

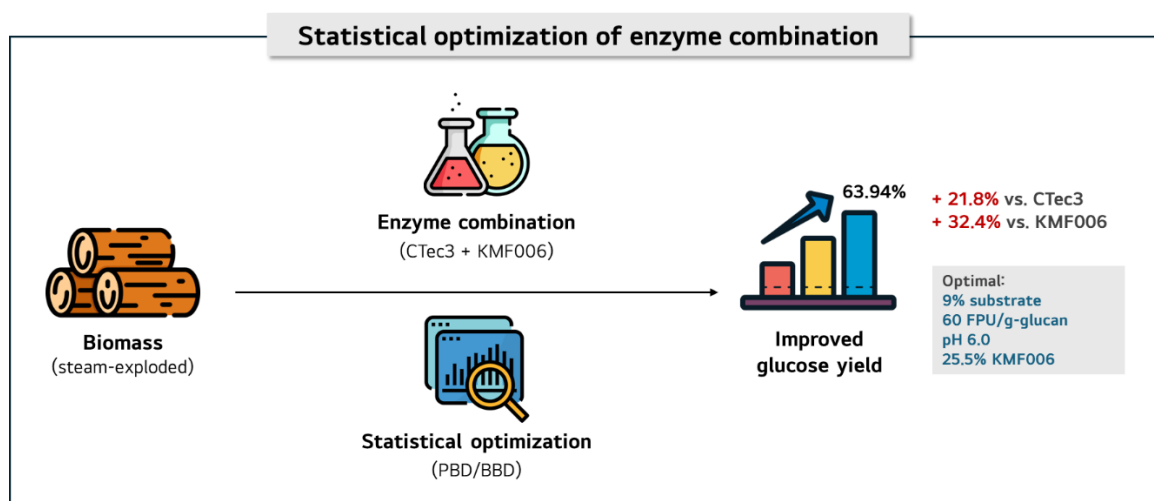
Statistical Optimization of a *Trichoderma*-based Enzyme Combination for Saccharification of Steam-exploded Lignocellulosic Biomass

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GRAPHICAL ABSTRACT



Statistical Optimization of a *Trichoderma*-based Enzyme Combination for Saccharification of Steam-exploded Lignocellulosic Biomass

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This study aimed to determine the optimal enzyme combination conditions for improving the saccharification efficiency of softwood biomass (*Larix kaempferi*). For this purpose, cellulase derived from *Trichoderma* sp. KMF006 was combined with a commercial enzyme (Cellic® CTec3). Comparative hydrolysis experiments with individual enzymes showed that *L. kaempferi* exhibited a lower glucose yield than hardwood, suggesting the need for a complementary enzyme combination. A Plackett-Burman Design (PBD) was used to identify significant variables, including substrate concentration, enzyme loading, pH, and the KMF006 blending ratio. The significant factors were further optimized using a Box-Behnken Design (BBD). The optimal conditions were determined to be a substrate concentration of 9% (w/v), enzyme loading of 60 FPU/g-glucan, pH of 6.0, and the KMF006 blending ratio of 25.5%. The predicted maximum glucose yield under these conditions was 63.9%, representing a 21.8% increase compared to CTec3 alone and a 32.4% increase compared to KMF006 alone. These results suggest that up to 25% of the commercial enzyme dosage can be substituted with KMF006 without compromising hydrolysis performance. Overall, this study demonstrates the feasibility of an enzyme combination approach for enhancing softwood saccharification.

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Keywords: Enzyme combination; Cellulase production; Softwood saccharification; *Trichoderma* sp.

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INTRODUCTION

Lignocellulosic biomass (LCB) is the most abundant renewable organic resource on the planet and has gained increasing attention as a sustainable raw material for the production of bio-based products (Srivastava *et al.* 2020; Ilić *et al.* 2023). LCB generally consists of cellulose (40 to 50%), hemicellulose (20 to 40%), and lignin (20 to 30%), which are intricately combined through physical and chemical interactions to form structural barriers that limit enzyme accessibility (Contreras *et al.* 2020). Among the types of LCB, softwood generally contains a higher lignin content and exhibits more extensive lignin cross-linking than hardwood, rendering it more recalcitrant to enzymatic hydrolysis (Rahikainen *et al.* 2011; Raulo *et al.* 2021). As a result, softwood often shows lower bioconversion efficiency even when enzyme dosages are comparable to those sufficient for other substrates, a limitation largely attributed to its lignin content (Raulo *et al.* 2021). In particular, lignin residues from softwood have been reported to exert stronger inhibitory effects on enzymatic hydrolysis than lignins derived from hardwoods or grasses (Nakagame *et al.* 2010). To overcome such recalcitrance, pretreatment processes are

required to disrupt these structural and compositional impediments, followed by enzymatic hydrolysis to release fermentable sugars (Du *et al.* 2020). The effectiveness of pretreatment is often quantified by the severity factor, which integrates temperature and residence time into a single index. Values between 4.0 and 5.0 are generally considered favorable, as they reflect a balance between sufficient structural disruption and minimal sugar degradation (McMillan *et al.* 2011; Nitsos *et al.* 2013; Balan *et al.* 2020).

The enzymatic hydrolysis of LCB is mainly carried out by three core cellulases, namely endoglucanase (EG), cellobiohydrolase (CBH), and β -glucosidase (BGL), which cleave internal bonds, remove terminal units, and convert cellobiose into glucose, respectively (Contreras *et al.* 2020; Du *et al.* 2020). However, cellulase production remains one of the most expensive steps in the overall LCB bioconversion process, contributing up to approximately 20% of the total cost (Srivastava *et al.* 2020). Commercial cellulase products can be advantageous in terms of consistency and proven activity, but they often show limited substrate specificity and reduced efficiency under high-solids loading conditions (Adsul *et al.* 2020). In contrast, microbial-derived cellulases have shown high adaptability to specific substrates (Lopes *et al.* 2018; Srivastava *et al.* 2020). Therefore, enzyme combinations involving both commercial and microbial enzymes have been reported to improve saccharification yields in various types of LCB (Suwannarangsee *et al.* 2012; Wang *et al.* 2012; Braga *et al.* 2014; Ji *et al.* 2014). These mixtures often exhibit synergies that surpass the theoretical sum of the individual enzyme performance (Contreras *et al.* 2020). Nevertheless, optimization of such combinations is complicated by the interaction effects of variables, such as enzyme loading, pH, enzyme blending ratio, and substrate composition. Therefore, statistical experimental design is usually required to establish optimal conditions (Lopes *et al.* 2018; Contreras *et al.* 2020).

Trichoderma sp. strain KMF006 is a fungal strain identified and characterized by this research group, which has been previously reported to produce a wide range of cellulolytic enzymes with high hydrolytic activity (Myeong and Yun 2024; Myeong *et al.* 2025). In particular, the EG and BGL activities of this fungus greatly contribute to the conversion of cellulose to glucose. Previous studies have demonstrated the saccharification performance of the KMF006 cellulase using *Quercus variabilis* (hardwood) and *Larix kaempferi* (softwood) as substrates under steam-exploded conditions, where notably lower glucose yields were observed for softwood (Myeong and Yun 2024). In this study, this trend was re-evaluated through direct comparison with commercial enzymes under standardized hydrolysis conditions. These findings underscored the persistent challenge of softwood recalcitrance, which has not been fully addressed by conventional commercial preparations.

Previous studies have demonstrated that enzyme combinations, in which commercial cellulases are supplemented with microbial-derived preparations, can substantially improve saccharification yields. Such effects have been reported across different lignocellulosic substrates (Suwannarangsee *et al.* 2012; Wang *et al.* 2012; Braga *et al.* 2014). Building on this strategy, the present study aims to evaluate the enzymatic hydrolysis performance of enzyme combinations using *L. kaempferi* as a representative substrate. The combination consisted of cellulases derived from *Trichoderma* sp. KMF006 and a commercial enzyme (Cellic® CTec3). KMF006, a cellulase-producing strain developed by our group, exhibits high EG and BGL activities that enhance cellulose-to-glucose conversion (Myeong and Yun 2024; Myeong *et al.* 2025), providing complementary potential when combined with commercial preparations. To determine optimal hydrolysis conditions, a Plackett-Burman Design (PBD) was employed to screen

key factors affecting glucose yield, followed by response surface optimization using a Box-Behnken Design (BBD). This approach demonstrates the effectiveness of integrating a newly developed microbial enzyme with a commercial preparation. It also emphasizes a practical strategy for improving saccharification efficiency in softwood, with potential applicability to other lignocellulosic feedstocks.

EXPERIMENTAL

Selection of Biomass and Steam Explosion Pretreatment

To encompass both major categories of lignocellulosic biomass, *L. kaempferi* and *Q. variabilis* were selected as representative softwood and hardwood species, respectively. Steam explosion pretreatment was carried out using a customized batch-type pilot unit (Youlim High Tech, Daegu, Republic of Korea), which was constructed based on the Masonite steam explosion technology. For each treatment, approximately 10 to 20 kg of air-dried wood chips were loaded into the reactor and exposed to saturated steam at 225 °C and 25 kgf·cm⁻² for 13 min. After the holding time, the reactor was rapidly depressurized to induce explosive decompression of the biomass structure. The pretreated biomass was subsequently cooled to 40 °C and filtered to recover the solid fraction.

The severity factor of the pretreatment was calculated using Eq. 1, as proposed by Overend and Chornet (1987),

$$\text{Log}R_0 = \log(t \cdot \exp((T-100)/14.75)) \quad (1)$$

where R_0 is the severity factor, t is the residence time (min), and T is the reaction temperature (°C). The severity factor calculated under the given conditions was 4.79, which falls within the range previously reported to result in high enzymatic hydrolysis efficiency (McMillan *et al.* 2011; Nitsos *et al.* 2013; Balan *et al.* 2020). The solid recovery after steam explosion pretreatment was approximately 87 to 98%.

The chemical composition of the pretreated biomass was determined in accordance with the National Renewable Energy Laboratory (NREL) technical report “Determination of Structural Carbohydrates and Lignin in Biomass” (NREL/TP-510-42618) (Sluiter *et al.* 2008). Extractives were determined according to the NREL protocol (NREL/TP-510-42619) using a two-step extraction with water (HPLC grade) and ethanol (Sluiter *et al.* 2005). These compositional analyses were carried out at Gyeongsang National University (Republic of Korea), and the resulting dataset was provided for the present study. The results are presented in Table 1.

Table 1. Chemical Composition of Biomass Pretreated by Steam Explosion

Biomass	Component Ratio (%)					
	Glucose	Xylose	Arabinose	Lignin ^a	Extractive	Total ^b
<i>Larix kaempferi</i> (softwood)	46.76	7.12	0.42	29.66	18.26	102.22
<i>Quercus variabilis</i> (hardwood)	61.76	4.09	0.17	17.06	17.82	100.90
Steam-explosion pretreatment conditions: 225°C and 25 kgf/cm ² for 13 min						
^a The lignin values are reported as total lignin (mainly acid-insoluble fraction)						
^b Minor deviations from 100% are due to rounding errors						

Enzyme Preparation

To assess the enzymatic hydrolysis performance of each cellulase before enzyme combination, a comparative analysis was conducted between the previously derived cellulase from *Trichoderma* sp. strain KMF006 and the commercial enzyme Cellic® CTec3 (Novonesis A/S, Bagsværd, Denmark). Although the activity of KMF006 has been previously reported (Myeong and Yun 2024; Myeong *et al.* 2025), this experiment aimed to directly compare its effectiveness against the commercial counterpart under identical conditions.

The KMF006 cellulase was produced from *Trichoderma* sp. strain KMF006, a fungal strain previously reported to exhibit high cellulolytic activity (Myeong and Yun, 2024). The strain was first precultured on malt extract agar (MEA), then transferred to potato dextrose broth (PDB) for liquid culture. Subsequently, cellulase was produced in a 7-L stirred-tank bioreactor (working volume of 4 L) using a defined medium composed of yeast extract (10 g·L⁻¹), KH₂PO₄ (5 g·L⁻¹), K₂HPO₄ (5 g·L⁻¹), MgSO₄·7H₂O (3 g·L⁻¹), and microcrystalline cellulose (Avicel, 20 g·L⁻¹), with an initial pH of 5.0. After autoclaving at 121 °C for 30 min and cooling to room temperature, 5% (v/v) preculture was inoculated. The culture was incubated at 31.3 °C with agitation at 150 rpm and aeration of 2 L·min⁻¹ for 17 d (Myeong and Yun 2024).

After fermentation, the culture broth was filtered and concentrated, and cellulase activity was determined based on filter paper units (FPU) according to established protocols (Myeong and Yun, 2024). Briefly, the filtrate was collected using Whatman No. 1 filter paper and concentrated to 1/60 of its original volume using Amicon® Stirred Cells (UFSC40001; Millipore Corp., Darmstadt, Germany) equipped with a 10-kDa polyether sulfone membrane.

Comparative Enzymatic Hydrolysis

Steam-exploded lignocellulosic biomass derived from *L. kaempferi* and *Q. variabilis* was used as the substrates. The moisture content of each pretreated biomass was measured using a halogen moisture analyzer (Mettler-Toledo International Inc., Columbus, OH, USA). Based on the measured moisture content (75 to 85%), the substrate amount was adjusted to achieve a final solid concentration of 7% (w/v). The prepared substrates were placed into glass tubes and sterilized by autoclaving at 121 °C for 30 min.

After cooling to room temperature, 0.1 M sodium citrate buffer (pH 5.0) and each enzyme preparation were added to reach a final enzyme loading of 40 FPU·g-glucan⁻¹. To prevent microbial contamination during hydrolysis, 0.05 mL of 2 % (w/v) sodium azide solution (final concentration of 0.02%, w/v) and polysorbate 80 (Tween 80, 100 mg·g-glucan⁻¹) were also added. The enzymatic hydrolysis reaction was carried out in a total reaction volume of 5 mL at 50 °C and 250 rpm for 72 h. After incubation, the reaction mixtures were heated at 100 °C for 30 min to terminate enzyme activity, followed by centrifugation at 13,000 rpm for 10 min. The supernatants were collected and filtered through a 0.2 µm syringe filter prior to glucose quantification.

Glucose concentrations were analyzed by HPLC and used to calculate glucose yield (GY, %) according to Eq. 2,

$$\text{Glucose Yield (GY, \%)} = (P_{glu}/S_{glucan}) \times 0.9 \times 100 \quad (2)$$

where P_{glu} is the amount of glucose released from enzymatic hydrolysis (mg·mL⁻¹), and S_{glucan} is the glucan content in the substrate (mg·mL⁻¹). The factor 0.9 reflects the

stoichiometric conversion from glucan to glucose under complete hydrolysis (Sluiter *et al.* 2008).

The glucan concentration of the substrate (S_{glucan}) was calculated based on the amount of substrate added, the solid content, and the glucan percentage in the dry matter, as shown in Eq. 3.

$$S_{glucan} \text{ (mg/mL)} = \frac{\text{Substrate (g)} \times (\text{solid (\%)/100}) \times (\text{glucan (\%)/100})}{\text{Total volume (mL)}} \times 1000 \quad (3)$$

The glucan composition of each biomass was determined *via* acid hydrolysis following the NREL protocol (NREL/TP-510-42618) (Sluiter *et al.* 2008), and the solid content was estimated based on the measured moisture content.

Analytical Procedure

High-performance liquid chromatography (HPLC) was performed to quantify the glucose concentration produced during enzymatic hydrolysis. Supernatants obtained from the hydrolyzed samples were filtered through a 0.2 μm syringe filter (PTFE-W; Biofact, Daejeon, South Korea) prior to analysis. The samples were diluted 10 to 20 fold to ensure accurate quantification. Sample preparation followed the NREL protocol (NREL/TP-510-42618) (Sluiter *et al.* 2008). The analysis was performed using an HPLC system equipped with a refractive index detector (RID-20A; Shimadzu, Kyoto, Japan). Glucose was separated using an Aminex® HPX-87P column (Bio-Rad, Hercules, CA, USA), with deionized water as the mobile phase. The flow rate was maintained at 0.6 $\text{mL} \cdot \text{min}^{-1}$, and the injection volume was 20 μL . Each run was completed within 20 min. The glucose concentration was calculated based on a standard calibration curve.

Screening of Significant Factors using Plackett-Burman Design (PBD)

A Plackett-Burman Design (PBD) was employed to identify significant factors affecting glucose yield (%) prior to optimization. The variables considered in the screening included substrate concentration (% w/v), enzyme loading (FPU), pH, the blending ratio between KMF006 and Cellic® CTec3 (% v/v), and the concentration of polysorbate 80 (Tween 80, $\text{mg} \cdot \text{g-glucan}^{-1}$). Each factor was evaluated at three levels (low, center, and high), as summarized in Table 2. The experimental design consisted of 20 combinations, including one center point, which were duplicated to yield a total of 42 experimental runs. The experiments were conducted in two blocks (Table A1).

Based on the PBD results, variables with statistically significant effects on glucose yield ($p < 0.05$) were selected for subsequent optimization using the Box-Behnken Design (BBD). All experimental designs and statistical analyses were performed using Minitab statistical software version 21 (Minitab LLC., State College, PA, USA).

Table 2. Experimental Levels of Variables Applied in the Plackett-Burman Design (PBD) for Enzyme Blending Optimization

Code	Variables	Unit	Low (-)	Center (0)	High (+)
A	Substrate concentration	% (w/v)	5	7	9
B	Enzyme loading	FPU	20	40	60
C	pH	-	4	5	6
D	KMF blending ratio	% (v/v)	25	50	75
E	Tween 80 concentration	mg/g-glucan	0	50	100

Optimization of Enzyme Combination using Box-Behnken Design (BBD)

To optimize the saccharification performance of the enzyme combination, a Box-Behnken Design (BBD), a type of Response Surface Methodology (RSM), was employed. The selected variables—substrate concentration (% w/v), enzyme loading (FPU), pH, and the blending ratio of KMF006 (% v/v)—were identified as significant in the prior PBD analysis. In contrast, polysorbate 80 was excluded from the subsequent optimization as it showed no statistically significant effect on glucose yield ($p > 0.05$) in the screening phase. The experimental levels of each factor are presented in Table 3.

A total of 27 experimental conditions were generated, each conducted in duplicate, resulting in 54 experimental runs. These were organized across three blocks (Table A2). Based on the BBD results, regression analysis was performed to evaluate the goodness-of-fit and statistical significance of the predictive model. The derived regression equation was subsequently used to determine the optimal conditions for maximizing saccharification yield. Experimental design and statistical analysis were conducted using Minitab statistical software version 21 (Minitab LLC) and Design-Expert version 13 (Stat-Ease Inc., Minneapolis, MN, USA).

Table 3. Experimental Levels of Variables Used in the Box-Behnken Design (BBD) for Enzyme Blending Optimization

Code	Variables	Unit	Low (-)	Center (0)	High (+)
A	Substrate concentration	% (w/v)	5	7	9
B	Enzyme loading	FPU	20	40	60
C	pH	-	4	5	6
D	KMF blending ratio	% (v/v)	25	50	75

RESULTS AND DISCUSSION

Comparative Saccharification Performance of Individual Enzymes

To evaluate the saccharification performance of the cellulase derived from *Trichoderma* sp. KMF006, comparative enzymatic hydrolysis was performed using steam-exploded *L. kaempferi* and *Q. variabilis* as substrates. The chemical compositions of the steam-exploded biomasses are summarized in Table 1. *Q. variabilis* showed a higher glucan content (61.8%) and a lower lignin content (17.1%) compared to *L. kaempferi*, indicating more favorable structural properties for enzymatic hydrolysis. As shown in Fig. 1, both enzymes achieved high glucose yields when *Q. variabilis* was used as the substrate. The glucose yield obtained with the commercial enzyme (Cellic® CTec3) was $78.8 \pm 5.7\%$, whereas independent two-sample Student-t tests indicated that there was no statistically significant difference in the yield obtained with KMF006 cellulase at $85.9 \pm 4.3\%$ ($p > 0.05$). These results indicate that the cellulase developed from KMF006 achieved similar levels of glucose recovery performance to the commercial enzyme.

In contrast, saccharification of *L. kaempferi* significantly reduced the conversion efficiency. The glucose yield of Cellic® CTec3 was $52.5 \pm 5.3\%$ and that of KMF006 was $48.3 \pm 0.3\%$. According to independent two-sample Student-t tests, there was no statistically significant difference between them ($p > 0.05$). This performance reduction was attributed to the unfavorable structural properties of *L. kaempferi*, such as its low glucan content (46.8%) and high lignin content (29.7%), which greatly increase the resistance to enzymatic degradation. Based on these findings, *L. kaempferi* was selected as the target

substrate for subsequent screening and optimization studies, given its potential for improved saccharification through enzyme combination formulation.

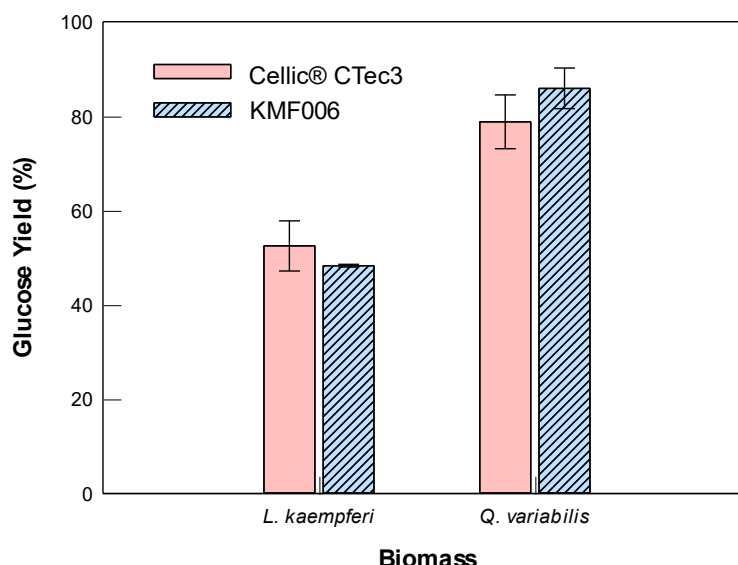


Fig. 1. Glucose yield (%) from steam-exploded lignocellulosic biomass hydrolyzed with Cellic® CTec3 and KMF006

Screening of Significant Factors Affecting Saccharification Yield

Larix kaempferi, which exhibited a lower glucose yield compared to *Q. variabilis* when treated individually with either Cellic® CTec3 or KMF006 (Fig. 1), was selected as the target substrate to enhance saccharification efficiency *via* enzyme combination formulation. Prior to optimization, a Plackett-Burman Design (PBD) was employed to identify significant factors that influenced glucose yield. The results of the saccharification experiments conducted based on the PBD are presented in Table A1 and Fig. 2a. Depending on the levels of the factors tested, the glucose yield ranged from 31.8% to 63.4%. These values exceeded those obtained with individual enzyme treatments (48.3 to 52.5%, Fig. 1) under certain conditions, suggesting that enzyme blending has the potential to improve saccharification performance.

Figure 2b shows the standardized effects of each variable in a Pareto chart. The baseline at $p = 0.05$ (corresponding to a t -value of 2.03) was used to identify the statistically significant factors. Enzyme loading (B) showed the greatest standardized effect (7.69), followed by substrate concentration (A, 5.66), pH (C, 4.53), and the blending ratio of KMF006 (D, 2.40). In contrast, polysorbate 80 concentration (E) was considered statistically insignificant, with a standardized effect value of 0.97, which was lower than the significance threshold.

Accordingly, Fig. 2c shows the main effect plots for four significant variables (A–D), excluding polysorbate 80. This chart connects the low and high levels of average glucose yields for each factor, while the dotted line represents the overall average yield across all experimental conditions and the red rectangle represents the average yield at the center point. Enzyme loading (B) showed the most prominent main effect, with a clear increasing trend in glucose yield as the level increased. Substrate concentration (A) and pH (C) also exhibited positive effects, with higher levels corresponding to higher yields. In contrast, the KMF006 blending ratio (D) had a negative effect, where the glucose yield decreased as the ratio increased. This suggests that maintaining an appropriate balance

between enzyme components is critical for achieving efficient saccharification. For all four factors, the center point (red square) clearly deviated from the straight line connecting the mean at low and high levels, suggesting that the relationship between these variables and the glucose yield may be nonlinear.

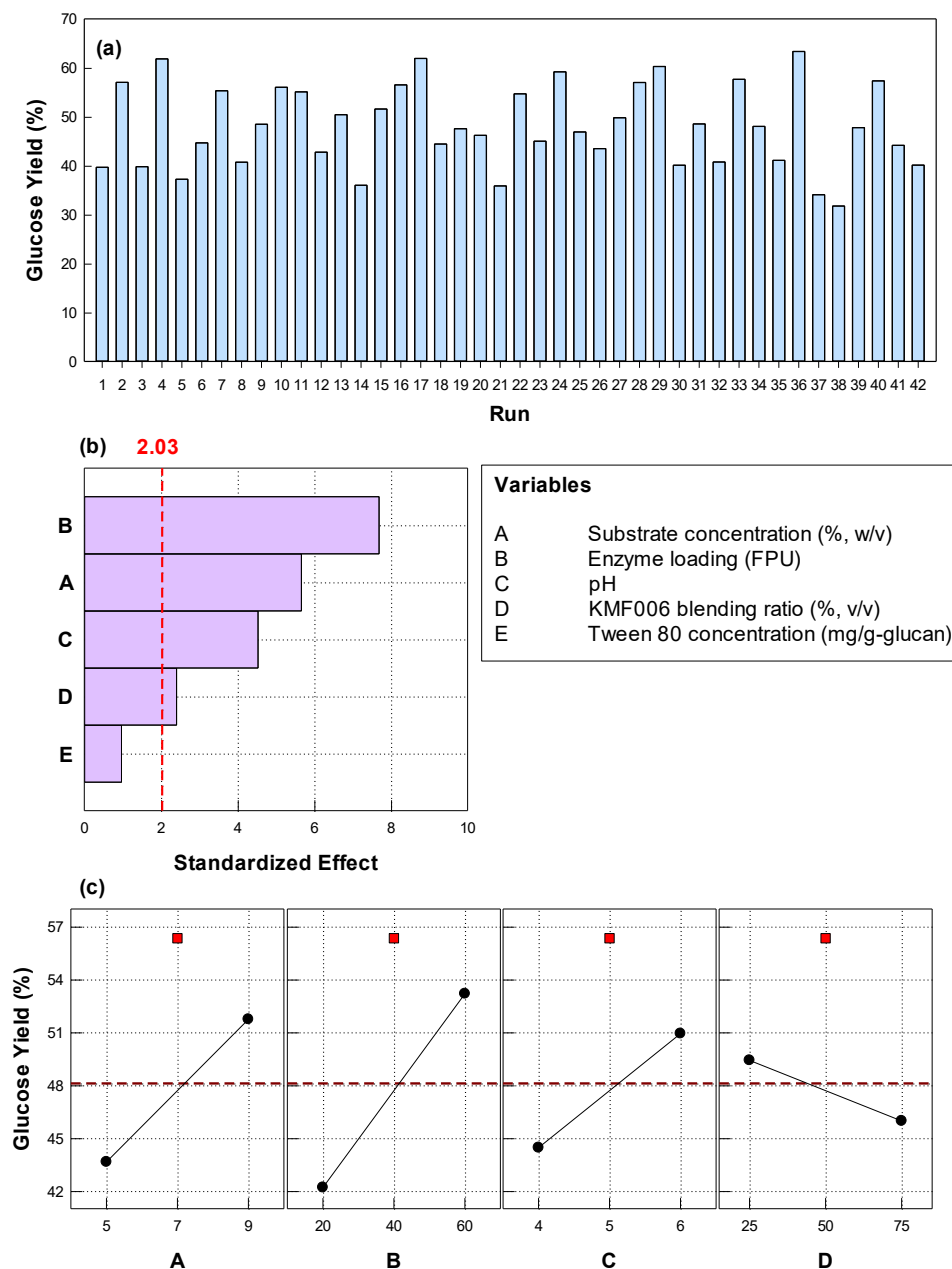


Fig. 2. Experimental results and effect analysis based on the Plackett-Burman Design (BBD). (a) Glucose yield (%) obtained from 42 experimental runs. (b) Standardized Pareto chart indicating the relative effect of each variable on glucose yield. The red dashed line at 2.03 represents the threshold for statistical significance ($\alpha = 0.05$). (c) Main effect plots for variables A–D. Each line represents the mean glucose yield at the low and high levels. Red squares indicate center point values, and the overall average is shown as a horizontal dashed line.

This nonlinearity was supported by the statistical results summarized in Table 4. The p -value for curvature was 0.001, indicating that a linear model alone could not

adequately describe the response and that a quadratic model was statistically required. Furthermore, all four variables, namely substrate concentration (A), enzyme loading (B), pH (C), and the KMF006 blending ratio (D), were found to be significant factors at the $p < 0.01$ level. On the other hand, the polysorbate 80 concentration (E) was not significant ($p = 0.192$). The block effect was also statistically insignificant ($p = 0.928$), suggesting that the effect of inter-block variability on glucose yield was minimal. The overall regression model was statistically significant ($p < 0.001$), with a coefficient of determination (R^2) of 87.45%, indicating that the model could acceptably explain the variability in the response.

Table 4. Regression Analysis of Plackett-Burman Design (BBD) for Glucose Yield (%)

Variation	DF	Adj SS	Adj MS	F-value	p-value
A	1	654.16	654.16	60.55	<.001
B	1	1206.26	1206.26	111.66	<.001
C	1	419.13	419.13	38.80	<.001
D	1	117.99	117.99	10.92	.002
E	1	19.15	19.15	1.77	.192
Block	1	0.09	0.09	13.18	.928
Curvature	1	142.36	142.39	0.01	.001
Model	7	2559.17	365.60	33.84	<.001
$R^2 = 87.45\%$ A: substrate concentration (% w/v), B: enzyme loading (FPU), C: pH, D: KMF006 blending ratio (% v/v), E: Tween 80 concentration (mg/g-glucan)					

Optimization of Enzyme Combination Using Response Surface Methodology (RSM)

Based on the results of the PBD, four significant factors (substrate concentration, enzyme loading, pH, and the blending ratio of KMF006) were selected for further optimization. To enhance the saccharification performance through enzyme combinations, a total of 54 experimental runs were performed to evaluate the glucose yield under each condition using the Box-Behnken Design (BBD) (Table A2). Consequently, glucose yield ranged from 36.5% to 61.7%, with the highest yield observed in Run 37 (substrate concentration: 9%, enzyme loading: 60 FPU, pH: 5.0, KMF006 blending ratio: 50%) and the lowest yield in Run 33 (substrate concentration: 7%, enzyme loading: 20 FPU, pH: 4.0, KMF006 blending ratio: 50%). These results highlight the significant effects of enzyme combination and condition optimization on saccharification efficiency.

Regression analysis results are presented in Table 5. The overall regression model was statistically significant ($p < 0.001$), with a coefficient of determination (R^2) of 92.5%, indicating a high level of explanatory power. The adjusted R^2 value and predicted R^2 value showed less than a 20% difference at 89.6% and 83.7%, respectively, supporting the predictive reliability of the model (Nisar *et al.* 2020; Abdullah *et al.* 2021). The lack-of-fit test results showed that the F -value was 1.33 and the p -value was 0.2638, indicating that the model adequately fit the experimental data (Nisar *et al.* 2020).

Table 5. Regression Analysis of Box-Behnken Design (BBD) for Glucose Yield (%)

Variation	DF	SS	Coefficient	F-value	p-value
A	1	162.47	3.44	45.88	<0.001
B	1	144.26	2.21	40.74	<0.001
C	1	170.07	3.52	48.03	<0.001
D	1	0.01	-0.02	0.00	0.9643
A ²	1	5.60	+0.7246	1.58	0.2164
B ²	1	42.39	-0.4984	11.97	<0.001
C ²	1	1.77	-0.4073	0.50	0.4841
D ²	1	14.52	-1.17	4.10	0.0501
AB	1	5.80	0.4256	1.64	0.2087
AC	1	2.40	0.5475	0.68	0.4158
AD	1	4.74	-0.7700	1.34	0.2546
BC	1	0.00	+0.0044	0.00	0.9896
BD	1	0.52	+0.1275	0.15	0.7037
CD	1	20.64	-1.61	5.83	<0.05
Block	2	9.83		1.39	0.262
Lack of fit	10	43.29		1.33	0.264
Model	14	1613.35		32.54	<0.001

R² = 92.49%, Adjusted R²: 89.65%, Predicted R²: 83.73%
A: substrate concentration (% w/v),
B: enzyme loading (FPU),
C: pH,
D: KMF006 blending ratio (% v/v)

The regression model included linear, quadratic, and interaction terms, and the resulting equation for glucose yield (%) is given in Eq. 4,

$$\begin{aligned}
 \text{Glucose Yield (\%)} = & 3.44A + 2.21B + 3.52C - 0.02D \\
 & + 0.7246A^2 - 0.4984B^2 - 0.4073C^2 - 1.17D^2 + 0.4256AB \\
 & + 0.5475AC - 0.7700AD + 0.0044BC + 0.1275BD - 1.61CD
 \end{aligned} \quad (4)$$

where *A* is substrate concentration (%), *B* is enzyme loading (FPU), *C* is pH, and *D* is KMF006 blending ratio (%).

Statistical significance testing showed that substrate concentration (*A*), enzyme loading (*B*), and pH (*C*) were all highly significant main effects ($p < 0.001$). Additionally, the quadratic term of enzyme loading (B^2) and the interaction between pH and the KMF006 ratio (CD) were significant at the $p < 0.05$ level. Although the blending ratio of KMF006 (*D*) was not significant as a main effect ($p = 0.9643$), its interaction with pH was statistically significant, suggesting that enzyme blending influences saccharification when combined with an appropriate pH condition. The significance of the B^2 term indicates that an excessive increase in enzyme loading may lead to diminishing returns or saturation, emphasizing the need for optimal dosing rather than linear escalation. These findings highlight the importance of interactive effects among variables and support the implementation of a comprehensive optimization strategy.

Figure 3 presents the optimization profiles derived from the regression model and visualizes the individual effects of each variable on glucose yield. The effects of enzyme loading (*B*), pH (*C*), and substrate concentration (*A*) were all positively correlated with saccharification, showing increasing trends. In contrast, the KMF006 blending ratio (*D*) exhibited a saturation curve, indicating that an excessive ratio could hinder further

improvements. The desirability function value for the optimal conditions was 1.0000, signifying perfect fulfillment of the objective (maximizing glucose yield). The optimized conditions predicted by the model were a substrate concentration of 9.0%, enzyme loading of 60 FPU, pH of 6.0, and the KMF006 blending ratio of 25.5%. The predicted maximum glucose yield under these conditions was 63.9%, showing a 21.8% improvement over Cellic® CTec3 alone and a 32.4% improvement over KMF006 alone (Fig. 1). These results demonstrate the potential of enzyme combination strategies to substantially improve saccharification performance.

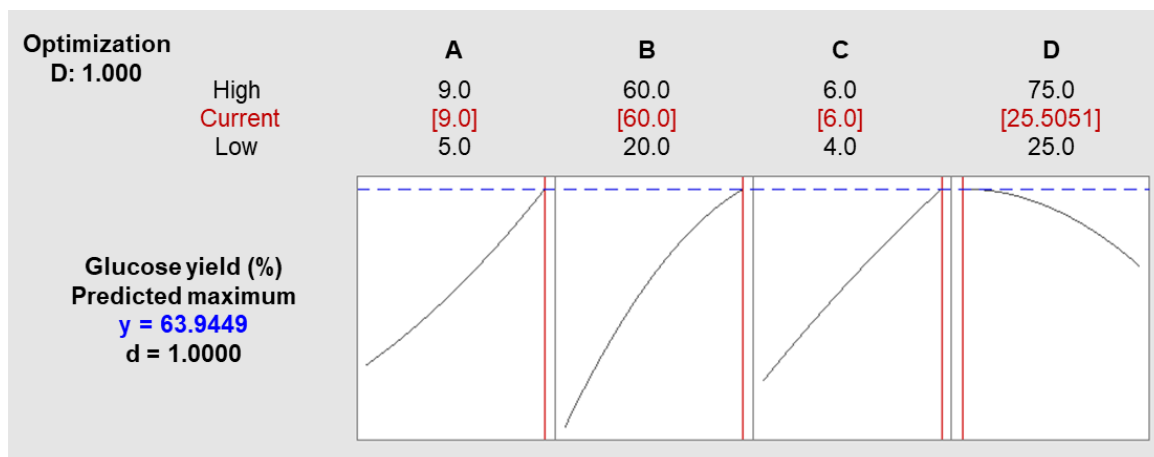


Fig. 3. Optimization profile derived from the Box-Behnken Design (BBD). The plot presents the predicted maximum glucose yield (63.94%) and the relative contribution of each factor to the response. A: substrate concentration (% w/v), B: enzyme loading (FPU), C: pH, D: KMF006 blending ratio (% v/v).

Figure 4 illustrates the response surface plots visualizing the interaction effects between pairs of variables on glucose yield. Each 3D surface plot shows predicted responses for two varying factors, while the remaining two variables were fixed at their optimal values (substrate concentration of 9.0%, enzyme loading of 60 FPU, pH of 6.0, and the KMF006 blending ratio of 25.5%). Figures 4a through 4c display interactions among substrate concentration (A), enzyme loading (B), and pH (C). Both substrate concentration and enzyme loading positively affected the glucose yield, and both achieved maximum yields at high levels (Fig. 4a). This supports previous findings that saccharification with a high substrate load can be cost-effective when enzyme activity is sufficiently maintained (Chen and Liu 2017; Baral *et al.* 2022). A significant effect of pH was observed in both Figs. 2b and 4c, where increasing pH levels led to enhanced glucose yields, with the maximum predicted yield at pH 6.0. Although Cellic® CTec3 and KMF006 individually showed optimal activity at pH 5.0 in 0.1 M sodium citrate buffer (data not shown), the optimal pH shifted when the two enzymes were combined. This result suggests that the optimal pH in a multi-enzyme system does not simply reflect the activity maxima of individual enzymes but rather emerges from their interactions. In contrast, at pH 4.0, the yield remained limited even when substrate or enzyme levels were increased, highlighting the important role of optimal pH in enzyme activity and saccharification efficiency.

Figures 4d through 4f illustrate how the blending ratio of KMF006 (D) interacted with other variables. In general, a blending ratio around 25% resulted in the highest glucose yield. Increasing the proportion of KMF006 beyond this point either reduced or led to a plateau in the yield. Remarkably, under high-level settings for the other variables, the yield

peaked when the KMF006 ratio was maintained at 25%. Given that the high cost of commercial enzymes is a major limiting factor in biorefinery operations (Klein-Marcuschamer *et al.* 2012; Siqueira *et al.* 2020; Singh *et al.* 2021), these results suggest that enzyme combination strategies can maintain saccharification efficiency while reducing dependence on costly commercial enzymes. The optimal blending ratio (25%) indicates that one-fourth of the commercial enzyme load can be substituted with KMF006, achieving comparable performance while reducing enzyme cost.

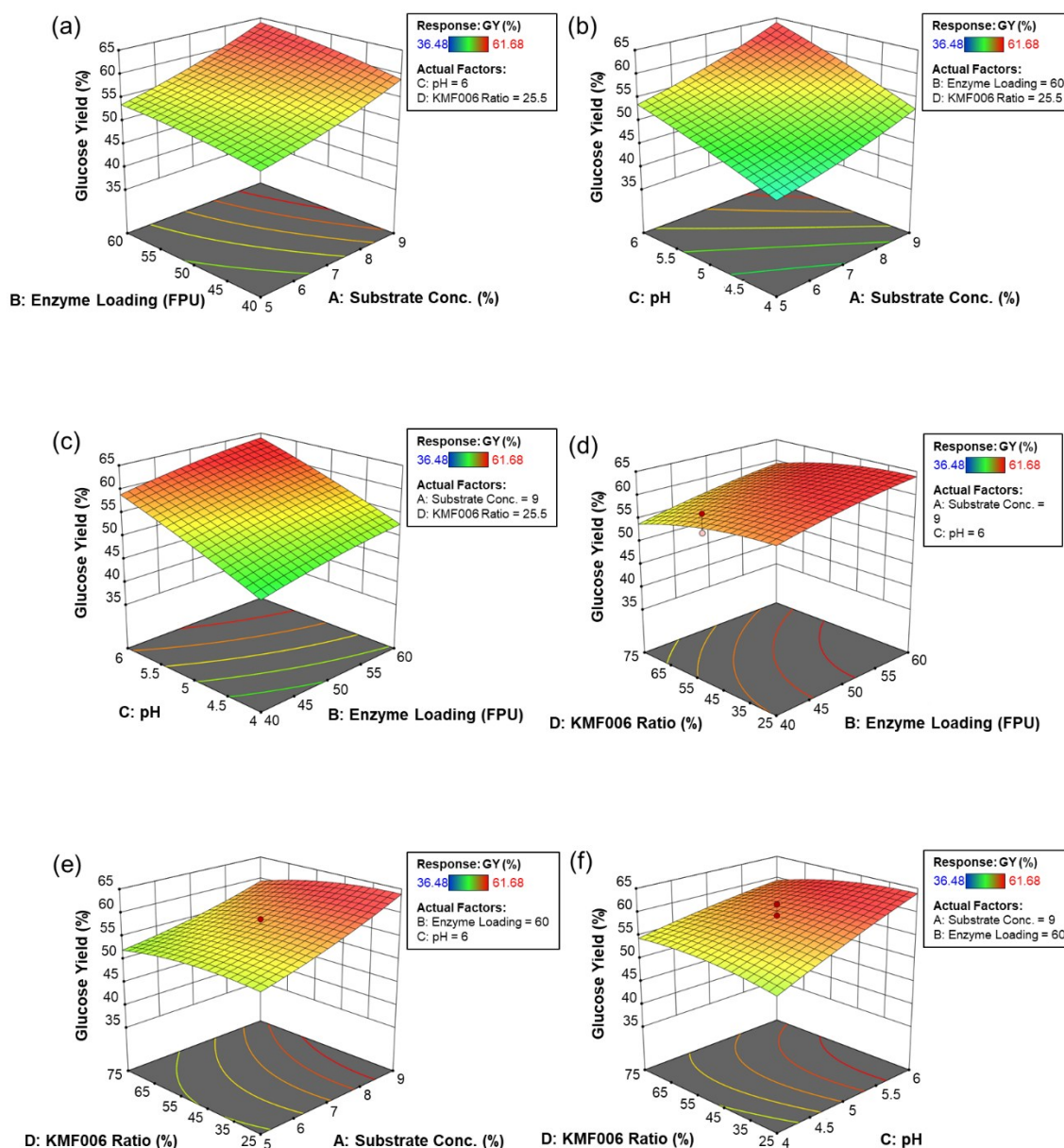


Fig. 4. Response surface plots illustrating the two-factor interaction effects on predicted glucose yield (%) based on the Box-Behnken Design (BBD). (a) substrate concentration and enzyme loading, (b) substrate concentration and pH, (c) enzyme loading and pH, (d) enzyme loading and KMF006 ratio, (e) substrate concentration and KMF006 ratio, and (f) pH and KMF006 ratio

Functional Characteristics of KMF006

Trichoderma sp. KMF006 strain used in this study possesses diverse cellulolytic activities that have been quantitatively characterized in previous investigations (Myeong and Yun, 2024; Myeong *et al.* 2025). KMF006 cellulase exhibited activity of 29.2 to 33.6 U·mL⁻¹ for EG, 3.46 to 4.0 U·mL⁻¹ for BGL, and 0.63 to 0.8 U·mL⁻¹ for CBH, with especially high activity of EG and BGL. These enzymatic activities are essential for the hydrolysis of cellulose to glucose and are considered to make an important contribution to the overall saccharification efficiency (Kim *et al.* 2025).

A previous study has demonstrated the saccharification capability of KMF006 cellulase under identical pretreatment conditions (Myeong and Yun, 2024). Under identical pretreatment conditions and an enzyme loading of 60 FPU·g-glucan⁻¹, the glucose yields of *Q. variabilis* (hardwood) reached 87.3% and that of *L. kaempferi* (softwood) reached 75.4% (Myeong and Yun 2024). Among these, the yield of *Q. variabilis* was similar to the value observed in this study (85.9%), showing that KMF006 cellulase maintained consistent performance on hardwood substrates. Furthermore, the yield levels observed in this study were higher than those reported in previous studies using hardwood-derived substrates. For instance, a study using chemical pulp from hardwoods reported glucose yields of 66 to 69% after 12 h of hydrolysis (Li *et al.* 2019). Another study using poplar sawdust showed glucose yields of 66.8% to 82.5% after 72 h of enzymatic hydrolysis (Lai *et al.* 2020), while a separate investigation using the same substrate reported yields of 63 to 73% (Chu *et al.* 2019). Similarly, a glucose yield of approximately 62% was also reported for enzymatic hydrolysis of eucalyptus wood chips (Fujii *et al.* 2009).

In contrast, *L. kaempferi* exhibited remarkably lower saccharification performance, with a glucose yield of 48.3% observed in this study (Fig. 1). Despite the identical pretreatment conditions, the previous investigation with a 60 FPU·g-glucan⁻¹ enzyme loading reported a yield of 75.4% (Myeong and Yun 2024). This discrepancy seems to be attributable to differences in enzyme capacities, suggesting that enzyme loading is a critical factor in achieving efficient saccharification, especially on recalcitrant substrates such as softwood. Softwood biomass is generally difficult to degrade due to its high lignin content (approximately 30%). In addition, extensive condensation reactions within the lignin structure further inhibit enzyme penetration and reduce catalytic activity (Rahikainen *et al.* 2011; Raulo *et al.* 2021). These structural properties contribute to the low saccharification yields observed in softwood. This also suggests that sufficient enzyme loading, and the use of supplementary enzymes may be necessary to improve hydrolytic efficiency.

Comparison of saccharification performance between KMF006 cellulase and the commercial enzyme Cellic® CTec3 revealed no statistically significant difference in either hard- or softwood substrates. For *Q. variabilis*, KMF006 achieved a glucose yield of 85.9%, whereas Cellic® CTec3 yielded 78.8%. For *L. kaempferi*, both enzymes showed similar performance, with KMF006 having a yield of 48.3% and Cellic® CTec3 having a yield of 52.5%. This result indicates that KMF006 possesses a saccharification efficiency equivalent to that of the commercial enzyme, suggesting its potential to partially replace costly commercial enzymes.

Overall, KMF006 exhibited stable and efficient saccharification performance for hardwood biomass and demonstrated a consistently high level of efficiency. When evaluated alongside the commercial enzyme, its performance was found to be within a similar range. These characteristics are associated with its high activities of EG and BGL, which are important contributors to cellulose hydrolysis. While the present study did not

directly demonstrate the mechanistic basis of synergy, the enzymatic profile of KMF006 suggests that it could serve as a complementary component when combined with commercial cellulase preparations. In addition, its applicability to softwood substrates may be further improved through appropriate process optimization. Taken together, these findings indicate that KMF006 holds potential as a useful element in enzyme combination strategies targeting diverse lignocellulosic substrates.

Enzyme Combination Strategy

Previous studies have been conducted to improve the efficiency of biomass saccharification. However, the high production cost of purified enzymes remains a major obstacle to industrial implementation (Raulo *et al.* 2021). As one strategy to overcome this limitation, the combination of commercial enzymes with crude enzymes derived from microorganisms has received increasing attention (Adsul *et al.* 2020). Enzyme combination can enhance overall saccharification yield not only through additive effects, in which individual enzyme activities are simply combined, but also through synergistic effects, where the combined action exceeds the expected sum of each component (Kuthiala *et al.* 2022).

Commercial enzyme formulations are supplied with consistent quality and typically include core cellulases, such as EG, CBH, and BGL, which provide stable baseline activities (Adsul *et al.* 2020). However, since they are generally produced for broad applicability, their performance can be limited by substrate specificity or under high-solids conditions (Adsul *et al.* 2020). In contrast, crude enzymes derived from microorganisms often contain a wide range of accessory enzymes, which may complement biomass degradation (Lopes *et al.* 2018). Nevertheless, such preparations are also known to suffer from inconsistent composition and unstable activity levels (Lopes *et al.* 2018). Based on this complementarity, combining the stability of commercial enzymes with the substrate adaptability of microbial-derived enzymes has been proposed in several recent studies as an effective approach (Lopes *et al.* 2018; Kuthiala *et al.* 2022).

Indeed, multiple studies have demonstrated improved saccharification performance through such enzyme combinations. For example, the co-application of Spezyme CP (Genencor, Palo Alto, CA, USA) with enzymes derived from *Aspergillus fumigatus* significantly improved the saccharification of corn stover (Wang *et al.* 2012). Similarly, blending Celluclast® 1.5 L (Novonosis) with a crude enzyme extract from *Aspergillus oryzae* enhanced the cellulose conversion efficiency in sugarcane bagasse (Braga *et al.* 2014). In another study, Suwannarangsee *et al.* (2012) achieved high hydrolysis yields for alkali-pretreated rice straw by combining Celluclast® 1.5 L (Novonosis) with *Aspergillus aculeatus* and expansin from *Bacillus subtilis*. These studies collectively indicate that enzyme blending can compensate for the limitations of individual enzyme systems and help tailor saccharification strategies to specific biomass types.

A similar strategy was employed in the present study, in which Cellic® CTec3 was combined with an experimental enzyme preparation (KMF006) derived from *Trichoderma*. This combination resulted in a glucose yield that was 21.8% higher than that obtained with Cellic® CTec3 alone, and 32.4% higher than with KMF006 alone, when applied to *L. kaempferi*, a recalcitrant softwood substrate. These results experimentally confirm that enzyme combination can improve hydrolysis efficiency through complementary action between commercial and crude enzyme sources, and they are consistent with the synergistic effects reported in previous studies. Furthermore, the present findings suggest that KMF006 could be used to partially replace commercial cellulase in enzyme

combinations, thereby maintaining hydrolytic efficiency while reducing overall enzyme costs, which is a critical factor for industrial feasibility.

Limitations and Future Directions

While this study systematically optimized the enzyme combinations using a Box-Behnken design, the predicted maximum glucose yield of 63.9% for *L. kaempferi* was not experimentally validated. Although all experimental runs under the PBD and BBD frameworks were carried out and analyzed, a separate confirmatory experiment at the predicted optimum was not performed. This limitation prevents direct evaluation of the model's predictive accuracy. Statistical optimization enabled the identification of significant main effects and interactions, particularly the synergistic role of KMF006 blending ratio and pH, thereby offering a valuable framework for process refinement and hypothesis-driven validation.

The enzymatic properties of the KMF006 preparation were previously evaluated based on core cellulases (EG, BGL, and CBH) (Myeong and Yun 2024; Myeong *et al.* 2025), but the presence and role of accessory enzymes were not analyzed. Accessory enzymes such as xylanase, LPMO, and non-catalytic proteins (*e.g.*, expansin, swollenin) are known to enhance hydrolysis efficiency by improving substrate accessibility (Polizeli *et al.* 2005; Qin *et al.* 2013; Lenfant *et al.* 2017). Since crude microbial enzymes often contain diverse auxiliary components (Kuthiala *et al.* 2022), compositional profiling of KMF006 would provide critical insight into the enzymatic basis of synergistic effects observed in this study. In addition, evaluating the enzyme activity profiles of blends of KMF006 with the commercial preparation could also be valuable for elucidating the mechanistic basis of the observed synergistic effects.

Although this study evaluated enzyme activity at a 5 L scale and successfully applied statistical optimization to identify critical factors affecting saccharification, further efforts are required to assess industrial applicability. Transitioning to larger bioreactor systems is essential for scaling up optimized conditions. Such scale transitions often introduce new challenges, as microbial enzyme production can be sensitive to fermentation parameters, and enzyme activity or composition may fluctuate. In addition, the experimental conditions applied in this study were selected to facilitate statistical modeling and do not directly represent an economically viable set of conditions. Therefore, future work should focus on validating saccharification performance under conditions that better reflect industrial practice, such as lower enzyme loading or higher substrate loadings. This will enable a clearer assessment of the practical applicability of the KMF006 preparation and its potential contribution toward reducing process costs.

CONCLUSIONS

1. This study identified the optimal conditions for an enzyme combination designed to improve the saccharification efficiency of softwood biomass (*L. kaempferi*) by combining cellulase derived from *Trichoderma* sp. KMF006 with the commercial enzyme (Cellic® CTec3). Comparative hydrolysis with individual enzymes confirmed the limited glucose yield from softwood, emphasizing the need for an effective enzyme combination strategy.

2. A Plackett-Burman Design was employed to screen key influencing factors, followed by optimization through a Box-Behnken Design. At the optimum conditions—9% substrate concentration, 60 FPU enzyme loading, pH 6.0, and a 25.5% KMF006 blending ratio—the glucose yield increased by 21.8% and 32.4% compared to Cellic® CTec3 and KMF006 alone, respectively. These results suggest that up to one-quarter of the commercial enzyme can be replaced without compromising enzymatic performance.
3. This study has presented valuable strategies for the enzymatic hydrolysis of recalcitrant biomass such as softwood. In particular, it provides quantitative support for the complementary action between commercial and microbial-derived enzymes, optimized through statistical approaches. These findings provide preliminary evidence that enzyme blending can enhance hydrolysis efficiency under specific conditions and may reduce dependence on commercial enzymes. While further validation is required at larger scales and across diverse substrates, this combination-based approach offers a potentially useful strategy for improving saccharification of recalcitrant lignocellulosic biomass.

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REFERENCES CITED

- Abdullah, R., Tahseen, M., Nisar, K., Kaleem, A., Iqtedar, M., Saleem, F., and Aftab, M. (2021). “Statistical optimization of cellulases by *Talaromyces thermophilus* utilizing *Saccharum spontaneum*, a novel substrate,” *Electron. J. Biotechnol.* 51, 79-87. DOI: 10.1016/j.ejbt.2021.03.007
- Adsul, M., Sandhu, S. K., Singhania, R. R., Gupta, R., Puri, S. K., and Mathur, A. (2020). “Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels,” *Enzyme Microb. Technol.* 133, article 109442. DOI: 10.1016/j.enzmitec.2019.109442
- Balan, R., Antczak, A., Brethauer, S., Zielenkiewicz, T., and Studer, M. H. (2020). “Steam explosion pretreatment of beechwood. Part 1: Comparison of the enzymatic hydrolysis of washed solids and whole pretreatment slurry at different solid loadings,” *Energies (Basel)* 13(14), article 3653. DOI: 10.3390/en13143653
- Baral, P., Kumar, V., and Agrawal, D. (2022). “Emerging trends in high-solids enzymatic saccharification of lignocellulosic feedstocks for developing an efficient and industrially deployable sugar platform,” *Crit. Rev. Biotechnol.* 42(6), 873-891. DOI: 10.1080/07388551.2021.1973363
- Braga, C. M. P., Delabona, P. S., Lima, D. J. S., Pzixão, D. A. A., Pradella, J. G. C., and Farinas, C. S. (2014). “Addition of feruloyl esterase and xylanase produced on-site improves sugarcane bagasse hydrolysis,” *Bioresour. Technol.* 170, 316-324. DOI: 10.1016/j.biortech.2014.07.115

- Chen, H. Z., and Liu, Z. H. (2017). "Enzymatic hydrolysis of lignocellulosic biomass from low to high solids loading," *Eng. Life Sci.* 17(5), 489-499. DOI: 10.1002/elsc.201600102
- Chu, Q., Song, K., Wang, J., Hu, J., and Chen, X. (2019). "Improving enzymatic saccharification of hardwood through lignin modification by carbocation scavengers and the underlying mechanisms," *Bioresour. Technol.* 294, article 122216. DOI: 10.1016/j.biortech.2019.122216
- Contreras, F., Pramanik, S., Rozhkova, A. M., Zorov, I. N., Korotkova, O., Sinitsyn, A. P., Schwaneberg, U., and Davari, M. D. (2020). "Engineering robust cellulases for tailored lignocellulosic degradation cocktails," *Int. J. Mol. Sci.* 21(5), article 1589. DOI: 10.3390/ijms21051589
- Du, J., Liang, J., Gao, X., Liu, G., and Qu, Y. (2020). "Optimization of an artificial cellulase cocktail for high-solids enzymatic hydrolysis of cellulosic materials with different pretreatment methods," *Bioresour. Technol.* 295, article 122272. DOI: 10.1016/j.biortech.2019.122272
- Fujii, T., Fang, X., Inoue, H., Murakami, K., and Sawayama, S. (2009). "Enzymatic hydrolyzing performance of *Acremonium cellulolyticus* and *Trichoderma reesei* against three lignocellulosic materials," *Biotechnol. Biofuels* 2(1), article 24. DOI: 10.1186/1754-6834-2-24
- Ilić, N., Milić, M., Beluhan, S., and Dimitrijević-Branković, S. (2023). "Cellulases: From lignocellulosic biomass to improved production," *Energies (Basel)* 16(8), article 3598. DOI: 10.3390/en16083598
- Ji, L., Yang, J., Fan, H., Yang, Y., Li, B., Yu, X., Zhu, N., and Yuan, H. (2014). "Synergy of crude enzyme cocktail from cold-adapted *Cladosporium cladosporioides* Ch2-2 with commercial xylanase achieving high sugars yield at low cost," *Biotechnol. Biofuels* 7(1), article 130. DOI: 10.1186/s13068-014-0130-x
- Kim, S., Ha, I., Lee, Y. Y., Lee, J., and Yun, J. (2025). "Development of a high-performance *Trichoderma* mutant for enhanced cellulase production through UV-induced random mutagenesis," *J. Fungi* 11(6), article 439. DOI: 10.3390/jof11060439
- Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B. A., and Blanch, H. W. (2012). "The challenge of enzyme cost in the production of lignocellulosic biofuels," *Biotechnol. Bioeng.* 109(4), 1083-1087. DOI: 10.1002/bit.24370
- Kuthiala, T., Thakur, K., Sharma, D., Singh, G., Khatri, M., and Arya, S. K. (2022). "The eco-friendly approach of cocktail enzyme in agricultural waste treatment: A comprehensive review," *Int. J. Biol. Macromol.* 209, 1956-1974. DOI: 10.1016/j.ijbiomac.2022.04.173
- Lai, C., Jia, Y., Yang, C., Chen, L., Shi, H., and Yong, Q. (2020). "Incorporating lignin into polyethylene glycol enhanced its performance for promoting enzymatic hydrolysis of hardwood," *ACS Sustain. Chem. Eng.* 8(4), 1797-1804. DOI: 10.1021/acssuschemeng.9b05724
- Lenfant, N., Hainaut, M., Terrapon, N., Drula, E., Lombard, V., and Henrissat, B. (2017). "A bioinformatics analysis of 3400 lytic polysaccharide oxidases from family AA9," *Carbohydr. Res.* 448, 166-174. DOI: 10.1016/j.carres.2017.04.012
- Li, Y., Li, B., Mo, W., Yang, W., and Wu, S. (2019). "Influence of residual lignin and thermal drying on the ultrastructure of chemical hardwood pulp and its enzymatic hydrolysis properties," *Cellulose* 26(3), 2075-2085. DOI:10.1007/s10570-018-2184-6
- Lopes, A. M., Ferreira Filho, E. X., and Moreira, L. R. S. (2018). "An update on

- enzymatic cocktails for lignocellulose breakdown,” *J. Appl. Microbiol.* 125(3), 632-645. DOI: 10.1111/jam.13923
- McMillan, J.D., Jennings, E.W., Mohagheghi, A., and Zuccarello, M. (2011). “Comparative performance of precommercial cellulases hydrolyzing pretreated corn stover,” *Biotechnol. Biofuels* 4(1), 29. DOI: 10.1186/1754-6834-4-29
- Myeong, S., Lee, Y. Y., and Yun, J. (2025). “Optimization and bioreactor scale-up of cellulase production in *Trichoderma* sp. KMF006 for higher yield and performance,” *Int. J. Mol. Sci.* 26(8), 3731. DOI: 10.3390/ijms26083731
- Myeong, S., and Yun, J. (2024). “Culture of *Trichoderma* sp. with biochar to produce high-activity cellulase in a laboratory,” *BioResources* 19(2), 2029-2044. DOI: 10.15376/biores.19.2.2029-2044
- Nakagame, S., Chandra, R. P., Saddler, J. N. (2010). “The effect of isolated lignins, obtained from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis,” *Biotechnol. Bioeng* 105(5), 871-879. DOI: 10.1002/bit.22626
- Nisar, K., Abdullah, R., Kaleem, A., and Iqtedar, M. (2020). “Statistical optimization of cellulase production by *Thermomyces dupontii*,” *Iran. J. Sci. Technol. Trans. A Sci.* 44(5), 1269-1277. DOI: 10.1007/s40995-020-00932-1
- Nitsos, C. K., Matis, K.A., and Triantafyllidis, K. S. (2013). “Optimization of hydrothermal pretreatment of lignocellulosic biomass in the bioethanol production process,” *ChemSusChem* 6(1), 110-122. DOI: 10.1002/cssc.201200546
- Overend, R. P., and Chornet, E. (1987). “Fractionation of lignocellulosics by steam-aqueous pretreatments,” *Philos. Trans. R. Soc. Lond. A Math. Phys. Sci.* 321(1561), 523-536. DOI: 10.1098/rsta.1987.0029
- Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti, R., Terenzi, H. F., Jorge, J. A., and Amorim, D. S. (2005). “Xylanases from fungi: Properties and industrial applications,” *Appl. Microbiol. Biotechnol.* 67(5), 577-591. DOI:10.1007/s00253-005-1904-7
- Qin, Y. M., Tao, H., Liu, Y. Y., Wang, Y. D., Zhang, J. R., and Tang, A. X. (2013). “A novel non-hydrolytic protein from *Pseudomonas oryzae* enhances the enzymatic hydrolysis of cellulose,” *J. Biotechnol.* 168(1), 24-31. DOI: 10.1016/j.jbiotec.2013.07.028
- Rahikainen, J., Mikander, S., Marjamaa, K., Tamminen, T., Lappas, A., Viikari, L., and Kruus, K. (2011). “Inhibition of enzymatic hydrolysis by residual lignins from softwood—study of enzyme binding and inactivation on lignin-rich surface,” *Biotechnol. Bioeng.* 108(12), 2823-2834. DOI: 10.1002/bit.23242
- Raulo, R., Heuson, E., Froidevaux, R., and Phalip, V. (2021). “Combining analytical approaches for better lignocellulosic biomass degradation: A way of improving fungal enzymatic cocktails?,” *Biotechnol. Lett.* 43(12), 2283-2298. DOI: 10.1007/s10529-021-03201-2
- Singh, A., Bajar, S., Devi, A., and Pant, D. (2021). “An overview on the recent developments in fungal cellulase production and their industrial applications,” *Bioresour. Technol. Rep.* 14, 100652. DOI: 10.1016/j.biteb.2021.100652
- Siqueira, J.G.W., Rodrigues, C., Vandenberghe, L.P. de S., Woiciechowski, A.L., and Soccol, C.R. (2020). “Current advances in on-site cellulase production and application on lignocellulosic biomass conversion to biofuels: A review,” *Biomass Bioenergy* 132, 105419. DOI: 10.1016/j.biombioe.2019.105419
- Sluiter, A., Ruiz, R., Scarlata, S., Sluiter, J., and Templeton, D. (2005). *Determination of Extractives in Biomass* (NREL/TP-510-42619), National Renewable Energy Laboratory, Golden, CO, USA.

- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008). *Determination of Structural Carbohydrates and Lignin in Biomass* (NREL/TP-510-42618), National Renewable Energy Laboratory, Golden, CO, USA.
- Srivastava, N., Elgorban, A. M., Mishra, P. K., Marraiki, N., Alharbi, A. M., Ahmad, I., and Gupta, V. K. (2020). “Enhance production of fungal cellulase cocktail using cellulosic waste,” *Environ. Technol. Innov.* 19, article 100949. DOI: 10.1016/j.eti.2020.100949
- Suwannarangsee, S., Bunternngsook, B., Arnthong, J., Paemanee, A., Thamchaipenet, A., Eurwilaichitr, L., Laosiripojana, N., and Champreda, V. (2012). “Optimisation of synergistic biomass-degrading enzyme systems for efficient rice straw hydrolysis using an experimental mixture design,” *Bioresour. Technol.* 119, 252-261. DOI: 10.1016/j.biortech.2012.05.098
- Wang, D., Sun, J., Yu, H.L., Li, C.X., Bao, J., Xu, J.H. (2012). “Maximum saccharification of cellulose complex by an enzyme cocktail supplemented with cellulase from newly isolated *Aspergillus fumigatus* ECU0811,” *Appl. Biochem. Biotechnol.* 166(1), 176-186. DOI: 10.1007/s12010-011-9414-9

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APPENDIX

Table A1. Experimental Matrix of the Plackett-Burmann Design (PBD) Along With the Corresponding Glucose Yield (%) for Each Run

Run	A	B	C	D	E	Glucose Yield (%)
1	-	-	-	-	+	39.67
2	+	+	-	+	+	57.04
3	-	-	+	+	-	39.80
4	+	+	+	+	-	61.84
5	-	-	-	-	-	37.23
6	-	-	+	-	+	44.68
7	0	0	0	0	0	55.33
8	-	+	-	+	-	40.73
9	-	+	+	+	+	48.47
10	-	+	+	-	+	56.04
11	+	+	-	-	-	55.10
12	+	-	-	+	+	42.77
13	+	-	+	-	+	50.43
14	-	-	-	+	-	36.02
15	-	+	+	-	-	51.58
16	+	+	-	-	+	56.52
17	+	+	+	-	-	61.92
18	+	-	+	+	-	44.44
19	+	-	+	+	+	47.56
20	-	+	-	+	+	46.21
21	+	-	-	-	-	35.87
22	+	-	+	-	+	54.69
23	-	+	-	+	+	45.02
24	+	+	-	-	-	59.18
25	+	-	+	+	+	46.90
26	-	-	-	-	+	43.49
27	+	+	-	+	+	49.81
28	-	+	+	-	-	57.02
29	+	+	+	+	-	60.27
30	+	-	-	-	-	40.09
31	+	-	+	+	-	48.55
32	+	-	-	+	+	40.77
33	+	+	-	-	+	57.66
34	-	+	+	+	+	48.04
35	-	-	+	+	-	41.09
36	+	+	+	-	-	63.35
37	-	-	-	+	-	34.08
38	-	-	-	-	-	31.76
39	-	+	+	-	+	47.79
40	0	0	0	0	0	57.35
41	-	-	+	-	+	44.16
42	-	+	-	+	-	40.12

Table A2. Experimental Matrix of the Box-Behnken Design (BBD) Along With the Corresponding Glucose Yield (%) for Each Run

Run	A	B	C	D	Glucose Yield (%)
1	-	0	+	0	48.10
2	0	-	0	-	42.88
3	-	0	-	0	43.38
4	0	-	0	+	41.11
5	0	0	0	0	51.44
6	-	0	+	0	53.20
7	0	+	0	-	52.69
8	+	0	+	0	60.49
9	+	0	-	0	51.56
10	0	-	0	+	42.97
11	+	0	-	0	48.88
12	-	0	-	0	45.65
13	0	-	0	-	39.59
14	0	0	0	0	51.73
15	+	0	+	0	56.60
16	0	+	0	+	56.19
17	0	+	0	+	52.38
18	0	+	0	-	52.23
19	+	0	0	-	52.38
20	-	0	0	+	47.94
21	0	0	0	0	48.92
22	0	+	+	0	56.61
23	0	-	+	0	45.72
24	+	0	0	+	51.50
25	+	0	0	-	52.30
26	+	0	0	+	49.59
27	-	0	0	-	47.00
28	0	-	-	0	38.49
29	-	0	0	+	48.63
30	0	-	+	0	45.41
31	0	+	+	0	58.72
32	0	+	-	0	50.91
33	0	-	-	0	36.48
34	-	0	0	-	47.00
35	0	0	0	0	50.32
36	0	+	-	0	48.33
37	+	+	0	0	61.68
38	+	+	0	0	59.33
39	0	0	+	+	47.93
40	-	-	0	0	38.34
41	-	+	0	0	54.04
42	0	0	-	+	47.23
43	0	0	+	-	55.01
44	0	0	0	0	47.32
45	-	-	0	0	38.42
46	+	-	0	0	43.62
47	+	-	0	0	44.73
48	0	0	-	-	45.86
49	0	0	+	-	52.60
50	0	0	-	-	43.60
51	0	0	+	+	49.33

Run	A	B	C	D	Glucose Yield (%)
52	0	0	-	+	44.73
53	0	0	0	0	51.29
54	-	+	0	0	48.57