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Full Length Article

Optimum Mixture and Synergy Analysis of Three Main Cellulases

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Abstract

Endo-1, 4-β-glucanase (Eg), 1, 4-β-D-glucan cellobiohydrolase B precursor (CbhB) and 1, 4-β-glucosidase (Bgl) are major components of cellulase. The three enzymes synergistically hydrolyze cellulose to glucose. Cellulases with a poor ratio of, or unbalanced enzyme components do not have a noticeable effect. Therefore, it is crucial to optimize the composition of cellulase mixtures and promote the synergy between key enzymes. We isolated and purified Eg, CbhB and Bgl to determine the optimal ratio and effect of these three cellulases. First, we carried out a pairwise-cooperation experiment to determine the optimal synergetic ratio. We then determined the optimal degradation ratio. The optimal synergistic effect of Eg (34.38%), CbhB (33.35%) and Bgl (32.27%) was found with Design-Expert. Best coordination was 4.199°, which is close to the theoretical value of 4.206°. The degradation of filter paper and absorbent cotton was studied with different combinations of Eg, CbhB and Bgl. The progressive and binding rates followed the order (Eg + CbhB + Bgl) > Eg + CbhB > Bgl, indicating that the three-enzyme combination promotes cellulase activity synergistically, providing a theoretical basis for industrial production.

Keywords: Endo-1, 4-β-glucose (Eg); 1, 4-β-D-glucan cellobiohydrolose (Cbh); 1, 4-β-glucosidase (Bgl); Synergy analysis

Introduction

Cellulase is a highly active biocatalyst that has received a great deal attention toward the effective use of cellulose resources. It is considered to be extremely efficient for use as a potential biological tool. Furthermore, it is a renewable and environmentally friendly resource (Zhu *et al*., 2013). The composition of cellulose is very complex, and it is usually combined with a variety of other components, such as lignin, pectin, and phenolic compounds. Industrial application of cellulase for the biodegradation of cellulose is limited by high cost and low enzyme activity (Kumar *et al*., 2009; Fang *et al*., 2010). Therefore, improvement of cellulase activity is crucial. The mixed cellulase that is responsible for cellulose degradation to glucose is comprised of at least three components, Endo-1, 4-β-glucanase (Eg), 1, 4-β-D-glucan cellobiohydrolase (Cbh) and 1, 4-β-glucosidase (Bgl). These components work synergistically in the process of hydrolysis and reduce product feedback inhibition (Galbe and Zacchi, 2002). The lack of synergy hydrolysis force and the low enzyme activity which makes separate cellulase cannot achieve the requirements of industrial production (Nidetzky *et al*., 1994). Most research has focused on how to improve cellulase's hydrolytic activity by (1) screening of new cellulase-producing bacterial strains, (2) expressing and transforming a single cellulase through random mutation and genetic engineering of bacteria. However, none of these studies have paid much attention to optimization of the ratio of components in cellulase (Jørgensen *et al*., 2007). In fact, synergy and multicomponent ratios should be given high priority, especially in light of studies that have confirmed that the degradation efficiency of cellulase is closely related to these parameters.

To clarify the relationship between the cellulase components, this study used three single cellulase components (Eg, Cbh precursor B [CbhB] and Bgl), which were cloned and successfully expressed in *Pichia pastoris*, separated and purified. The purified component enzymes were used in a pairwise synergy study. Based on the results, Design-Expert software was used to analyze the optimum ratio of the three kinds of cellulase. This was combined with microscopy, scanning electron microscopy (SEM) and thin-layer chromatography to determine the activity of the optimum mix ratio on selected substrates (Irwin *et al*., 1993; Kim *et al*., 1998; Gusakov *et al*., 2007). It is our hope that this study will provide some guidance for industrial production work.

Materials and Methods

Experimental Materials

Eg, CbhB and Bgl originated from *Bacillus subtilis C-36*,

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Aspergillus niger Asp-524 and Aspergillus oryzae gif-10, respectively, which were isolated and preserved in our laboratory. *Pichia pastoris* strain X33, *Pichia pastoris* GS115, plasmid pPIC9k and vector pPICZαA were obtained from Invitrogen. The experimental substrates were purchased from Sigma (USA).

Cellulase Expression and Purification

The recombinant bacteria were grown in BMGY liquid medium at 30°C, with vigorous shaking at 250 rev/min for 24 h; cells were harvested by centrifugation at 1,500×*g* for 10 min and then the precipitates were resuspended in BMMY medium to an OD_{600} of 1.5–2. Expression of the recombinant cellulases was induced by adding 1% (v/v) methanol to the BMMY medium every 24 h, and then detected by SDS-PAGE (Armstrong, 1983; Bajorath *et al*., 1993; Boyle *et al*., 1998; Cregg *et al*., 2009).

For purification of the isolated Eg we used the pPIC9k carrier. We targeted gene and vector pPICzαA the band itself has His histidine tag in the construction of the early support with β-glucosidase and exoglucanase. The fused expression, to enable it to take advantage of His-tag protein with Ni^{2+}-specific adsorption capacity. Purification involved filtration, concentration, desalting the column (GE, USA) to wash away contaminating proteins, and protein content in the collected eluate peaks was determined at an absorbance of 280 nm. Finally, enzyme activity was determined.

Determination of Protein Content and Enzyme Activity

Protein content was determined by the Bradford method; we used bovine serum albumin (BSA) as a standard (Bradford, 1976). A glucose standard curve was developed as follows: absorbance was measured at 540 nm and plotted using the regression equation. To determine enzyme activity, a standard line was used to isolate glucose content (Santiago-Hernández *et al*., 2007). One unit (U) of enzyme activity was the amount of enzyme required to produce 1 μmol of reducing sugar per minute (Jeoh *et al*., 2002). Glucose assays were performed according to the GOD–POD method, using the following formula to calculate the amount of glucose:

$$\mathrm{Glu}(\mathrm{mmol}\cdot \mathrm{L}^{-1}) = \frac{\text{Samples OD}-\text{Blank OD}}{\text{Standards OD}-\text{Blank OD}} \times \text{Calibrator concentration}(5.55\mathrm{mmol}\cdot \mathrm{L}^{-1}).$$

Pairwise Synergistic Effect between the three Cellulases

To determine the optimal pairwise combinations of the different cellulases, total enzyme content was held constant, and the cellulase was reacted with Whatman filter paper as the substrate, at component ratios of 100:0, 25:75, 50:50, 75:25, and 0:100. The amount of substrate was 50 mg, the reaction system was 300 μL and temperature and pH values were set according to a stability study at 50°C and 6.8, respectively. The dinitrosalicylic acid (DNS) method was used to measure enzyme activity according to reducing sugars produced (Pavón-Orozco *et al*., 2012).

Synergy of the Three Cellulases

We used Design-Expert software to study the relationship between the synergistic-response surface variables among the three types of cellulase. The total proportion of mixed cellulase was limited to 100%. The three types of cellulase were provided at 0–100% to establish a surface model of continuous variables; the ratios of the mixed components and their interactions were evaluated according to the table while adding the set proportions of cellulase. Using a standard method to measure the amount of reducing sugars and glucose levels, we analyzed the degree of coordination, and used the relationship between the ratios of coordination degrees to generate a response-surface plot, revealing the optimal mix ratio.

Validation Study of Synergy

To determine and verify the effects of the time course of the three types of cellulase, two model substrates were used: Whatman No. 1 filter paper and absorbent cotton. These were added to equal volumes of Eg, Bgl or CbhB, or mixtures of two or three of these, and changes in substrate morphology were observed microscopically after 24 h. In addition, the degraded product was analyzed by thin-layer chromatography and the filter surface was analyzed by SEM (Zhou *et al*., 2009).

The Optimum Mixing Ratio for Cellulase Activity

The filter paper (50 mg) or the microcrystalline cellulose Avicel (1%) was added to 0.015mol/L of phosphate-buffered saline. A specified amount of enzyme solution containing Eg, Bgl or CbhB, or a mixture at the optimum ratio was then added to the substrate. After the reaction, the resultant sugar was precipitated and measured by DNS method.

Determination of the Optimum Mixing Ratio for Cellulase Binding

A specified amount of enzyme solution, held at 4°C, was added to either insoluble Avicel or filter paper as the substrate. BSA was added at 0.1 mg/mL and the reaction mixture was shaken at 20 rev/min for 1 h. The mixture was then centrifuged and residual enzyme activity was measured in the supernatant, in three replicates (Hägglund *et al*., 2003; Geng *et al*., 2012).

Determination of the Optimum Temperature and Thermostability for the Optimum Mixing Ratio of Cellulase

To determine the optimum temperature, 100 μL of enzyme solution was reacted with the filter substrate at 30–90°C, in 10°C increments. Standard methods were used to assay

the amount of reducing sugars. Use the relative activity masterpiece. To determine thermal stability, 100 μL aliquots of enzyme solution were incubated for 10 min in 50, 60, 70 or 80°C then placed on ice for 5 min. Residual enzyme activity was measured by standard methods. Additionally, use the relative activity masterpiece.

Results

Cellulase Expression and Purification

To separate and confirm the induced expression of the recombinant cellulases (Eg, Bgl and CbhB), eluates were collected from a DEAE-cellulose column for Eg, and from a Ni^{2+} column for Bgl and CbhB. Protein content was measured at OD_{280}. The results are shown in Fig. 1. Eg, Bgl and CbhB all showed a single elution peak. We collected these peaks and measured their enzyme activity: 10 μL of enzyme solution was added to 10 μL of protein loading buffer and boiled for 10 min, centrifuged at 12,000 *g* for 1 min, and the supernatant was discarded. The pellets were subjected to SDS-PAGE. The results are shown in Fig. 2. Each cellulase showed a single band, at approximately 58 kDa, 100 kDa and 60 kDa for Eg, Bgl and CbhB, respectively, slightly different from the predicted molecular mass. However, each band showed activity toward its specific substrate. Because yeast is a eukaryotic host strain, expressed proteins may have a certain degree of glycosylation, resulting in the secretion of a protein with a higher than predicted molecular mass. Based on the figure, the purification results were good and the isolates were used for further analysis.

Standard protein and glucose curves were used to determine protein content before and after the purification. Total enzyme activity was measured and the specific activity calculated for the purified cellulase components. The results are shown in Table 1. The results showed good purification and high enzyme activity. The synergistic action of these components could now be explored.

Pairwise Synergistic Effects for the Three Cellulases

Pairwise combinations of CbhB and Eg, Eg and Bgl, and CbhB and Bgl were tested at five different ratios: 100:0, 75:25, 50:50, 25:75, 0:100. These solutions were reacted with the model filter substrate under standard conditions, and reducing sugar content was measured to determine synergy. The results are shown in Figs. 3, 4 and 5. Degree of synergy was determined for all ratios of the three enzyme component combinations.

For the Eg and CbhB combination, the maximum release of reducing sugars occurred at a 50:50 ratio, reaching 125.47 ± 8.41 mg/mL, with a maximum degree of synergy of 2.68 (Fig. 3). For the Eg and Bgl combination, maximum release of reducing sugars was at the 25:75 ratio (Eg to Bgl), amounting to 59.65 ± 2.91 mg/mL, and a maximum degree of synergy of 2.17 (Fig. 4).

Table 1: Summary of purification of three types of cellulose

Name	Eg		Bgl		CbhB	
	Crude	Purified	Crude	Purified	Crude	Purified
Total protein (mg)	114.2	1.2	495.3	0.9	278.9	1.4
Total activity (U)	925.0	213.1	1287.8	114.0	55.8	4.9
Specific activity (U/mg)	8.1	177.6	2.6	126.7	0.2	3.5
Recovery (%)	100%	23.0%	100%	8.9%	100%	8.8%
Purification (fold)	1.0	21.9	1.0	48.7	1.0	17.5

Note: Each value represents the mean of triplicate measurements

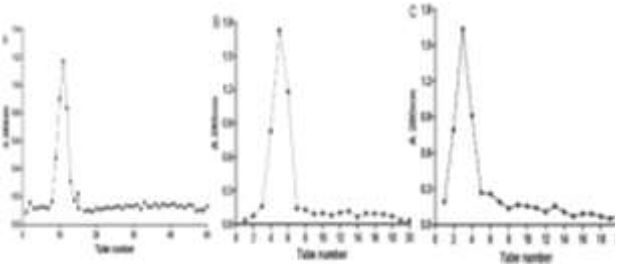

Fig. 1: The elution curve of three kinds of cellulase
A. Eg elution curve by DEAE-cellulose column chromatography. B. Bgl elution curve by Ni^{2+} column chromatography. C. CbhB elution curve by Ni^{2+} column chromatography

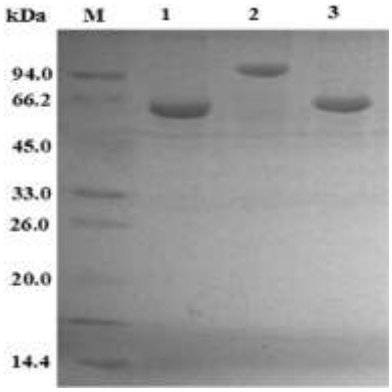

Fig. 2: SDS-PAGE of the three purified cellulases. Lanes: M, MW markers (low); 1, Eg; 2, Bgl; 3, CbhB

For the CbhB and Bgl combination, the maximum degree of synergy occurred at 75:25, respectively. Maximal release of reducing sugars was 59.43 ± 6.55 mg/mL, with maximum degree of synergy of 1.92 (Fig. 5). For 75% CbhB alone, release of reducing sugars amounted to 30.06 ± 2.91 mg/mL, while 25% Bgl alone showed no release of reducing sugars.

Synergy of the Three Cellulases

Synergy analysis of all three cellulases was performed using Design-Expert software to establish the ratios in the mix, setting a total combined ratio of 100%. The design features are shown in Table 2, modeled according to the relationship between ratio and degree of synergy based on Table 2.

Table 2: Summary of mixture design and quantities of glucose

Order	Eg (%)	CbhB (%)	Bgl (%)	Degree of synergy		Glucose production (mg/L)
				Experimental	Predicted	
1	100.00	0.00	0.00	1.000	0.998	4.1±1.13
2	0.00	0.00	100.00	1.000	1.142	0.0
3	16.67	16.67	66.67	3.275	2.878	55.5±1.20
4	0.00	100.00	0.00	1.000	0.839	7.7±0.85
5	50.00	0.00	50.00	1.688	1.828	41.6±2.23
6	16.67	66.67	16.67	2.344	2.856	26.5±4.59
7	0.00	50.00	50.00	1.714	1.695	42.5±1.63
8	66.67	16.67	16.67	2.920	2.954	44.6±4.04
9	33.33	33.33	33.33	4.266	4.194	35.5±4.53
10	50.00	50.00	0.00	2.229	2.066	8.6±0.26

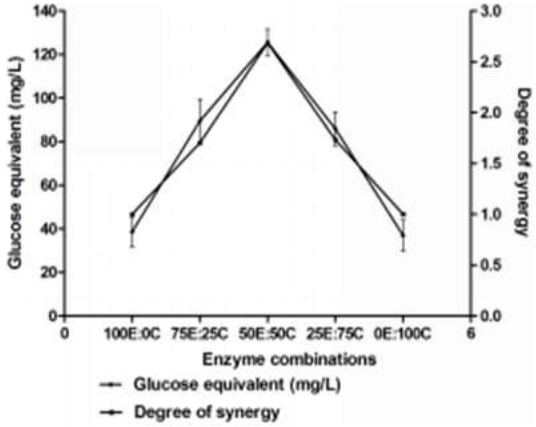

Fig. 3: Synergy between Eg and CbhB at different ratios. ● Glucose equivalent (mg/L), ■ degree of synergy. 100E:0C, 100% Eg; 75E:25C, 75% Eg:25% CbhB; 50E:50C, 50% Eg:50% CbhB; 25E:75C, 25% Eg:75% CbhB; 0E:100C, 100% CbhB. Synergy was quantified by reducing sugar release

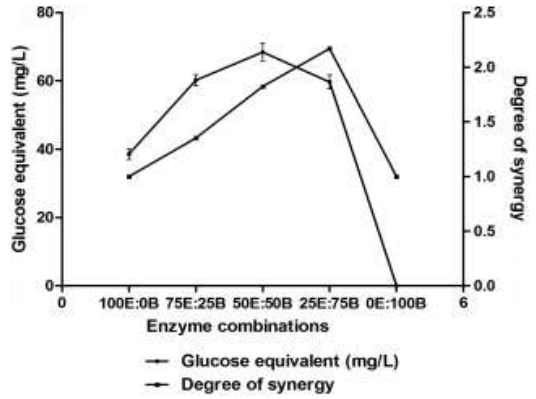

Fig. 4: Synergy between Eg and Bgl at different ratios. ● Glucose equivalent (mg/L), ■ degree of synergy. Explanation: 100E:0B, 100% Eg; 75E:25B, 75% Eg:25% Bgl; 50E:50B, 50% Eg:50% Bgl; 25E:75B, 25% Eg:75% Bgl; 0E:100B, 100% Bgl. Synergy was quantified by reducing sugar release

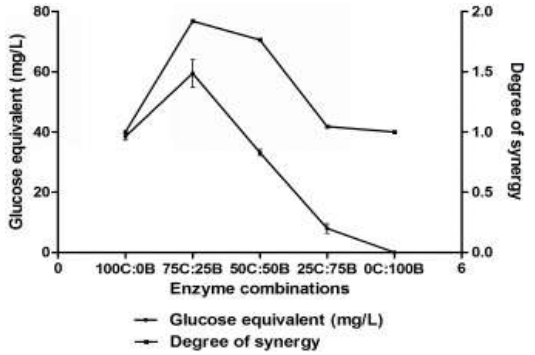

Fig. 5: Synergy between CbhB and Bgl at different ratios. ● Glucose equivalent (mg/L), ■ degree of synergy. Explanation: 100C:0B, 100% CbhB; 75C:25B, 75% CbhB:25% Bgl; 50C:50B, 50% CbhB:50% Bgl; 25C:75B, 25% CbhB:75% Bgl; 0C:100B, 100% Bgl. Synergy was quantified by reducing sugar release

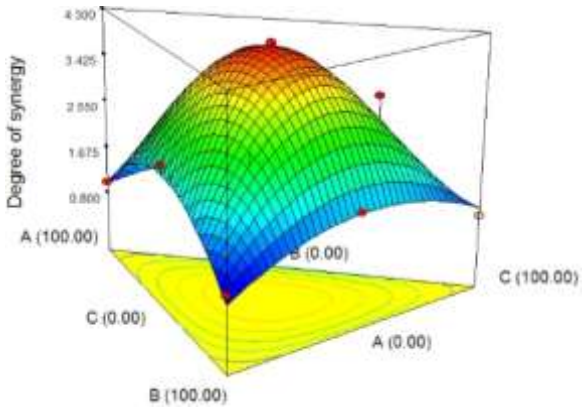

Fig. 6: Interaction of response surface for degree of synergy among the three types of cellulase. A, Eg; B, CbhB; C, Bgl

Fig 7: Degradation of filter paper and absorbent cotton with the three types of cellulase

(A) Degradation of filter paper after 24 h of incubation. (B) Degradation of filter paper after 48 h of incubation. (C) Degradation of absorbent cotton after 24 h. of incubation. (D) Degradation of absorbent cotton after 48 h. of incubation

Also used the Design-Expert to perform a stepwise regression and build a model according to the experimental value. The model stepwise regression equation was:

$$Y=0.99830\times A+0.83885\times B+1.14194\times C+4.59030\times AB+3.03248\times AC+2.81757\times BC+55.35529\times ABC \quad (1)$$

where A is Eg, B is CbhB, and C is Bgl. $R^2 = 0.9520$, $P < 0.05$. The results are shown in Table 3.

The effects of three different proportions of cellulase on the degree of synergy can be analyzed from the response surface model in Fig. 6. The three types of cellulase influenced each other, cooperating with one another to degrade the filter paper to reducing sugars.

The optimum ratio for the three cellulases in percentages was: Eg (34.38%), CbhB (33.35%) and Bgl (32.27%), with a predictive synergistic value of 4.206 degrees. The experimentally determined synergy of the composition was 4.199, very close to the theoretical value, validating the response surface analysis using the optimum ratio. As can be seen from Table 2, the amount of glucose produced was positively correlated with the amount of Bgl in the mixture. At 66.67% Bgl, glucose levels reached 55.5 ± 1.20 mg/L. However, the filter paper showed no degradation when Bgl was added alone.

Validation of Synergy

Eg, CbhB and Bgl were added separately, in that order, to the reaction system. Changes in the substrate were observed after 24 h and 48 h by microscopy and SEM. The results are shown in Figs. 7, 8, 9 and 10.

Fig. 7 shows the results of incubation with the three cellulases alone or in combinations of two or three added simultaneously. Overall, the filter paper showed more degradation at both time points (Fig. 7A, B) than (Fig. 7C, D). In fact, there was no degradation of cotton with any of the three cellulases or their combinations. Use of a single enzyme—Eg, CbhB or Bgl—could not degrade the filter paper effectively. Only Eg showed slight degradation of the filter paper after 24 h and 48 h, as reflected by solution turbidity. There were no significant visual changes with CbhB or Bgl, and the liquid did not become cloudy. The results of this analysis were in agreement with the previous activity and synergy degree tests. When two of the enzymes were mixed (in any combination), there was no clear change after 24 h, but a mixture of the three enzymes showed some degradation of the filter paper. In contrast, after 48 h, Eg+CbhB had degraded the filter paper to a paste, and Eg+Bgl and CbhB+Bgl had also degraded it to a gelatinized state. This phenomenon reflects the varying degrees of synergy between the enzymes. For the cotton group, there were no substantial changes after 24 h with any of the three cellulases. After 48 h, the rapid emergence of a large degree of filter papers past condition reaction. The effect was most obvious when the filter paper sank to the bottom of the tube, with no large particles upon shaking.

Table 3: Estimated regression coefficients and ANOVA for the experimental results

Source	Degree of freedom	Sum of squares	Mean squares	*F* value	*P* value
Model	6	10.23	1.71	9.92	0.0434
Linear-Mixture	2	0.027	0.013	0.078	
Residual	3	0.52	0.017		
Total	9	10.75			
					$R^2 = 0.9520$

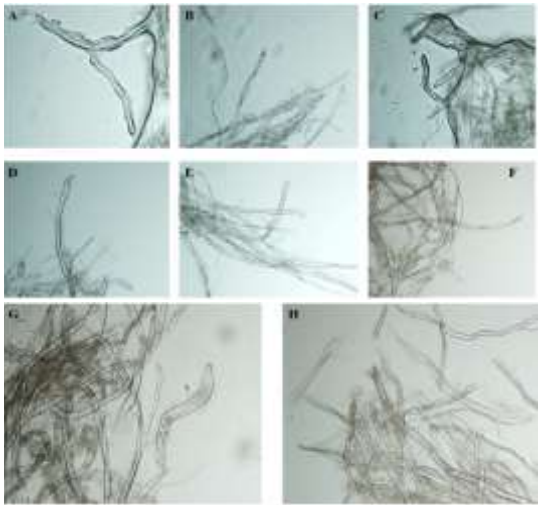

Fig. 8: Microscopic analysis of filter paper after 24 h of incubation. (A) CK (no cellulase, just water); (B) Eg; (C) CbhB; (D) Bgl; (E) Eg+CbhB; (F) Eg+Bgl; (G) CbhB+Bgl; (H) Eg+CbhB+Bgl. All photographs are ×400 magnification

To explore the more subtle morphological changes in the filter paper and cotton, they were examined under the microscope after 24 h (Figs. 8 and 9).

The filter paper showed clear differences after 24 h reaction with the three enzymes (Fig. 8). The original microstructure of the paper consisted of extremely tight spaces and a clear crystal structure. When Eg or CbhB was added, the structure loosened, there were many small fibers around the emergence and the filter paper began to break apart. This result was most pronounced when Eg, CbhB and Bgl were added together, with a large number of breakages and cellulose flock appearing. However, when Bgl was added alone, there were no obvious microstructural changes in the filter.

A more detailed SEM study was conducted on the synergistic effects of the cellulases on filter paper degradation after 24 h (Fig. 10). The filters with the Bgl or blank (no enzyme) solutions were not degraded. However, the other two cellulases, separately, caused a certain degree of degradation, with the filter paper becoming more layered, the surrounding fiber filaments larger, and the structure looser. When the three cellulase enzymes were mixed, the degree of degradation was highest.

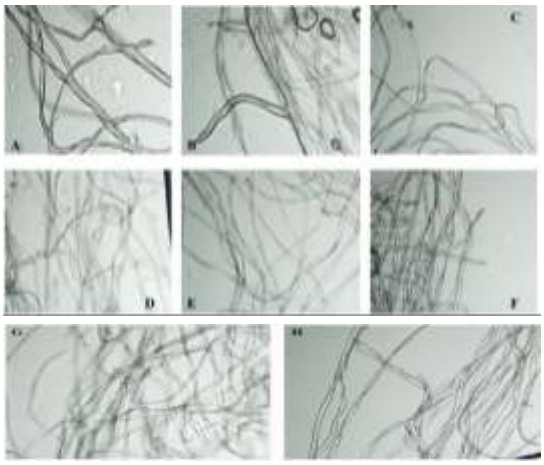

Fig. 9: Microscopic analysis of absorbent cotton after 24 h of incubation. (A) CK (no cellulase, just water); (B) Eg; (C) CbhB; (D) Bgl; (E) Eg+CbhB; (F) Eg+Bgl; (G) CbhB+Bgl; (H) Eg+CbhB+Bgl. All photographs are ×400 magnification

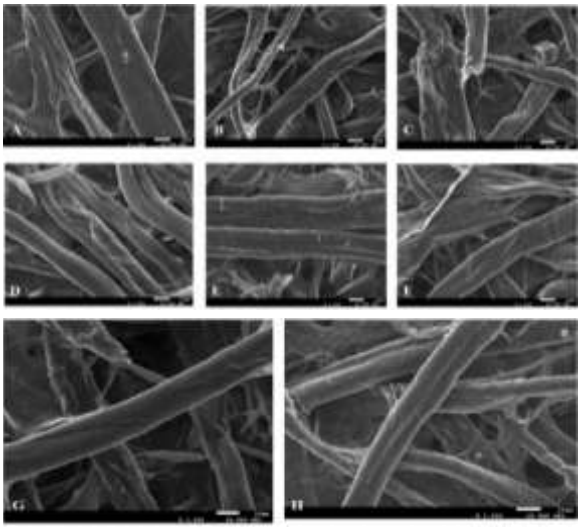

Fig. 10: SEM of filter paper after 24 h of incubation. (A) CK (no cellulase, just water); (B) Eg; (C) CbhB; (D) Bgl; (E) Eg+CbhB; (F) Eg+Bgl; (G) CbhB+Bgl; (H) Eg+CbhB+Bgl. All photographs are ×1000 magnification

This was consistent with the results of the visual analysis and microscopic observation showing varying degrees of synergy among the cellulases.

We centrifuged the reaction solutions of all cellulase enzyme combinations after 48 h with the filter paper at 10,000 *g* for 10 min to remove the sediment. The supernatant (5–10 μL) was subjected to thin-layer chromatography analysis of the degradation products (Fig. 11). When Eg was added alone, it produced a small amount of reducing sugar. The disaccharide content was higher, trailing the rest of the oligosaccharides to form a band,

Table 4: Cellulase processing on Avicel and filter paper

Enzyme	Avicel [a]	Filter paper [a]
Eg	21.2	5.9
CbhB	13.5	3.4
Bgl	-	-
Eg+CbhB+Bgl	44.7	12.1

[a]Processing ratio is defined as μmol soluble reducing sugar divided by μmol; insoluble sugar; - Not detected

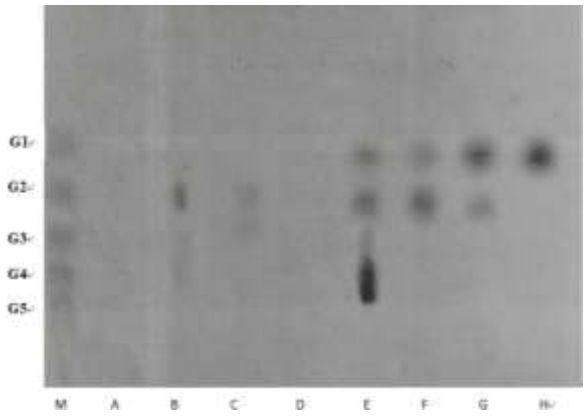

Fig. 11: Thin-layer chromatography of the products of filter paper degradation after 48 h of incubation. Lanes: M, glucose standard (G1: glucose; G2: cellobiose; G3: cellotriose; G4: cellotetraose; G5: cellopentaose). A, CK (no cellulase, just water); B, Eg; C, CbhB; D, Bgl; E, Eg+CbhB; F, Eg+Bgl; G, CbhB+Bgl; H, Eg+CbhB+Bgl

but not enough to produce. When CbhB was added alone, there was a small amount of disaccharide. There were no oligosaccharides when Bgl was added alone. When Eg was added with CbhB, monosaccharides, disaccharides, oligosaccharides, and a large number of other fibers appeared. In the pairwise combinations, those with Bgl produced a large number of monosaccharides and disaccharides, but no oligosaccharides. When Eg, CbhB and Bgl were added together, only simple sugars (in large amounts) were generated.

The Optimum Mixing Ratio of Cellulase Activity

Progressive cellulase performance is best described by the release of reducing sugars when it is adsorbed to a solid substrate. To better demonstrate the synergy among the three enzymes, Eg, CbhB and Bgl were added at their optimum mixing ratio. Results are given in Table 4. Eg and CbhB showed some progressive activity on insoluble substrate, with Eg being superior to CbhB on both plain and microcrystalline microfiber filter paper. However, Bgl did not show any progress on these two substrates. The degree of progress on both microcrystalline cellulose and paper followed the order (Eg + CbhB + Bgl) > Eg > CbhB > Bgl. When the three enzymes were mixed, their degradation progress increased significantly, indicating that they

promote each other's roles: the amount of progress with all three enzymes was higher than the sum of their individual rates of progress. This suggests that Bgl promotes their continued degradation of insoluble substrate, increases the progress, and plays a key role in the overall degradation.

Determination of Optimum Mixing Ratio for Cellulase Binding

The binding rate was measured separately with a ceramic microfiber substrate and filter elements; the results are given as ratio of residual activity (Table 5). Addition of Bgl effectively improved Eg and CbhB's binding ability.

Determination of the Optimum Temperature and Thermostability for the Optimum Mixing Ratio of Cellulase

In the temperature range of 30–90°C, in 10°C increments, Eg, CbhB, Bgl at their optimal ratios were reacted with substrate, and the optimum reaction temperature was determined. The results are shown in Fig. 12. Except for CbhB, the enzymes' optimum reaction temperature was 50°C. At that temperature, CbhB activity reached about 75%, then as the temperature further increased, enzyme activity declined rapidly.

The solutions were incubated for 10 min at 50, 60, 70 and 80°C, and residual activity was measured, then thermal stability was determined. The results, shown in Fig. 13, indicate that the thermal stability of the optimum mixing ratio for cellulase (mixture) is not much different from those of the single enzyme components; when the temperature exceeded 70°C, the residual activity was about 40%.

Discussion

Three cellulase proteins were isolated and purified through chromatographic columns, producing single bands of 58 kDa, 100 kDa and 60 kDa for Eg, Bgl and CbhB, respectively (Fig. 2). Their specific activities were 177.6 mg/mL, 126.7 mg/ mL and 3.5 mg/mL, respectively. The three purified cellulases were used in a pairwise synergy study, and results showed strong synergy among all cellulases. Eg+CbhB reached maximum synergy (maximum degree of coordination of 2.68) at a ratio of 50:50, possibly due to Eg shearing the β-1,4 glycoside bonds of cellulose from internal, dispersing filaments and releasing smaller fragments of cellulose to produce more crystalline cellulose chain ends for CbhB (Riedel *et al*., 2006). Thus, these two cellulases can effectively promote the hydrolysis of cellulose. Maximum synergy of Eg+Bgl occurred at a ratio of 25:75 (maximum degree of coordination was 2.17), and with CbhB+Bgl at 75:25 (maximum degree of coordination 1.92), However, only Bgl, alone, showed no release of reducing sugars. The degrees of coordination might have increased due to CbhB or Eg degradation of paper to generate a large amount of disaccharides,

Table 5: Binding of cellulase to insoluble substrates

Enzyme	Binding rate (%[a])	
	Avicel	Filter paper
Eg	44.9 ± 4.7	26.5 ± 1.6
CbhB	28.0 ± 3.0	13.0 ± 0.6
Bgl	-	-
Eg+CbhB+Bgl	73.9 ± 3.9	44.9 ± 4.4

± Standard deviations

[a]Percentage of enzyme bound to the substrate; - Not detected

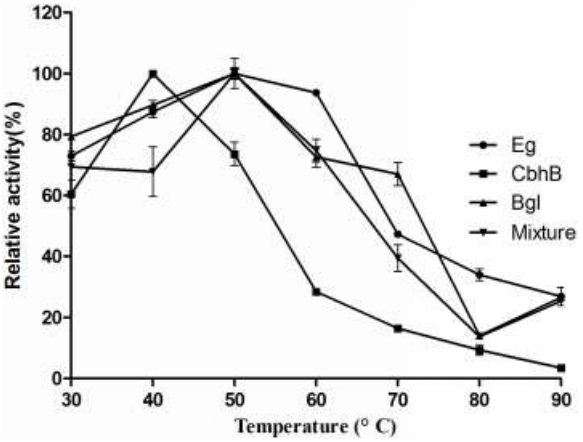

Fig. 12: Optimal temperature for the three types of cellulase. Mixture: Eg+CbhB+Bg

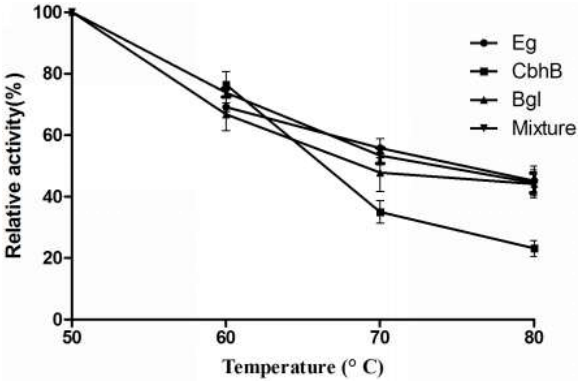

Fig. 13: Thermostability of the three types of cellulase. Mixture: Eg+CbhB+Bgl

allowing Bgl to play its role in reducing sugar release, and resulting in decreased release of reduced disaccharides, such as glucose and disaccharide sugar, and lowering of the anti-substrate inhibition of cellulase activity caused by disaccharide sugar, promoting further hydrolytic reactions. Results of this study are consistent with those of Saibi and Gargouri (2011). Based on these results and Design-Expert software, we obtained the optimum synergy ratio of Eg (34.38%), CbhB (33.35%) and Bgl (32.27%), which had a predicted synergy value of 4.206, and a practical value of 4.199, close to the predicted value. Current research in this area is focused on the best optimization of multiple sources

of cellulase, such as Zhou *et al*. (2009) who optimized six different sources of Eg and found the optimal mixing ratio for steam-exploded corn core degradation. Liu *et al*. (2011) increased hydrolysis of substrate by optimizing the thermal stability of cellulase derived from *Clostridium thermocellum*. Many studies have also focused on improving the hydrolysis of lignocellulose, or improving cellulase synergistic activity by adding xylan enzymes. Our experiment assessing glucose content at optimal enzyme ratios showed a positive correlation between the amount of glucose produced and the amount of Bgl added, thus proving that Bgl can reduce the inhibition of Eg and CbhB activity caused by the disaccharide product. This study demonstrates that cellulase activity can be improved by synergy, establishing a theoretical foundation for industrial applications.

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