yeast surface revealed that bioethanol was produced from swollen cellulose, and pretreated rice straw yielded fermentation efficiencies of 95.1±0.45% and 92.3±0.29%, respectively. Molaverdi et al. (2022) [40] conducted ethanol production from rice straw through cellulose restructuring and high solid loading fermentation by Mucor indicus and reported an ethanol yield of 73.7%.

Compared with commercial enzymes, the YSD method resulted in greater cellulose hydrolysis and ethanol yields. Simultaneous saccharification and fermentation using Thermotolerant yeast with engineered enzymes has efficient ethanol fermentation activity and can be widely used in the industrial production of bioethanol, which has greater potential as an industrial microbial strain. The consolidated bioprocessing approach, consisting of saccharification and fermentation at relatively high temperatures, was also used in the present study. Similar results were also reported in the study conducted by Hasunuma et al. (2012) [41], who focused on the consolidated bioprocessing of saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast via surface display.

Conclusion:

In conclusion, protein engineering of cellulase can increase the hydrolytic activity of cellulases against cellulose for efficient glucose production, which is essential for bioethanol production. Compared with commercial enzymes, the surface display of engineered enzymes on thermotolerant yeast has promising ethanol efficiency. This thermotolerant surface-displayed strain can be utilized as an optimal strain for bioethanol production.

Authors' Contributions:

Dr Chand Pasha: Planned project, methodology, Supervision, Paper writing
Shaik Muzammil Pasha: Methodology, Data curation, Analysis
Vadlamudi Nikhil: Analysis, Paper writing
Rebeca Cortez-Zambrano: Final draft correction, Validation.
Arifullah Mohammed: Manuscript correction, Statistical analysis, Writing and Review.
Gooty Jaffer Mohiddin: Final draft correction, Validation, Proofreading, Review and Editing.

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Data availability statement:

The datasets supporting the conclusions of this article are included within the article and its supplementary files. Additional raw data generated during the study are available from the corresponding author upon reasonable request.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Permissions

Permissions to collect rice straw from privately owned agricultural land at Jallapally Farm, Pothangal Mandal, Nizamabad District, Telangana, India, was obtained from the respective landowner prior to collection.

Conflict of Interest:

The authors declare no conflicts of interest.

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