Statical Optimization of Cellulase Enzymes production by *Trichoderma harzianum* PP400831 Using Response Surface Methodology and Their Application in Production of 2G Bioethanol

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Abstract

The development of second-generation (2G) bioethanol from lignocellulosic sources, such as sugarcane bagasse, is very important as a viable alternative to conventional fossil fuels. However, the high cost associated with enzymatic hydrolysis, which breaks down cellulose into fermentable sugars, poses a key challenge. This study focused on enhancing cellulase enzyme production by a novel, locally isolated strain, *Trichoderma harzianum* PP400831, using statistical optimization BBD-RSM to improve enzyme activity. Optimization efforts resulted in maximal endoglucanase and exoglucanase activities of 4.01 IU/mL and 2.64 IU/mL, respectively after 9 days at 2% cellulose mixture concentration and 0.15% tween 80. After saccharification of pretreated (SCB) by the crude enzymes and fermentation of produced reduced sugar by *S. cerevisiae* MN901244 yielded an ethanol concentration of 25.63 g/L. This work represents a significant step toward developing a cost-effective, sustainable, and high-performing cellulase production process for second-generation bioethanol.

Key words: *Trichoderma* sp., Cellulase enzyme, Sugarcane bagasse, Ethanol production

Introduction

Intensive industrialization and transportation development in contemporary urban centers are main source of the intensive global need for sustainable energy sources. Biomass-derived fuel such as bioethanol offers a sustainable pathway for significantly decreasing the global demand for fossil fuel, in addition to its ability to decrease the harmful effects of fossil fuel on environment. Bioethanol production offers a substantial reduction in greenhouse gas emissions, ranging from 68% to 91% compared to fossil fuels **[1]**. Emissions associated with bioethanol production are estimated to be between 23 and 85 g CO_2eq/MJ, which is notably less than those of fossil fuels **[2].** Where Fossil fuels (specifically gasoline and diesel) generally emit between 90 and 95 g CO2eq/MJ on a full life-cycle basis according to European Environment Agency (EEA) - European Union.

The global production of bioethanol has many challenges, such as high production costs and feedstock availability, according to **[3,4]** they recorded that bioethanol can be produced from various feedstocks categorized into four generations: First Generation (1G) that includes traditional crops like corn and sugarcane which competed with food resource, Second Generation (2G) where Lignocellulosic biomass is a feedstock which does not compete with food supply, Third Generation (3G) considered algal biomass as high yield potential finally, Fourth Generation (4G) which used engineered feedstocks

One of promising lignocellulosic feedstock is sugar cane bagasse (SCB) which considered as a byproduct of sugar industry that helps not only in reducing production cost but also enhance waste valorization through biofuel production, it contains approximately 32-55% cellulose, 26.7-32% hemicellulose, and 19-24% lignin **[5]**. The efficient utilization of (SCB) for bioethanol production depend on effective pretreatment processes due to its complex composition.

The chemical pretreatment of sugarcane bagasse (SCB) is crucial for enhancing bioethanol production by breaking down its complex lignocellulosic structure. Various methods, including alkaline, acid, and organosolv pretreatments, have been explored to optimize sugar yields and fermentation efficiency. The study by **Zohri et al. [6]** successfully employed a base-acid sequential pretreatment method using 0.2% sodium hydroxide (NaOH) and 98% sulfuric acid (H_2SO_4) to process lignocellulosic biomass. The alkaline step primarily focuses on lignin removal, which is crucial for swelling the biomass and increasing the porosity and enzymatic accessibility of the cellulose fibers. The subsequent acid step is highly effective at dissolving the hemicellulose component.

Abubakar et al. [7] recorded that sulfuric acid at concentrations around 0.5 M produced reducing sugars (up to 270.90 g/L) when applied at 120°C. According to **Ruan et al. [8]** 1% NaOH for 60 min, and a solid-to-liquid ratio of 1:15 pretreatment has shown significant lignin removal up to 82.3 % and 85.33 % cellulose conversion rate during enzymatic hydrolysis.

A crucial step after chemical treatment of lignocellulosic materials is the enzymatic hydrolysis of cellulose material into fermentable sugars which still considers as one of the main barriers of the lignocellulose bioconversion due to the high cost **[9]**. Usually complex of cellulase enzymes were employed. Cellulase complexes typically consist of multiple enzymes, including cellobiohydrolases, endoglucanases, and β-glucosidases, which work in harmony to break down cellulose into glucose.

Microbial production of cellulase enzymes has become motivating idea due to low cost, and the broad diversity of enzyme-producing strains. Fungi, particularly *Trichoderma*, *Aspergillus*, dominate industrial cellulase production because of their high secretion capacity and ability to grow on cheap lignocellulosic substrates **[10].**

Trichoderma harzianum is a filamentous fungus well known for cellulase production due to high enzyme yield and its ability to utilize various substrates effectively such as avicel, carboxymethyl cellulose, and banana pseudostem **[11,12]**. The cellulase enzyme system produced by *T. harzianum* includes Exoglucanase, endoglucanase, and β-glucosidase **[11]**. The production of these cellulase enzymes is influenced by various environmental and cultural factors. The type and concentration of Carbon source, pH, temperature and incubation period are critical factors in enzyme production. **[11,13]** detected that *Trichoderma harzianum* produces cellulase optimally at a pH of 6.5 and a temperature of 25°C after 120 h. However, other studies have reported slightly different optimal conditions, such as a pH of 5.0 and a temperature of 40°C, highlighting the potential for strain-specific variations **[14]**. **Khokhar et al. [15]** found that untreated wheat straw as carbon source enhance cellulase activity in *T. reesei* compared to other strains. Also, **Ike and Tokuyasu [16]** demonstrate that sucrose after hydrolyzed to glucose and fructose elevate cellulase activity achieving comparable enzyme activities to glucose. Typically, utilization of glycerol as a carbon source has shown to double cellulase production compared to glucose **[17].** Moreover, Addition of surfactants during cellulase enzyme production can improve enzyme stability, activity and provide better substrate interaction. Tween-20 has been noted to enhance cellulase production, although galactose was found to be a more effective inducer than glucose or Tween-20 **[15]**.

Optimization process remains strain-specific and crucial for industrial application. There is a persistent need to integrate highly optimized, low-cost microbial cellulase production with an effective, sequential chemical pretreatment to maximize sugar yield from SCB. Furthermore, studies often lack a comprehensive, end-to-end evaluation that connects the optimized enzyme production, through

efficient hydrolysis of chemically pretreated SCB, to the final fermentation efficiency into ethanol.

Therefore, this study investigates this critical gap by detecting the optimization conditions of cellulase enzyme production by a locally strain, *T. harzianum* PP400831. We then evaluate the capacity of this optimized enzyme system in the saccharification of cellulose-rich biomass obtained after the established base-acid pretreatment **(Z.B.A.P.)** of sugarcane bagasse. Ultimately, the study explores the subsequent integrated conversion of the released fermentable sugars into ethanol by *Saccharomyces cerevisiae* MN901244, offering a holistic, optimized bioconversion strategy.

Materials and Methods

A- Screening for cellulase enzymes production

Seven isolates of *Trichoderma harzianium* were obtained from previous studies in our Laboratory **[18, 19]**. Cultures were grown on Czapek's agar medium which composed of (g/L): glucose, 20; $NaNO_3$, 2; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; agar agar, 15 per one liter of distilled water. for 7 days at 28 °C to be used in further experiments.

To evaluate the cellulase enzyme production by fungal isolates, the method detailed by **[20]** was employed. The process involved adjusting the pH to 5.0. An agar block from a one-week-old fungal colony, cultivated on Czapek's medium, was placed in the center of plates containing Mandel's medium. This medium consisted of: urea (0.3 g/L), $(NH_4)_2SO_4$ (1.4 g/L), KH_2PO_4 (2.0 g/L), $MgSO_4$ (0.3 g/L), yeast extract (0.25 g/L), peptone (0.75 g/L), carboxymethyl cellulose (10 g/L), and agar (15 g/L). Plates were incubated at 30°C. After seven days, each isolate's three plates were stained with 1% Congo red for 20 min, then destained using a 1 M NaCl

solution for 20 min, following the protocol by **[21]**. The hydrolysis capacity was quantified by calculating the ratio of the clear zone diameter to the colony diameter, a method attributed to **[22]**.

B- Optimization of enzyme production using Response Surface Methodology

This study utilized *Trichoderma harzianum* PP400831, a strain previously isolated in previous study by **Abd El-latif et al. [23]** from onion rhizosphere in our lab and characterized both morphologically and molecularly, with its genetic sequence deposited in the gene bank (accession PP400831). This strain was found to be a highly producer of cellulase enzyme. To optimize the production of endogluconase and exogluconase activity, Response Surface Methodology was applied using the Box-Behnken design **[24]** via Design Expert Software. The fermentation process involved inoculating 250 mL Erlenmeyer flasks, each containing 50 mL of sterilized Mandel's medium (where cellulose and ammonium sulfate concentrations varied according to experimental design), with spore suspensions from 7-day-old cultures grown on Czapek's agar. Flasks were then incubated for 7 days at 30˚C and 150 rpm. After incubation, the culture media were filtered using Whatman "No. 1" paper to separate the mycelia from the liquid filtrates. Three independent variables were investigated for the chosen isolate, as detailed in table 1. Based on these inputs, the Box-Behnken design generated 17 experimental runs. The measured values for exogluconase, endogluconase activity from these runs were re-entered into the RSM program, which then predicted the most favorable production conditions by analyzing the interactions among the variables. The determined optimal condition was as follows:

Table (1): Values of independent variables in the Box-Behnken design for *Trichoderma harzianum* PP400831

Parameters (Independent variables)	**Units**	**Coded levels**

		-1	0	+1
Concentration of cellulose mixture (A)	%	2	2.5	**3**
Incubation period (B)	Day	4	6.5	**9**
Tween 80 concentration (C)	%	0.1	0.15	0.2

C- Production of crude enzymes

Spore suspension of *Trichoderma harzianum* PP400831 in ratio 10% was inoculated to five liters of production medium to produce cellulase enzyme according to the best predicated conditions as following: incubation period 9 days, cellulose mixture concentration 2.02% and tween 80 0.15%. Saturated solution of ammonium sulphate (70%, w/v) was used to precipitate protein. Addition of ammonium sulphate occurred at 4˚C with gentle stirring and kept overnight to precipitate protein after that it collected by centrifugation at 10.000 rpm for 15 min at 4˚C **[25]**. Lypholiziation of the collected protein (precipitate) was obtained to get the crude enzyme in solid state.

D- Collection and preparation of Sugarcane bagasse

In 2024, sugarcane bagasse samples were collected from the Abo-Qurqas sugar factory in El-Minia Governorate, Egypt. Five kg of each sample were transported to the lab in sterile plastic bags, washed, and dried at 70°C for 24 h in a Venticell oven, model MMM Medcenter. The samples were then refrigerated at 3-5˚C until analysis. To prepare for pretreatment, the bagasse samples were milled to reduce the particle size to 1 cm (by a bagasse grinder, Faculty of Sugar and Integrated Industries Technology, Assiut University), and one kilogram of SCB was treated to obtain enough cellulose-rich substrate for various experiments.

E- Base-acid pretreatment method by Zohri *et al.* 2019 (Z.B.A.P.)[6]

To pretreat the substrate, 80 g were combined with 11 g of NaOH dissolved

in 800 mL of distilled water, and the mixture was autoclaved at 120°C for 40 min. Subsequently, an equimolar quantity (14.5 ml, assay 98%) of sulfuric acid in 200 mL H_2O was introduced to the reaction, followed by re-autoclaving at 105°C for 40 min. The resulting substrate underwent washing with tap water and was then post-treated with 1% NaOH at room temperature to facilitate lignin dissolution. Following filtration and repeated washing with water, the solid cellulose-rich substrate was dried at 70°C for 24 h.

F- Saccharification of pretreated sugar cane bagasse by crude cellulase enzyme

Different weights of crude enzymes (0.2, 0.25, 0.3 and 0.35 g/g cellulose-rich substrate) produced by *T. harzianum* PP400831were used to hydrolyze 10% cellulose-rich substrate from sugarcane bagasse pretreated by (Z.B.A.P) method. Then reducing sugar concentrations were measured by dinitrosalysilic acid method **[26],** The hydrolysis yield was calculated by **[27]** equation:

Hydrolysis yield % = glucose amount in 100 mL / (1.1) (C) (x)

Where, C is the cellulose fraction percentage in one gram of substrate; X is the amount of substrate in 100 mL solution and 1.1 is the correlation factor due to the addition of water molecules into cellulose.

G-Kinetics studies on crude cellulase enzymes

The Michaelis constant (Km) and maximum velocity (Vmax) were determined. To calculate the Michaelis constant for cellulase, varying quantities of crude enzymes (0.1, 0.15, 0.2, 0.25, 0.3, and 0.35 g/g cellulose-rich substrate) derived from *T. harzianum* PP400831 were employed to hydrolyze a 10% cellulose-rich substrate generated from sugarcane bagasse treated by (Z.B.A.P.) method. The reaction was conducted for 30 min at 50 °C, and cellulase activity was evaluated according to **[28]**. The Km and Vmax values of cellulase enzymes

were determined using the. Lineweaver-Burk plot constructed using cellulose-rich substrate concentrations and cellulase activity.

H- Ethanol bioproduction

In this study two commercial cellulase enzymes (the first one is Sternzym cellulase C 21032 was obtained from Stern enzyme GmbH and Co. KG, Germany and derived from *Trichoderma reesei* was used at concentration 0.2 g/g, and the other is Cellic C tec 2. Cellulase was obtained from Novozymes, Denmark and derived from different fungal isolates was used at concentration 0.1 mL/g) were used separately and mixture of them (sternzyme C 21032, 0.1 g/g; Cellic C, 0.05 mL/g) in addition the crude cellulase enzyme which produced previously was used at a concentration of 0.3 g/g to produced reducing sugar from pretreated sugar cane bagasse. The produced solution containing reduced sugar was enrichment with peptone, 5g/L; malt extract, 3g/L and yeast extract, 3 g/L then ethanol production was done at 28˚ C for 72 h using *Saccharomyces cerevisiae* MN901244 was previously isolated from molasses in the same laboratory and recorded as highly ethanol producer **[29]**.

I- Analytical Methods

The determination of endogluconase activity involved mixing 0.5 mL of culture supernatant with 0.5 mL of 1% CMC (dissolved in pH 5 citrate buffer) at 50°C for 30 min. Following this, 2 mL of dinitrosalicylic acid reagent was introduced **[26]**, and the sample was boiled in a water bath for 10 minutes, cooled, and its absorbance was read at 540 nm. An enzyme unit signifies the amount capable of liberating 1 µmol of reducing sugar equivalents per minute **[28]**. Exogluconase activity was ascertained through the identical protocol, with the distinction of employing 1% microcrystalline cellulose in place of 1% CMC.

$$\text{Enzyme activity IU/mL} = C \times V_t / V_e \times t$$

C μmol of glucose equivalent released (from DNS standard curve), V_t, total assay volume (mL) typically includes enzyme + substrate which is 1ml, V_e, volume of enzyme used (mL) 0.5 ml, t, incubation time (min) which is 30 min at 50°C.

Quantitative analysis of ethanol concentration (g/L) was carried out with a High-Performance Liquid Chromatography system (HPLC). After centrifuging the fermented broth, 5μl of the resulting supernatant was injected into an Agilent 1100 HPLC system. For separating phenolic compounds, an ODS HYPERSIL 250x4.6mm 5μm HPLC Column was utilized. A DAD 1100 diode array detector was employed for compound detection. The mobile phase consisted of demineralized water, flowing at a consistent rate of 0.5 mL/min and a temperature of 80 °C.

J- Statistical analysis

Statistical computations were carried out with Statistica IBM SPSS 26, incorporating analysis of variance. Homogeneity among groups was assessed via the Tukey test, maintaining Values of $p < 0.05$ were considered statistically significant. Data presented are mean values derived from three distinct experimental series.

Results

This study included production of crude cellulase enzyme by *Trichoderma harzianum* PP400831 which used for saccharification of pretreated (SCB). Then it used for production of ethanol by *Saccharomyces cerevisiae* MN901244 figure 1.

A. Screening for cellulase enzymes production

Seven *Trichoderma harzianum* isolates were screened for their ability to

produce cellulase enzymes was presented in table 2. It was clear that all isolates could produce cellulase enzymes and *Trichoderma harzianum* PP400831 showed the highest clear zone that was 8.13 cm with hydrolysis capacity 1.33.

B. Optimization of Cellulase enzymes production

Box-Behnken experimental design (BBD) of Response Surface Methodology (RSM) was employed for optimizing the levels of cellulose mixture concentration, incubation period and tween 80 concentration as an inducer (independent variables) in addition to, detect the effect of their interactions on production of cellulase enzymes (endogluconase and exogluconase activities) by *T. harzianum* PP400831. Seventeen experiments with different combinations of the factors were shown in table 3. The predicted and experimental results were presented in table 3, The statistical analysis of dependent variables was carried out by using (ANOVA) table 4.

Second-order quadratic model equation (Eq. 1) (coded units) was used to express endogluconase and exogluconase (activities), which produced.

$$Y_{\text{Endogluconase (activity)}} = +2.41 - 0.1213A + 0.7088B - 0.3125C - 0.7900AB + 0.0575AC + 0.2475BC - 0.0030A^2 - 0.0180B^2 - 0.0055\,C^2$$

$$Y_{\text{Exogluconase (activity)}} = +1.88 - 0.0462A + 0.2213B - 0.0800\,C - 0.4725\,AB - 0.0150AC + 0.0750BC + 0.0372A^2 + 0.0122B^2 + 0.0248C^2$$

$Y_{\text{Endogluconase (activity)}}$ is the activity of produced endogluconase IU/ml, $Y_{\text{Exogluconase (activity)}}$ is the activity of produced exogluconase IU/ml.

The results from the Analysis of Variance (ANOVA) for both endogluconase and exogluconase activities (Table 4) confirm that the generated regression models are statistically significant. The calculated F-values were significantly higher than the critical value, and the model p-value was very small (0.0001), indicating that the experimental parameters had a highly significant effect on the enzyme activities.

The detailed ANOVA results (Table 4) reveal the specific effects of the factors on each response. The factors of endogluconase activity which are A, B, and C were found to be significant while the square effects (A^2, B^2, C^2) and all interaction terms (AB, AC, BC) were non-significant. In case of exogluconase activity the factors B and C had a significant linear effect, while factor A was non-significant. Only the interaction effect AB was significant, with all other interaction and square terms being non-significant.

The coefficient of determination (R^2) further demonstrated the model fit to the experimental data. R^2 values were calculated as 0.9737 for endogluconase and 0.9500 for exogluconase activity (Table 5). These values are very close to 1, signifying that the models can explain 97.37% and 95% of the variability in the respective enzyme activities, which is excellent. This strong correlation is visually confirmed in Figure 2, where the plots of predicted versus actual results show all experimental points lying very close to the regression line, confirming the high agreement between the model predictions and the actual observed values.

Figure 2 a,b shows 3D plots of the interaction effect of the three independent variables on endogluconase and exogluconase activity which include cellulose mixture concentration (2 – 3 %) and each of incubation period (4 – 9 days) and Tween 80 concentration (0.1 – 0.2 %). It clearly noticed that endogluconase and exogluconase activity increased by increasing incubation period, and concentration of tween 80 as inducer. The maximum activity of endoglucansae was 4.01 IU/ml produced after 9 days incubation period with 2% cellulose mixture concentration and 0.15 % tween 80. In case of exogluconase activity, the maximum activity was 2.64 IU/ml at the same conditions.

C. Verification of the models

After the analysis of the previous results of the seventh runs, the program

predicated the optimum conditions. The predicted optimum conditions were obtained as the incubation period 9 days, cellulose mixture concentration 2.02 % and tween 80 concentration; 0.15 %. Triplicate experiments were carried out under the recommended optimum conditions. Table 6 showed the experimental and the predicated values under optimum conditions, it was clear that the validity of the final experiment is relatively high 93.54 and 96.59 % for endogluconase and exogluconase activity, respectively by calculating the ratio of experimental / predicated values where experimental values were close to the predicted values of the response surface model.

D. Kinetics studies on crude cellulase enzymes

The most important kinetic properties were detected which include the Km and Vmax values of cellulase enzymes produced by tested strain using cellulose rich substrate produced after pretreatment of SCB. Km was 4.114 μmol of substrate and Vmax was 81.3 μmol of sugar/min/mL of enzyme figure 3.

E. Pretreatment of Sugar Cane Bagasse:

SCB pretreatment was carried out using base-acid pretreatment method described by **[19]** and referred to it here by Z.B.A.P. method. The chemical composition of native and pretreated SCB was determined, Cellulose percent was raised from 42.1 in native to 87.6 % after pretreatment by Z.B.A.P. methods figure 4. Hemicellulose content in pretreated SCB was decreased to 4.9 % compared to 25.4 % in the native sample. Lignin was also reduced from 22.1 % in native to 5.7 % in pretreated SCB.

The morphological characteristics of native and pretreated sugarcane bagasse SCB utilizing the Z.B.A.P. method were analyzed through scanning electron microscopy (SEM). SEM pictures illustrate the distinction between native bagasse Image (1) and the treated sample (Image 2). Image (1) illustrates the

compact architecture of native SCB. The compact structure resulting from the connectivity of cellulose and hemicellulose biopolymers embedded inside the lignin matrix is illustrated at four distinct magnifications. Image (2) illustrates the cellulose-rich SCB generated by the Z.B.A.P. process, demonstrating the disintegration of the dense, stiff structure into cellulose fibers that manifested as irregular, uniformly elongated strips, indicating the liberation of disaggregated cellulose from the lignocellulosic matrix in loose bundles.

F. Ethanol Fermentation:

The crude enzyme in addition to the commercial enzymes was used for hydrolysis 10 % cellulose-rich substrate at 50 ˚C for 60 h. Table 7 showed reducing sugar yield % and hydrolysis % resulted by using them, the highest reducing sugar produced by all enzymes was after 48 h and the mixture of commercial enzymes [Sternzyme C 21032 (0.1 g/g) ; Cellic C tec 2 (0.05 mL/g)] produced the highest R.S yield was 8.53 ± 0.02 % corresponding to hydrolysis % as 88.48 ± 0.21%, followed by Cellic C tec 2 enzyme at concentration 0.1 mL/g substrate produced R.S. yield 7.83 % with hydrolysis % as 81.22. Using sternzyme C 21032 (0.2 g/g), the highest R.S. yield was 6.64 % with hydrolysis % of 68.88. finally, the crude enzyme which produced by *T. harzianium* PP400831 at concentration 0.3 g/g produced R.S yield 6.54 % corresponding to hydrolysis yield of 67.87 %.

The highest reducing sugar samples which produced from saccharification of 10% solid load of pretreated SCB by using tested enzymes were fermented by *S. cerevisiae* MN901244 and the results were shown in table 8. Results showed that using mixture of the two commercial enzymes gave the highest ethanol yield which was 35.27 g/L that presented as 80.91 % of theoretical value. Followed by ethanol concentrations 32.35, 26.35 and 25.63 g/L were corresponding as 80.85, 77.66 and

76.96 % of theoretical value, respectively produced from reducing sugar produced by Cellic C tec 2, Sternzyme C 21032 and T. *harzianium* PP400831 crude cellulase enzymes, respectively.

Discussion

Trichoderma sp. is a filamentous fungus widely recognized for its ability to produce many important enzymes. Recently one of the most important enzymes are cellulase enzymes, which are essential for the hydrolysis of cellulose into soluble sugars. Cellulases are a group of enzymes that include endoglucanases, exoglucanases, and β-glucosidases, working synergistically to break down cellulose into glucose. The production of these enzymes has gained significant attention due to their potential applications in biofuel production, biorefineries, and the conversion of plant biomass into valuable products.

This study presents a novel and integrated approach toward sustainable biofuel production. We first utilized Box-Behnken Design coupled with Response Surface Methodology (BBD-RSM) to optimize the fermentation conditions for maximal cellulase enzyme production by our local isolate, *T. harzianum* PP400831. The resulting cost-effective enzyme cocktail was highly efficient, demonstrating saccharification performance on pretreated sugarcane bagasse comparable to that of high-cost commercial enzymes. This successful enzymatic hydrolysis efficiently yielded fermentable sugars, which were subsequently converted into ethanol using *S. cerevisiae* MN901244 strain, validating the complete process efficacy.

The production of cellulase enzymes by *Trichoderma harzianum* is a significant area of research due to its potential applications in biofuel production and waste management. It was recorded that *Trichoderma* sp is wide spread in various ecosystem **[30]** and it has an important role in biodegradation of

agriculture waste. In addition to, it is known as a source of many important industrial enzymes **[31]**.

In this study, seven isolates of *Trichoderma harzianum* were tested for their capacity to produce cellulase enzymes. Isolate T. *harzianum* PP400831 was the most effective, exhibiting the largest clear zone of 8.13 cm and the highest hydrolysis capacity of 1.33. These findings contrast with those reported by **Florencio et al. [32]**, who observed slightly higher hydrolysis capacity values of 1.74 for *T. harzianum* CEN139 and 1.63 for *T. harzianum* CEN 241.

The optimization of cellulase enzyme production by *Trichoderma* species using Response Surface Methodology (RSM) has been extensively studied, revealing various effective strategies. RSM is employed to systematically evaluate the influence of multiple variables on enzyme production, leading to enhanced yields through adjustments of growth conditions.

The current study successfully used the Box-Behnken Design (BBD) within Response Surface Methodology (RSM) to optimize the production of cellulase enzymes by *Trichoderma harzianum* PP400831. The resulting model recommended optimal conditions were 9-days incubation period, 2.02 % cellulose mixture concentration, and 0.15% Tween 80 concentration which yielded enzyme activities of 3.71 IU/mL for endoglucanase and 2.64 IU/mL for exoglucanase enzyme.

Various studies have optimized conditions for cellulase production, focusing on factors such as pH, temperature, and substrate type. The findings indicate that *T. harzianum* can efficiently produce cellulolytic enzymes, making it a valuable organism for industrial applications. **Poor et al. [11]** found that the ideal conditions for cellulase production by *T. harzianum* include incubation period 120 h,

temperature 25 °C and pH level 6.5. Other study suggests variations, such as optimal temperatures around 40 °C and pH of 5.0 for different *Trichoderma* strains **[14]**.

The study by **Saravanan et al. [33]** used (RSM) to determine the optimal medium composition, achieving a maximum cellulase activity of 7.8 IU/mL using mango peel as a substrate. The specific optimized concentrations found were, 25.30 g/L Avicel, 23.53 g/L Soybean, 4.90 g/L cake flour, and $CaCl_2 \cdot 6H_2O$ at 0.95 g/L.

The ability of *T. harzianum* to utilize diverse substrates like Avicel, carboxymethyl cellulose, cellobiose, and Whatman grade 1 filter paper and banana pseudostem has been well documented **[11, 12, 13]**. Avicel and CMC are frequently favored among these, because of their high cellulose content and their strong cellulase-inducing properties. A study by **Triwahyuni et al. [34]** found that wheat bran was the superior substrate for cellulase production by the *Trichoderma* T004 strain, achieving the highest activity of 0.52 FPU/mL. Conversely, using rice bran or Empty Fruit Bunches (EFBs) resulted in significantly lower enzyme activity. Moreover, agro-industrial waste such as peach-palm has shown significant potential, with *T. stromaticum* AM7 achieving a 31.58-fold increase in cellulase production through optimized conditions **[35]**.

Cekmecelioglu et al. [36] demonstrated that *Trichoderma reesei* produces its best cellulase levels when grown on a combination of distillers dried grains with soluble and mango peel, the medium is supplemented with specific nutrients designed to maximize enzyme activity. In a relevant study, **Guruk and Karaaslan [14]** detected that the activity of cellulase enzyme was directly proportional to the substrate concentration, hitting its maximum at 2 % CMC. Any concentration greater than 2 % CMC failed to further enhance the activity, pointing to enzyme saturation.

Numerous studies **[11, 14]** have demonstrated that longer incubation times,

specifically extending up to 120 h, are beneficial for maximizing cellulase production. In line with this, the study by **Guruk and Karaaslan [14]** found that cellulase activity consistently peaked on the fifth day across all their isolates. Among these, the *Trichoderma* isolate (T1) achieved the highest activity, measuring 0.008 IU/mL.

Tween 80, a non-ionic surfactant, enhances the hydrolytic efficiency of cellulases, particularly by reducing the inhibition caused by lignin and hemicelluloses, thus maintaining high enzyme activity during hydrolysis **[37]**. Additionally, it stimulates the secretion of extracellular proteins, including cellulases, in certain fungi, leading to increased enzyme production **[38]**. **Lee et al. [39]** demonstrated that adding surfactants can significantly enhance fungal morphology, by increasing substrate accessibility and boosting cellulase production as much as 177 %. To find the ideal culture conditions for *Trichoderma harzianum* KUC1716, they tested various surfactants and concentrations. Their results showed that 1.0 % Tween 80 was the most effective, yielding the maximum cellulase production and activity levels.

Table 9: Comparative Analysis of the current study and others for production of cellulase enzyme by *Trichoderma sp.*

Producer	Production conditions	Activity of produced enzyme	References
Trichoderma harzianum PP400831	9 days, a cellulose mixture concentration of 2.02%, and a Tween 80 concentration of 0.15% as inducer. (Submerged fermentation)	endoglucanase activity of 4.01 IU/mL and exoglucanase activity of 2.64 IU/mL	Current study
T. harzianum PK5	Copra meal as C-source and KNO_3 as N-source, pH4, T 30 °C, moisture concentration of 125% (v/w), inoculum size of 8%. (Solid state fermentation)	Total cellulase activity 252.54 ± 7.73 U/gds	**Antia et al. (2024) [40]**
T. harzianum LMLBP07	untreated banana Pseudostem moisture content 75%, T 40 °+C, 120 h.	Total cellulase activity: 21.75 ± 3.9 FPU/g d.s	**Legodi et al. (2023) [41]**

13-5	(Solid state fermentation)		
T. viride DW4	a mineral salts medium with corncobs as the sole carbon source, pH5, 5 days	Total cellulase activity (3.41 ± 0.46 IU/mL)	**Kamaluddeen et al. (2023) [42]**
Trichoderma reesei NCIM 1186 and *Penicillium citrinum* NCIM 768	steam pre-treated wheat bran with 70 % moisture content, inoculated with 10^6 spores, T 30 °C and pH 5, 6 days. (Solid state fermentation).	Total cellulase activity of 6.71 FPU/gds	**Lodha et al. (2020) [43]**
Trichoderma reesei	40g/L paper sludge as carbon source and 67.5 g/L pasteurised blood as nitrogen source substituted in Mandels medium	Total cellulase activities up to 28.1 FPU/mL	**Weiss et al. (2020) [44]**

The kinetic characterization of the crude cellulase enzyme produced in this study revealed a Michaelis constant (Km) of 4.114 mol. This value signifies the substrate concentration was 4.114 mol required for the enzyme to operate at half of its maximum potential activity. Furthermore, the determined maximum reaction velocity Vmax was 81.3 mol/min/L, indicating that the maximum enzyme capacity when fully saturated with substrate is the production of 81.3/ mol of sugar per minute per mL of enzyme solution.

Sugarcane bagasse is an excellent raw material for bioethanol because it's abundant at sugar mills and is already pulverized after the juice is extracted. The main goal of chemical pretreatment is to prepare the cellulose for enzymatic breakdown. This is achieved by removing lignin and hemicellulose, decreasing cellulose crystalline structure, and increasing its porosity. Effective pretreatment must also meet four key criteria which are, high sugar yield, preserve the cellulose, avoid creating compounds that inhibit fermentation, and be economically viable.

For this research, we pretreated sugarcane bagasse (SCB) using the method

described by **Zohri et al. [6]**, which we refer to as Z.B.A.P. This method proved highly effective as showed in figure 5, where the cellulose content in the pretreated SCB more than doubled, rising from 42.1 % in the raw material to an impressive 87.6% after pretreatment. Simultaneously, both the hemicellulose and lignin were drastically reduced. Hemicellulose dropped from 25.4 % to 4.9 %, and lignin decreased from 22.1 % to just 5.7 %. The findings from the current study are consistent with that established by **Rezende et al. [27]** where they initially reported that raw (SCB) contains 35.2 % cellulose, 24.5 % hemicellulose, and 22.1 % lignin. Following a two-step pretreatment with 1% H_2SO_4 and 2 % NaOH, they observed similar component shifts where the cellulose content increased significantly to 84.7 %, while hemicellulose dropped sharply to 3.3 % and lignin decreased to 9.5 %. other studies confirm that combined chemical pretreatments significantly increase the cellulose concentration in sugarcane bagasse (SCB). For instance, **Cui et al. [45]** used nitric acid followed by sodium hydroxide, which dramatically raised the SCB cellulose content from 54.43 % to 93.53 %. Similarly, **Igbojionu et al. [46]** employed a two-stage process (3 % NaOH then 0.3 % maleic acid) on SCB initially containing 40.4 % cellulose, 20.9 % hemicellulose, and 22.5 % lignin. Their method effectively purified the biomass, resulting in a pretreated material that was 80.1% cellulose, with residual hemicellulose and lignin levels of only 4.0 % and 3.7 %, respectively. Meanwhile, **Nath et al. [47]** used a sequential alkali and organosolv method, successfully increasing the cellulose content from 42.5 % to 65.94 %. They also quantified the solid yield, noting that 1kg of raw SCB produced 461 g of the cellulose-rich substrate after pretreatment.

The morphological characteristics of both native and pretreated sugarcane bagasse (SCB) by Z.B.A.P. method were examined using Scanning Electron Microscopy (SEM). The SEM images visually confirm the success of the

pretreatment. Image 1 (Native SCB) shows a solid, interconnected structure where cellulose and hemicellulose are tightly embedded within the lignin, resulting in a highly aggregated surface. Conversely, Image 2 (Treated SCB) displays a drastically different morphology where the structure is characterized by loosely packed, disaggregated cellulose fibers appearing as irregular uniform long strips.

The findings indicate that the Z.B.A.P. technique effectively disrupted the lignocellulosic matrix, which is crucial for enhancing cellulose accessibility to enzymes. This result contrasts somewhat with other chemical pretreatment outcomes of **Rezende et al. [27]** who used sequential acid/base treatment on SCB. They found that even after pretreatment, the cellulose fibers, though released from the rigid structure, were still tightly aggregated and formed attached bundles. However, **Zohri et al. [6]** who used a one-step method involving both sodium hydroxide and sulfuric acid, also observed a successful outcome. They specifically reported that their pretreated SCB had a disaggregated morphology, unlike the aggregated structure of the native substrate.

The saccharification of the pretreated sugarcane bagasse (SCB) using a 10 % solid load was detected. The highest reducing sugar yield was achieved using a mixture of the two commercial enzymes, following this, individual commercial enzymes and the crude enzyme generated progressively lower R.S. yields. These results are supported by other biomass hydrolysis studies, such as that by **Santos et al. [48]**, who tested cellulase enzymes derived from marine *Aspergillus sydowii* CBMAI 934 on cellulose extracted from alkaline-pretreated sugarcane bagasse, they achieved a maximum saccharification of 78%.

Following saccharification, the released reducing sugars were used to produce ethanol via fermentation with *S. cerevisiae* MN901244. The highest

ethanol yield was 35.27 g/L, achieved using the sugars derived from the expensive commercial enzyme mixture (10 % solid load). Critically, the sugars produced by our crude cellulase enzyme generated a comparable and significant ethanol concentration of 25.63 g/L from the same concentration of substrate.

The crude enzyme cocktail synthesized in this study achieved an ethanol yield of 25.63 g/L, representing approximately 73 % of the yield obtained using high-cost commercial standard enzymes. While this result is promising, it remains lower than the 90 g/L benchmark typical of commercial corn ethanol production (based on GREET model assumptions from the HigbyBarrett 2024 report). However, it is important to note that corn-based production relies on edible substrates, potentially impacting food and feed security. The lower ethanol concentration observed here can be attributed to several factors. primarily the recalcitrant nature of sugarcane bagasse (SCB). The complex lignocellulosic structure of SCB requires pretreatment and hydrolysis, processes which often result in sugar loss and lower initial substrate concentrations. Furthermore, the hydrolysate likely contained oligomeric sugars that were not fully assimilated during fermentation, thereby limiting the final conversion efficiency [49].

The formation of inhibitory compounds (e.g., furfural, HMF, organic acids) from the pre-treatment stage may be present, these compounds can significantly reduce cell viability and metabolic efficiency, capping the final yield. Finally, simple batch fermentation process which employed in this study could play a role in limitation the overall productivity compared to industrially preferred modes such as fed-batch or continuous fermentation.

So, the achieved yield, when viewed through the lens of using a cost-free, non-food-chain waste material for simultaneous waste problem-solving and

biofuel generation, represents a promising and environmentally sustainable path toward lignocellulosic ethanol production.

Table (10): **Comparison of bioethanol production from various substrates using different enzymatic saccharification systems.**

Substrate	Enzymes used for Saccharification	Ethanol concentration (g/L)	References
10 % pretreated SCB	EG, EXG produced by *Trichoderma harzianum* PP400831	25.63	Recent study
20 g/L CMC	BGL1, EG produced by *Trichoderma viride*.	4.6	Gong et al. **[50]**
Barley straw pretreated with laccases complexes	BGL1 produced by *Saccharomyces fibuligera,* EG produced by *Clostridium thermocellum*	2.3	Hyeon et al. **[51]**
Alkaline peroxide pretreated wheat straw	BGL1 , EG , CBHI produced by *T. reesei*.	24	Zhang et al. **[52]**
20 g/L of NaOH pretreated corn cob (43% cellulose)	BGL1 produced by *Sachharomyces fibuligera*, EG produced by *T. reesei*.	4	Davison et al. **[53]**

BGL1 (β-glucosidase), EG (endoglucanase), EXG (exogluconase), CBHI (cellobiohydrolase I)

However, these yields compare favorably to other studies, where **Shaibani et al. [54]** produced only 1.36 g/L of ethanol from 5% alkali-pretreated SCB using the commercial enzyme Celluclast for saccharification and *Saccharomyces cerevisiae* for production of ethanol. Similarly, **Chandel et al. [55]** achieved 8.13 g/L of ethanol after hydrolyzing acid/base-pretreated SCB with Celluclast and Novozym 188 enzymes, followed by fermentation with *S. cerevisiae*. However, a higher yield of 75.57 g/L was reported by **Gao et al. [56]** when fermenting a high solid loading (33 %) of alkali-pretreated bagasse hydrolyzed by Cellic C Tec2. **Subramanian and Suresh [57]** recorded that, continuous fermentation utilizing

the *E. coli* FBR strain consistently achieved an ethanol concentration of approximately 19.2 g/L when using wheat straw hydrolysate (WSH) as the feedstock.

Based on our calculations, processing one ton of native SCB with the high-cost commercial enzyme mixture yields 215 L of ethanol. Crucially, by utilizing the cost-effective crude cellulase developed in this study, the ethanol concentration was 164.94 L per ton of SCB, demonstrating the excellent economic viability of the crude cellulase enzyme. Comparison with other studies confirms the variability in ethanol production per ton of bagasse. **Arnaldo and Adriano [58]** reported a yield of 149.3 L of ethanol from one ton of bagasse. Similarly, **Mesa et al. [59]** showed that one ton of pretreated SCB could produce between 142 kg and 151 kg of ethanol, which corresponding to a volume range of approximately 180 L to 192 L. In contrast, the sequential alkali and organosolv pretreatment used by **Nath et al. [47]** generated a significantly lower yield of only 27 kg (34.20 L) of ethanol per ton of bagasse.

Conclusion

The research successfully optimized cellulase enzymes production using the local strain *T. harzianum* PP400831. The ideal conditions were 9 days of incubation with 2 % cellulose and 0.15 % Tween 80 which resulted in enzyme activities of 4.01 IU/mL for endoglucanase and 2.64 IU/mL for exoglucanase. The produced cellulase enzymes demonstrated high efficacy in hydrolyzing pretreated sugarcane bagasse, comparable to expensive commercial enzymes. Following saccharification, the resulting reduced sugars were fermented to produced ethanol using *S. cerevisiae* MN901244, yielding 25.63 g/L. This final ethanol concentration represents approximately 73 % of the yield obtained when using commercial enzyme. Offering a sustainable solution for cost reduction in 2G bioethanol production from sugar cane

bagasse. Also, these findings highlight the potential for wider industrial application, promoting a more circular bioeconomy and reducing reliance on fossil fuels.

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Author contributions

M.A., A.Z., S.M. M.A.: investigation, draft writing, and software, S.M., M.A.: methodology, MA, A.Z.: write and review the article and approved the submitted version.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflict of interest

The authors declare no competing interests.

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Table (2): Screening for cellulolytic activity of *Trichoderma harzianum* isolates.

Tested Isolates	Colony diameter (Cm)	Clear zone diameter (cm)	HC value*
***T. harzianum* 1**	6.20 ±0.36	6.47±0.32	1.04±0.01
***T. harzianum* 2**	6.87±0.11	7.73±0.15	1.14±0.02
***T. harzianum* 3**	7.47±0.06	7.87±0.15	1.05±0.02
***T. harzianum* 4**	6.03± 0.06	7.37±0.21	1.22±0.04
***T. harzianum* PP400831**	6.10 ± 0.10	8.13±0.15	1.33±0.03

***T. harzianum* 70**	7.87±0.12	8.10±0.17	1.03±0.01
***T. harzianum* 80**	7.07±0.12	8.17±0.12	1.16±0.03

HC value: hydrolysis capacity which calculated as diameter of clear zone / diameter of colony.

Table (3): A Box-Behnken experimental design and the results of dependent variables (endogluconase and exogluconase (activity), by *Trichoderma harzianum 60*

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	A:C- Source (%)	B:incubation Period (days)	C:Tween 80 Concentration (%)	Endogluconase (activity) (IU/mL)	Exogluconase (activity) (IU/mL)
1	2.5	9	0.1	3.15	2.16
2	2.5	4	0.2	1.12	1.52
3	2.5	6.5	0.15	2.55	1.82
4	2	6.5	0.1	2.89	2.06
5	2.5	6.5	0.15	2.37	1.82
6	3	6.5	0.2	2.02	1.79
7	3	6.5	0.1	2.53	2
8	2.5	6.5	0.15	2.4	1.89
9	2.5	6.5	0.15	2.35	1.96
10	2	9	0.15	4.01	2.64
11	2	6.5	0.2	2.15	1.91
12	3	4	0.15	2.34	2.16
13	2.5	9	0.2	3.02	2.17
14	3	9	0.15	2.19	1.6
15	2.5	4	0.1	2.24	1.81
16	2.5	6.5	0.15	2.36	1.9
17	2	4	0.15	1	1.31

Table (4): ANOVA for the entire quadratic models of response (1) endogluconase activity, response (2) exogluconase activity,

Response (1) Endogluconase activity					
Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	7.67	9	0.8526	215.90	< 0.0001
A-incubation period	0.1176	1	0.1176	29.78	0.0009
B-Conc of cellulose mixture	4.02	1	4.02	1017.55	< 0.0001
C-Conc of Tween 80	0.7813	1	0.7813	197.82	< 0.0001
AB	2.50	1	2.50	632.11	< 0.0001
AC	0.0132	1	0.0132	3.35	0.1100
BC	0.2450	1	0.2450	62.04	0.0001
A^2	0.0000	1	0.0000	0.0096	0.9247
B^2	0.0014	1	0.0014	0.3454	0.5752
C^2	0.0001	1	0.0001	0.0323	0.8626
Residual	0.0276	7	0.0039		
Lack of Fit	0.0003	3	0.0001	0.0159	0.9968
Pure Error	0.0273	4	0.0068		
Cor Total	7.70	16			
Response (2) Exogluconase activity					
Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	1.39	9	0.1540	50.14	< 0.0001
A-incubation period	0.0171	1	0.0171	5.57	0.0503
B-Conc of cellulose mixture	0.3916	1	0.3916	127.47	< 0.0001
C-Conc of Tween 80	0.0512	1	0.0512	16.67	0.0047
AB	0.8930	1	0.8930	290.68	< 0.0001
AC	0.0009	1	0.0009	0.2930	0.6051
BC	0.0225	1	0.0225	7.32	0.0304
A^2	0.0058	1	0.0058	1.90	0.2103
B^2	0.0006	1	0.0006	0.2057	0.6639
C^2	0.0026	1	0.0026	0.8395	0.3900
Residual	0.0215	7	0.0031		
Lack of Fit	0.0074	3	0.0025	0.7031	0.5979
Pure Error	0.0141	4	0.0035		
Cor Total	1.41	16			

Table (5): Correlation coefficients that indicate the fitting of the mode of the dependent variables.

Dependent variables	R^2	R^2%
Endogluconase (activity)	**0.9737**	**97.37**
Exogluconase (activity)	**0.9500**	**95**

Table (6): Comparison of experimental values with predicted values at the optimized condition

	Predicated value	Experimental value	Ratio Experimental / Predicated %
Endogluconase units (IU/ml)	3.97	3.71	93.45
Exoglucnase units (IU/ml)	2.64	2.55	96.59

Table (7): Reducing sugar yield (R. S%) and hydrolysis % from 10 solid load of pretreated (SCB) by Z.B.A.P. method using *Trichoderma* crude enzyme, mixture of commercial enzymes (Sternzyme C 21032 and Cellic C tec), each one individualy for 60 h.

Enzymes	***T. harzianum;* 0.3 g/g**		**Sternzyme C 21032; (0.2 g/g)**		**Cellic C tec 2; (0.1 ml /g)**		Mix (**Sternzyme C 21032 (0.1 g/g); Cellic C tec 2 (0.05 mL/g)**	
parameters / Time (h)	**R.S%**	**Hydrolysis %**	**R.S%**	**Hydrolysis %**	**R.S %**	**Hydrolysis %**	**R.S %**	**Hydrolysis %**
12	**0.87±0.02**	**9.02±0.11**	**0.73 ± 0.04**	**7.57 ± 0.42**	**1.85±0.12**	**19.19 ± 1.25**	**1.67±0.09**	**17.32 ±0.94**
24	**2.58±0.04**	**26.76±0.2**	**2.65 ± 0.02**	**27.49 ± 0.21**	**3.96±0.03**	**41.08 ± 0.31**	**3.64±0.04**	**37.76 ± 0.42**
36	**4.82±0.03**	**50.00±0.31**	**4.75 ± 0.10**	**49.27 ± 1.04**	**6.33±0.02**	**56.66 ± 0.21**	**5.83±0.07**	**60.48 ± 0.73**
48	**6.54±0.02**	**67.84±0.45**	**6.64±0.08**	**68.88 ± 0.83**	**7.83±0.03**	**81.22±0.32**	**8.53±0.02**	**88.48± 0.21**

60	**6.42±0.03**	**66.59±0.73**	**6.51±0.07**	**68.43 ± 1.14**	**7.81± 0.02**	**81.09± 0.32**	**7.65±0.02**	**79.36 ±0.21**

Table (8): Fermentation Kinetics of ethanol production from reducing sugar resulted from enzymatic saccharified of 10 % pretreated SCB by *S. cerevisiae* MN901244 at 28°C & pH 5 for 72 h.

Enzyme / Kinetics parameters	Trich. Cellulase enzymes R.S. 6.54%	Sternzyme C 21032 R.S. 6.64%	Cellic C tec 2 R.S. 7.83%	Mixture of commercial Enzymes R.S. 8.53%
g/l	**25.63±0.21^{a}**	**26.35±0.16^{b}**	**32.35±0.47^{c}**	**35.27±0.24^{d}**
V\V%	**3.25±0.11**	**3.34±0.02**	**4.10±0.06**	**4.47±0.03**
%of theo.	**76.69±0.34**	**77.66± 0.47**	**80.85± 0.97**	**80.91±0.36**
$Y_{g/gIS}$	**0.39±0.00**	**0.40±0.00**	**0.41±0.00**	**0.41±0.00**
$Y_{g/gIsb}$	**0.26±0.00**	**0.26±0.00**	**0.32±0.00**	**0.35±0.00**
$Y_{g/g\ bagasse}$	**0.12±0.00**	**0.13±0.00**	**0.15±0.00**	**0.17±0.00**

g/L= Ethanol concentration (g/L)

V\V%= Ethanol concentration (V/V%)

% of theo.= Percentage of ethanol yield over the theoretical value of ethanol

$Y_{g/gIS}$ = Ethanol yield over the initial reducing sugar (g/g)

$Y_{g/gIsb}$ = Ethanol yield over the initial enzymatic saccharified PSCB (g/g)

$Y_{g/g\ bagasse}$= Ethanol yields over raw bagasse (g/g)

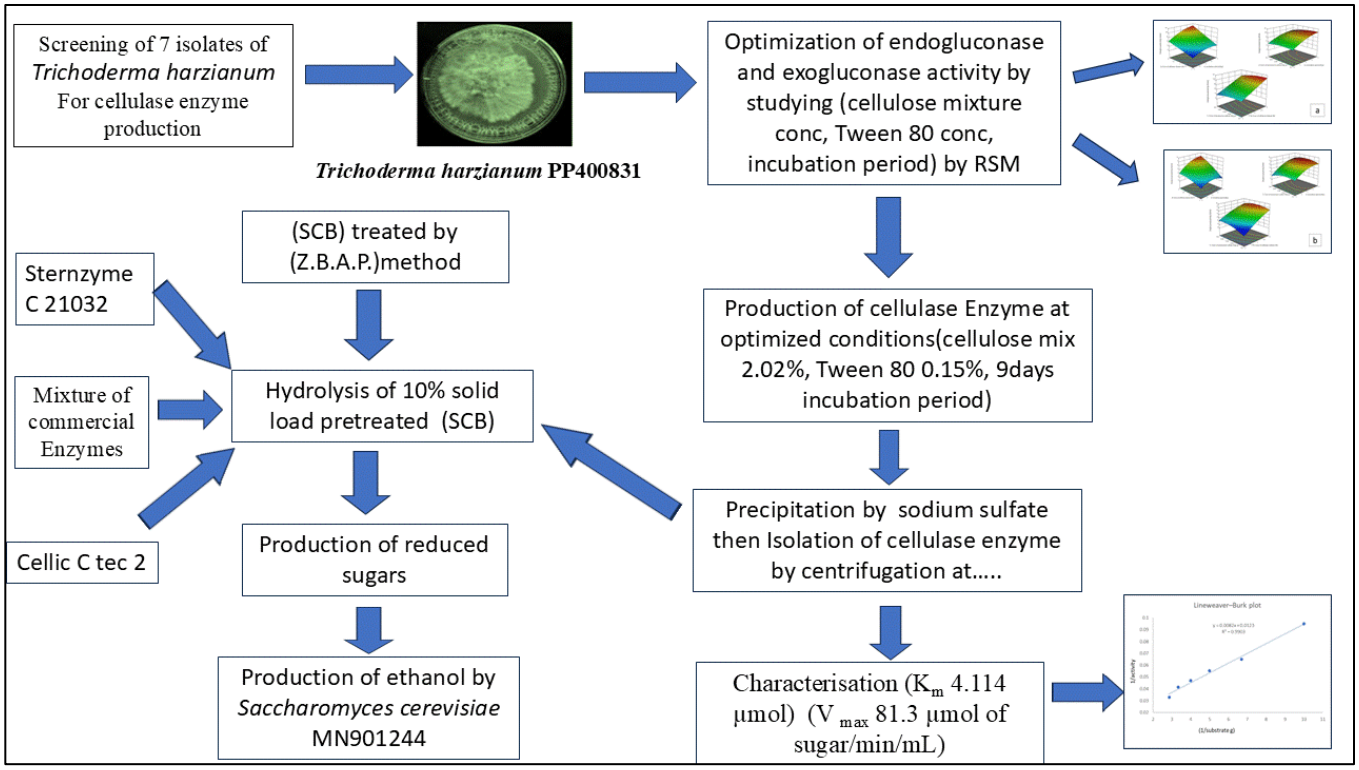


Figure (1): A design summarized the methodology which included in the manuscript

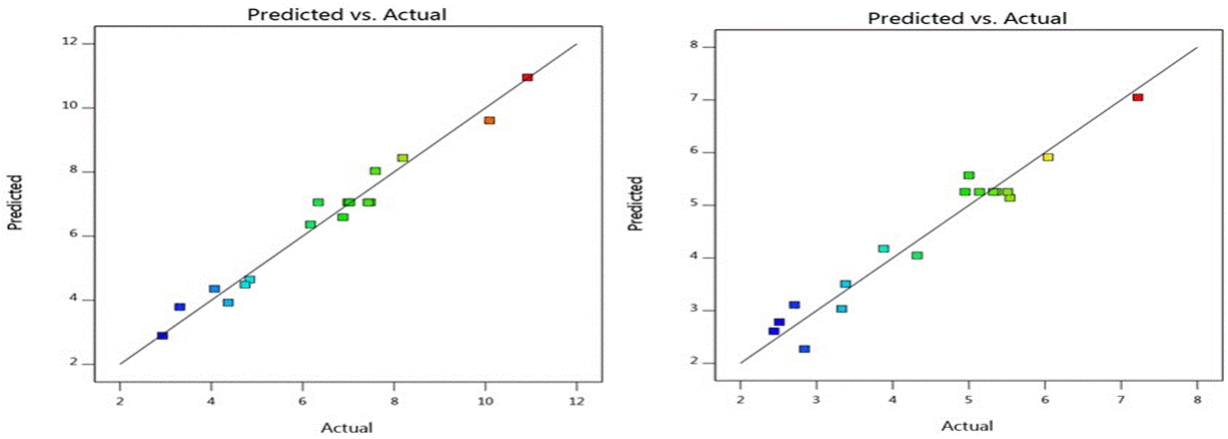
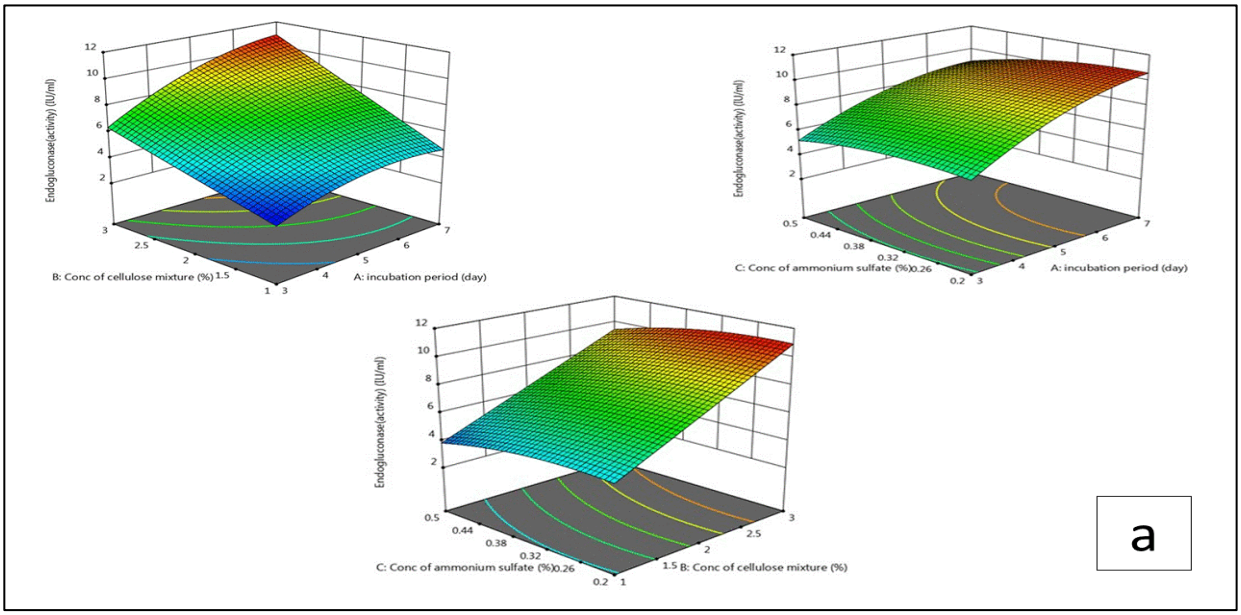


Figure (2): Predicted vs actual values for endogluconase and exogluconase activties.



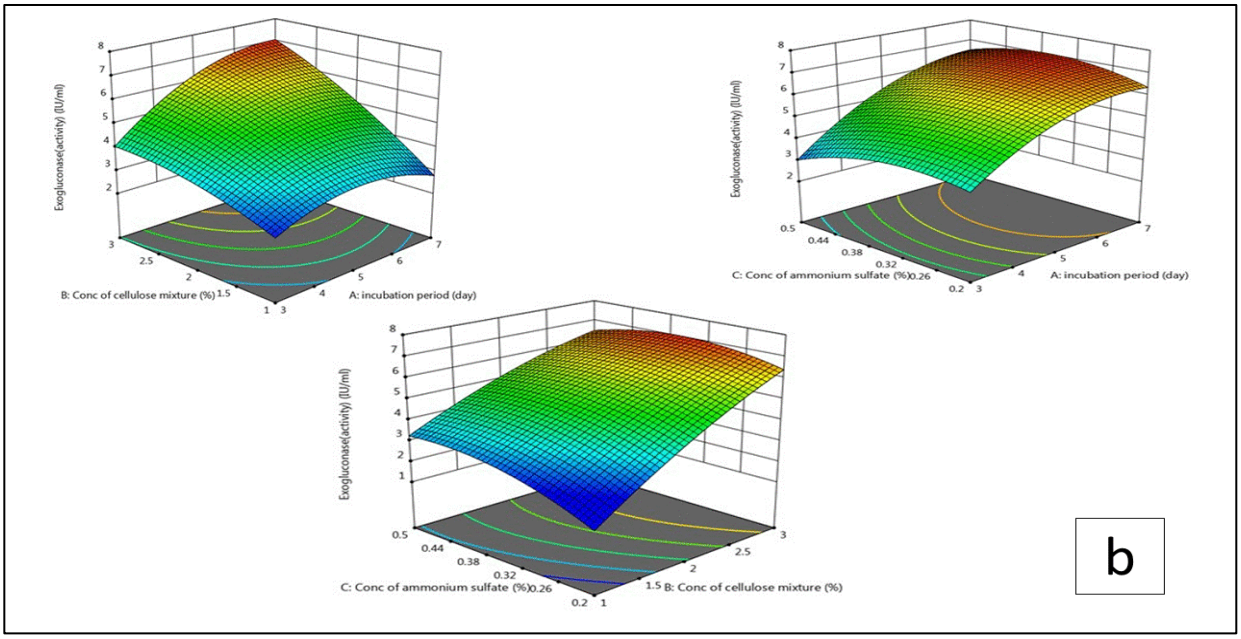


Figure (3): Response surface 3D plots indicated the effect of cellulose mixture concentration, incubation period and tween 80 concentration for evolution of endoglucanase and exoglucanase activities.

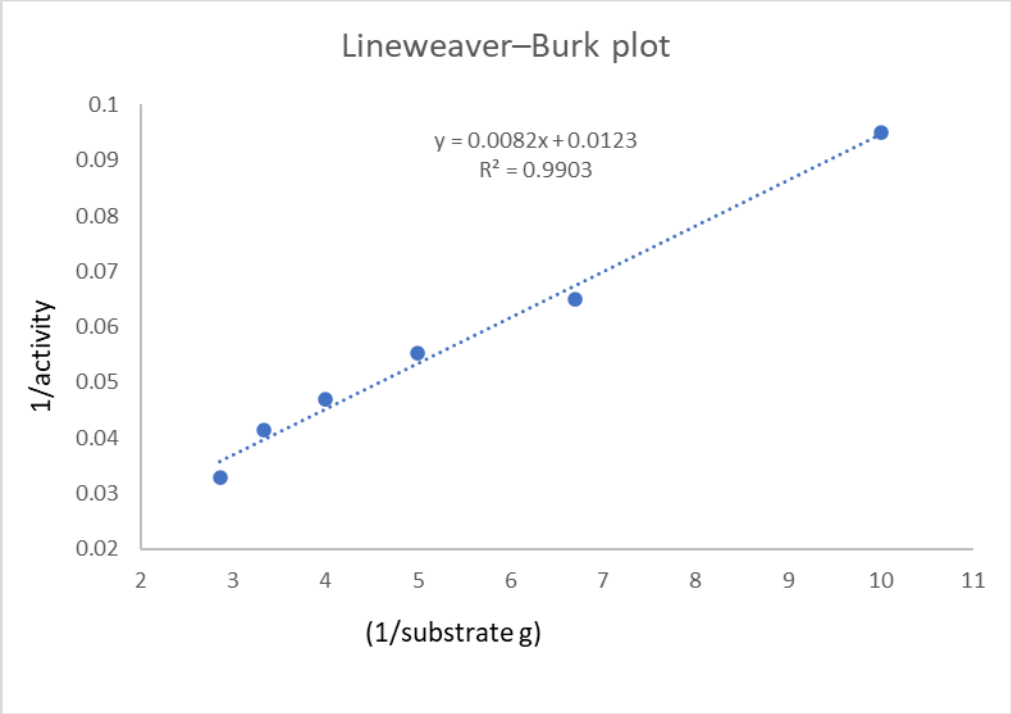


Figure (4): Kinetic studies of cellulase enzymes activity from *Trichoderma harzianum* (PP400831) shows Lineweaver–Burk plot

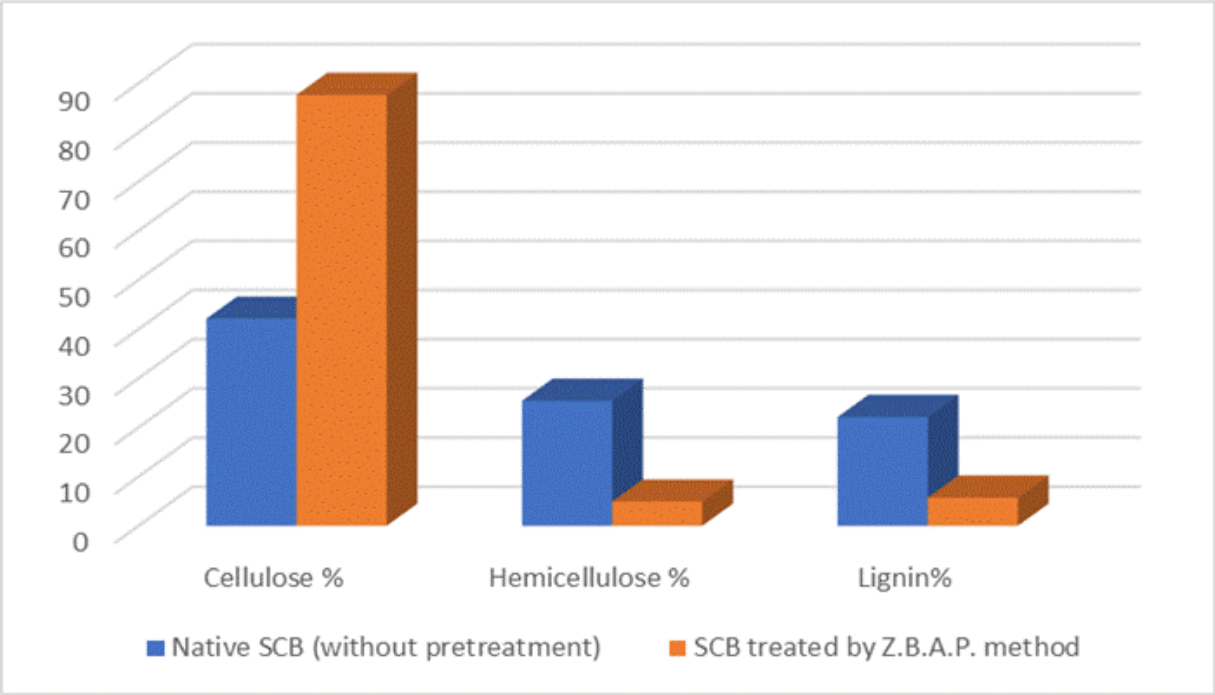


Figure (5): Cellulose, hemicellulose, and lignin content in native and cellulose-rich SCB produced by using Z.B.A.P. methods.

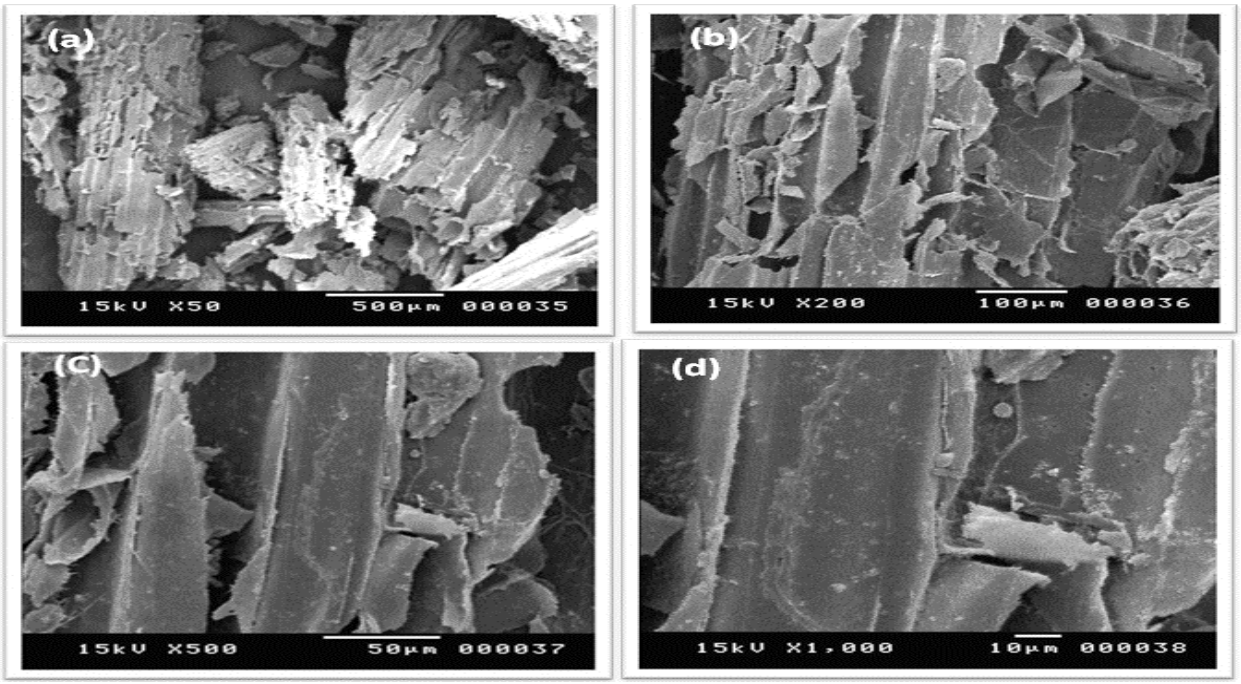


Fig. 6: SEM image of native SCB with different magnifications (**(a) ×50, (b) ×200, (c) ×500 and (d) ×10000**). illustrates the compact architecture of native SCB

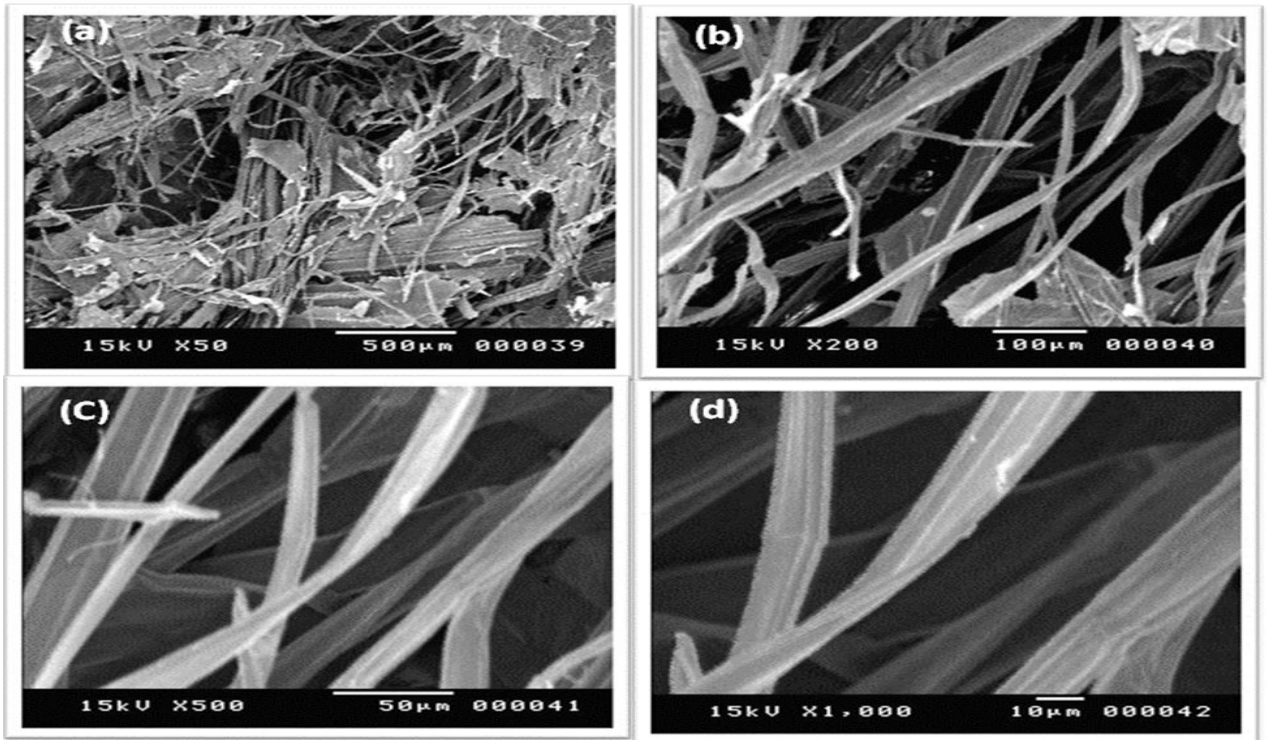


Fig. 7: SEM image of cellulose- rich SCB produced by Z.B.A.P. method with different magnification ((a) ×50, (b) ×200, (c) ×500 and (d) ×1000). The compact structure was broken down, resulting in irregular, long strips of cellulose fibers.