Statistical Optimization of lignocellulolytic enzyme production by *Fusarium oxysporum* through response surface methodology

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Abstract

The successful technique for producing a multienzyme complex for biomass conversion involves both the selection of microorganisms and the fermentation process parameters. This study describes the isolation, screening, and selection of biomass-degrading fungal species from corn-cultivated soil samples, as well as the refinement of the cultural conditions for a *Fusarium oxysporum* (IF 5) strain that was grown on several agro-industrial wastes (sugar cane bagasse, corn stover, corn cob, and rice straw) in solid state fermentation to produce a multienzyme complex. Evaluation of lignocellulosic substrate for enzyme synthesis indicated corn stover to be the most effective substrate, with activities of cellulase 16 ± 0.76 U/gds, xylanase 29 ±1.25 U/gds, laccase 4.91 ± 1.02 U/gds, lignin peroxidase 3.98 ± 0.87 U/gds, and manganese peroxidase 3.21 ± 0.98 U/gds. Response surface methodology (RSM) with Box-Behnken design (BBD) was employed to examine the interaction between variables (moisture content, inoculum size, inoculum volume, and incubation period). Maximum enzyme activity was found at a moisture content of 80%, a biomass size of 2 mm, an inoculum volume of 4 mL, and an incubation time of 15 days. The multiple correlation level R^2 was 0.9736 for cellulase, 0.9944 for xylanase, 0.9931 for laccase, 0.9934 for lignin peroxidase, and 0.9950 for manganese peroxidase. These findings suggest that *Fusarium oxysporum* (IF 5) is capable of synthesizing all five enzymes essential for the efficient conversion of biomass, found that the ideal circumstances for lignocellulolytic enzyme synthesis were extremely beneficial for the generation of bioethanol from lignocellulosic biomass.

Keywords: *Lignocellulolytic enzymes, submerged fermentation, Lignocellulosic waste, solid state fermentation, Response surface methodology.*

1.Introduction

The biological conversion of lignocellulosic materials to sustainable fuels and substances involves the degradation of structural polysaccharides such as cellulose and hemicellulose. Ligninolytic enzymes degrades the polysaccharides by attacking the glycoside hydrolases in lignocellulosic biomass. This enzyme facilitates the hydrolytic activity where the polysaccharides are converted into monomers. These monomers are subsequently fermented into bioethanol by yeasts. The enzyme complex (cellulases, hemicellulases, ligninases and pectinases) are required to degrade lignocellulose biomass due to its complexity (Malgas et al., 2017). It is thought that using these enzymes to biodegrade lignocellulose into monomers is the most efficient way to produce bioethanol.

Bioconversion and biodegradation of lignocelluloses into usable products is a significant environmental challenge. Typically, lignocellulosic biomass, such as corn stover and rice straw, is saccharified into monosaccharides and then fermented with yeast to make biobased products (Wettstein et al., 2012). Pretreatment is essential for the breakdown of lignin structure and the reduction of biomass recalcitrance; it also increases cellulase enzyme accessibility. Through physical, chemical, and biological pretreatment processes, the lignin content of wood can be lowered by around 50 percent.

The major disadvantages of the physical and chemical methods are costly equipment, high energy consumption, and the potential for secondary environmental contamination, whereas the biological treatment has the benefits of low cost, simple operation without special requirements, and environmental protection (Li et al., 2013; liang et al., 2010;).

A desirable method for converting lignocellulosic materials into biomass and producing lignocellulolytic enzymes is solid-state fermentation (SSF) (Sharma and Arora, 2010; Elisashvili et al., 2009). The residual biomass may be utilized as animal feed, and the enzymes can be recovered and processed to various degrees for use in other industries.

As lignocellulosic biomass decomposes biologically, certain bacteria, white-rot, soft-rot, and brown-rot fungus can degrade lignin and hemicellulose (Rasmussen et al. 2010; Wan and Li, 2012). The vast majority of microorganisms are incapable of attacking lignin, while certain fungi can concurrently degrade lignin and polysaccharides while losing carbohydrates, while others can solely degrade lignin. Due to their ability to produce proteolytic enzymes and reactive exoenzymes, their capacity to destroy lignin was unique. Collectively known as cellulases, the three primary hydrolyses are xylanases (EC 3.2.1.8), endo-1,4-D-glucanases (EC3.2.1.4), and exo-1,4-D-glucanases (EC 3.2.1.91). A variety of fungal strains also secrete the three extracellular enzymes lignin peroxidase (LiP) (EC

1.11.1.14), manganese-dependent peroxidase (MnP) (EC 1.11.1.13), and laccase (Lac) (1.10.3.2). It is essential to choose the appropriate strains in order to identify the strain that produces the most enzymes.

Fundamental research's ultimate goal is to lower the cost of enzymes by employing less expensive raw materials and to improve the fermentation in industry sectors (Lee et al. 2011). Because it ignores interactions between variables and parameters, the traditional conventional operational parameters (COS) technique of changing one element at a time cannot provide the ideal physical and nutritional conditions (Hye et al., 2008; Kammoun et al., 2008; Lotfy et al., 2011). The response surface technique, in contrast, enables simultaneous adjustment of a number of parameters at various levels in order to examine the interactions between two or more elements. This examination can produce precise optimization outcomes (Benzina et al., 2012).

This work describes the isolation, screening, and selection of lignocellulolytic degrading fungi, as well as the optimization of the factors for multienzyme complex production by a selected fungus, *Fusarium oxysporum* (IF 5), cultivated using various agroindustrial residues in solid state fermentation. The statistical response surface method (RSM) is also used to find the best process factor values for making these enzymes more efficiently.

2. Materials and Methods

2.1 Collection of samples

Various soil samples were collected from agricultural farm. Fifteen samples were gathered and stored in sterile polythene bags for future use.

2.2 Isolation of lignin degrading fungi

To isolate lignin-degrading fungi from soil samples, a minimal salt medium with lignin as a carbon source was utilized. (g/L) Na_2HP0_4: 2.4, K_2HPO_4: 2.0, $MgSO_4$: 0.1, NH_4NO_3: 5.0, $CaCl_2$: 0.1, Agar: 6.0, and Kraft lignin: 1 (g/L). Covering MSM-L agar plate with serially diluted soil samples. For the development of ligninolytic fungi, the plates were incubated at 28°C for 3-5 days (Chairattanamanokorn et al., 2006). To develop a pure culture, the selected colonies were cultivated repeatedly on potato dextrose agar (PDA) and kept at 4°C until required.

2.3 Screening for cellulolytic, hemicellulolytic, and ligninolytic enzyme production

Cellulase and xylanase activities were detected using CMC and xylan agar plates, respectively. After 7-8 days of incubation at 28 ± 2°C, the CMC / Xylan agar plates have been inundated with 0.1% Congo red solution for 10- 15 minutes and destained with 1M

NaCl for 10 minutes. Clear zone development surrounding fungal colonies indicates cellulose or xylose breakdown (Ghose et al., 1987).

The synthesis of manganese peroxidase and lignin peroxidase enzymes was evaluated using an Azure B agar plate test (Bandounas et al., 2011). A clean zone surrounding the colonies indicates that manganese peroxidase and lignin peroxidase are being produced.

Laccase enzyme production is evaluated using the tannic acid agar plate test and the guaiacol plate assay. The isolated fungi were streaked onto tannic agar as well as guaiacol agar plates, where they were cultured for 10 days at 25 °C (Monssef et al., 2015).

A yellow to brown zone around the colony of the fungal strain that oxidizes tannic acid indicates laccase enzyme production. The development of the orange or brown halo zone observed in guaiacol plates was due to laccase enzyme production (Sheikhi et al., 2012). Potential fungal strains have been chosen for further investigation.

2.4 Evaluation quantitatively of fungal isolates for lignocellulolytic enzyme synthesis

2.4.1 Production of enzymes during submerged fermentation (SMF)

The cellulase, xylanase, laccase, lignin peroxidase, and manganese peroxidase enzyme activities of fungal isolates with a wider zone width were measured during submerged fermentation. All isolated fungal strains were inoculated into 100 mL of minimal salt medium containing lignin and incubated at 120 rpm for seven days. Every day for seven days, samples were collected. The fermentation broth was spun for 30 minutes at 7,000 x g to extract the crude enzymes (Dinis et al. 2009).

2.4.2 Enzyme activity assay

The cellulase and xylanase activities were evaluated utilizing a customized form of the method described in Delabona et al. (2012) with some modifications. This was achieved by the combination of phosphate citrate buffer, CMC, and crude cellulase enzyme with acetate buffer, 1% xylan, and crude xylanase enzyme, respectively. In a water bath, the enzyme combination was heated over 30 minutes at 50°C. This reaction was halted by adding 3 ml of the 3,5-dinitrosalicylic acid (DNS) reagent. Using an enzyme-free control solution and a UV-Visible spectrophotometer, the absorbance was evaluated at 540 nm. As benchmarks for cellulase and xylanase activity, glucose and xylose were used as well. Under standard test conditions, one unit of enzyme activity corresponds to the quantity of enzyme that releases 1μmol of equivalent glucose and xylose per minute.

Veratryl alcohol is oxidized to veratraldehyde in the presence of hydrogen peroxide during the lignin peroxidase test. Enzyme extract, 1 mM veratryl alcohol, 0.2mM hydrogen

peroxide, and 0.1M citrate buffer comprised the reaction mixture. The mixture is incubated at 30°C for 10 minutes. Absorption is measured at 310 nm (Jing, 2010).

According to Huy (2017), the activity of manganese peroxidase was evaluated. Additionally, 1 mM manganese sulphate and 50 mM sodium malonate were added. The aforementioned procedure was initiated by mixing 0.1 mM of hydrogen peroxide and incubated at 35°C for 30 minutes. At 270nm, the complex form of manganese malonate was detected.

Monssef et al. (2015) have reported on the oxidation of guaiacol for the laccase test. The reddish-brown colour generated by laccase oxidation of guaiacol at 450 nm is used to measure enzyme activity. With 2 mM Guaiacol, 10 mM sodium acetate buffer, and 1 mL of enzyme extract, a reaction combination can be created. Enzyme activity is measured in units per microliters(U/mL).

2.5 Molecular identification of selected fungal isolate

Internal transcribed spacer (ITS) regions were amplified to identify the efficient fungal isolate for the synthesis of cellulase, xylanase, laccase, lignin peroxidase, and manganese peroxidase. Universal primers ITS 1 (5'TCCGTAGGTGAACCTGCGG3') (Forward) and ITS 2 (3'GCTGCGTTCTTCATCGATGC5') (Reverse) were used to amplify the ITS region. Sequencing was performed by a professional service provider (Macrogen, South Korea). The 18s rRNA sequences were compared to the database sequences on the internet using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) to find the sequence that was the most comparable. Multiple sequence alignment was performed on a sorted sequence of the strain's 15 most comparable neighbor sequences using CLUSTAL ω. The trial version of MEGA 11.0 software is utilized to calculate similarity indices and construct phylogenetic relationships using the neighbor-joining method.

2.6 Solid state fermentation

The potential of corn stover, corn cob, sugar cane bagasse, and rice straw as substrates for cellulase, xylanase, laccase, lignin peroxidase, and manganese peroxidase synthesis during fermentation was examined. To start fermentation, 10g of each substrate with particles between 1 mm and 3 mm in size were added to a mineral salt medium. To assess the influence of each parameter, including inoculum quantity (1.0 - 4.0 ml, with 10^{-7} CFU/g of substrate in 1.0 ml), particle density (1.0 - 4.0 mm), and moisture content (50 - 90%), the mineral salt medium was employed as a moistening agent. Before sterilization, the overall pH of the medium was maintained at 7. These components were completely mixed and kept at 28

± 2°C for fifteen days, with daily samples collected and evaluated. Each experiment was conducted in triplicate.

2.7 Optimization of fermentation attribute values via response surface methodology (RSM)

Response surface methodology applying Box and Behnken factorial design (Box and Behnken, 1960) with the quadratic polynomial was developed to assess the combined effect of four independent variables utilizing design - expert version 13 (Stat-Ease, USA) analysis software. The study made use of four independent variables (factors): A (Moisture content), B (Biomass size), C (Inoculum volume), and D (Incubation time). Using these dependent variables, the production of cellulase, xylanase, laccase, lignin peroxidase, and manganese peroxidase, as well as the best combination, were studied. If enzymatic hydrolysis is optimized, the effects of all the parameters and their interactions must be considered since they can influence enzymatic digestion (Pereira et al., 2021). Each independent variable was analyzed at three coded levels, -1, 0, and +1, corresponding to low, medium, and high values, respectively (Table 2.7). The contents of each flask were incubated at 28 ± 2°C for the length of the experiment, and fifteen days later, they were examined.

$$Y = \beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_{1,1}A^2+\beta_{2,2}B^2+\beta_{3,3}C^2+\beta_{4,4}D^2+\beta_{1,2}AB+\beta_{1,3}AC+\beta_{1,2}AB+\beta_{1,3}AC+\beta_1AD+\beta_{2,3}BC+\beta_{2,4}BD+\beta_{3,4}AD$$

Where,

Y represents cellulase, xylanase, laccase, lignin peroxidase, manganese peroxidase activity (response),

β_0 is the constant term;

β_1, β_2, β_3 and β_4 are the coefficient of linear term $\beta_{1,1}$, $\beta_{2,2}$, $\beta_{3,3}$ and $\beta_{4,4}$ is the coefficient of quadratic terms

Independent variables	**Symbol coded**	**Range and levels**		
		-1	0	+1
Moisture content (%)	A	75	80	85
Biomass size (mm)	B	1	2	3
Inoculum volume (ml)	C	3.5	4	4.5
Incubation time (days)	D	14	15	16

Table 2. 7 Exploratory range, level, and code for variables that are independent

3.Results and discussions

3.1 Lignocellulolytic fungi isolation

This research evaluates the potential for lignin degradation by a fungal strain using lignin. From soil samples, twenty-four fungi with ligninolytic potential were isolated. The isolates were given the designations IF1, IF2, IF3, IF4, IF5, ... IF24 (IF stands for Isolated Fungi). Through repeated subculturing, a pure culture of the isolates was maintained. *Myrothecium verrucaria*, a large fungal genus, was isolated by Su (2018) from ligninase-active soil, decaying leaves, and decayed wood. In addition, Barapatre and Jha (2017) found that *Aspergillus flavus* as well as *Emericella nidulans* may decrease lignin by 14.4% to 21.0% in different media.

Table 3. 1: Qualitative screening of the fungal isolates on CMC, Xylan, Azure B, Tannic acid, Guaiacol containing medium. (+) indicates release of the enzyme to degrade the respective substrate, (-) indicates the absence of enzymes.

S.No	Isolates	CMC	Xylan	Azure B	Tannic acid	Guaiacol
1	IF1	+	+	+	+	+
2	IF2	+	+	+	+	+
3	IF3	-	-	-	-	+
4	IF4	+	-	+	-	-
5	IF5	+	+	+	+	+
6	IF6	-	+	-	-	-
7	IF7	-	-	+	-	-
8	IF8	+	+	+	+	+
9	IF9	-	+	-	-	-
10	IF10	-	-	+	-	+
11	IF11	+	+	+	+	+
12	IF12	-	-	-	-	+
13	IF13	+	+	+	+	+
14	IF14	+	+	+	+	+
15	IF15	-	-	+	-	-
16	IF16	+	-	-	+	-
17	IF17	-	-	+	-	-
18	IF18	-	-	+	+	-
19	IF19	-	-	-	-	+
20	IF20	+	+	+	+	+
21	IF21	+	-	-	-	+
22	IF22	+	+	+	+	+
23	IF23	+	+	-	-	-

24	IF24	+	+	+	+	+

3.2 Evaluation of the isolated fungi's production of ligninolytic enzymes

The ability of an isolated fungus to produce cellulase, xylanase, laccase, manganese peroxidase, and lignin peroxidase was assessed. The fungus was chosen based on the clear zones surrounding the colonies. According to zone diameter, the ten fungi with the largest zones were chosen for further study. All the ten fungal isolates produced zone around the colonies in CMC, xylan, azure B, guaiacol and tannic acid agar plates. By generating several ligninolytic enzymes, lignocellulolytic fungi and bacteria are capable of decomposing lignocellulosic material on their own (Sanchez, 2009). Previous studies demonstrated that, in ligninolytic enzyme screening, fungi capable of generating laccase and/or peroxidase did not generate cellulase and/or xylanase, or vice versa (Singh and Chen, 2008). This study found the fungal strains that generate combinations of these enzymes.

3.3 Quantitative screening of fungal strains under submerged fermentation

The quantitative screening was performed to evaluate the capability of isolated colonies to produce enzyme complexes in a fermentation medium. As demonstrated in Table 1.1, the 10 fungal strains isolated from soil samples exhibited substantial enzyme activity. The fungal strain IF 5 with the highest enzyme activities for cellulase (15.58 ± 4.5 U/ml), xylanase (22.97 ± 0.03), laccase (4.86 ± 0.03), lignin peroxidase (3.97 ± 0.42) and manganese peroxidase (3.45 ± 0.05) was chosen for further study. Environment-isolated microorganisms exert a significant effect on the phenotypes they exhibit and also permit genotype-by-environment interactions to incorporate ecological variety (Dewitt et al., 1998). The genes for ligninolytic enzymes are present in the isolated fungal strains, and the presence of a particular nutrient influenced the enzyme's ability to be expressed (Amadi et al., 2016).

3.4 Molecular characterization of a potential fungal strain

The probable fungal strain IF 5 was selected for molecular identification. By amplifying the internal transcribed spacer (ITS 2) in its 18s rRNA sequence, the fungal strain was identified. The sequence that was used for BLAST searches in GenBank. The BLAST and Phylogenetic tree (Neighbor-joining joining method) of the fungal strain revealed a 18s rRNA sequence with a maximum 100% similarity to *Fusarium oxysporum*. A GenBank submission was made for the sequence (Accession Number: 0N724156). A phylogenetic tree shows how the strains are connected (Figure 3.4).

Name of the isolates	Cellulase U/ml	Xylanase U/ml	Laccase U/ml	LiP U/ml	MnP U/ml
IF 1	6.11±0.20^{d}	9.05± 0.02^{j}	1.92±0.03^{b}	0.28±0.01^{g}	0.14±0.12^{i}
IF 2	5.74±0.03^{e}	9.93±0.02^{d}	1.86±0.07^{c}	0.32±0.02^{f}	0.12±0.02^{j}
IF 5	19.58 ±4.5^{a}	22.97±0.03^{a}	4.86±0.03^{a}	3.97±0.42^{a}	3.45±0.05^{a}
IF 8	10.23±3.6^{c}	6.12±0.22^{i}	1.12±0.02^{i}	0.35±0.03^{d}	0.27±0.04^{h}
IF 11	8.58±0.01^{f}	7.97±0.03^{c}	0.86±0.01^{j}	0.97±0.02^{c}	0.45±0.04^{b}
IF 13	9.57±0.02^{g}	5.02±0.02^{b}	1.54±0.04^{f}	2.68±0.18^{b}	0.25±0.05^{c}
IF 14	7.12±0.47^{j}	9.48±0.03^{e}	1.64±0.04^{e}	0.35±0.04^{d}	1.04±0.01^{g}
IF 20	8.25±0.16^{b}	7.24±1.02^{h}	1.34±0.02^{h}	1.18±0.05^{f}	0.12±0.02^{f}
IF 22	10.14±0.49^{i}	11.25±0.32^{g}	1.47±0.03^{g}	0.21±0.54^{e}	1.13±0.04^{e}
IF 24	6.32±0.03^{h}	5.39±0.07^{f}	1.69±0.06^{d}	1.08±0.02^{h}	0.19±0.21^{d}

Table 3.3 Enzyme activities of the selected isolates

Figure 3.3 Enzyme activities of the selected isolates

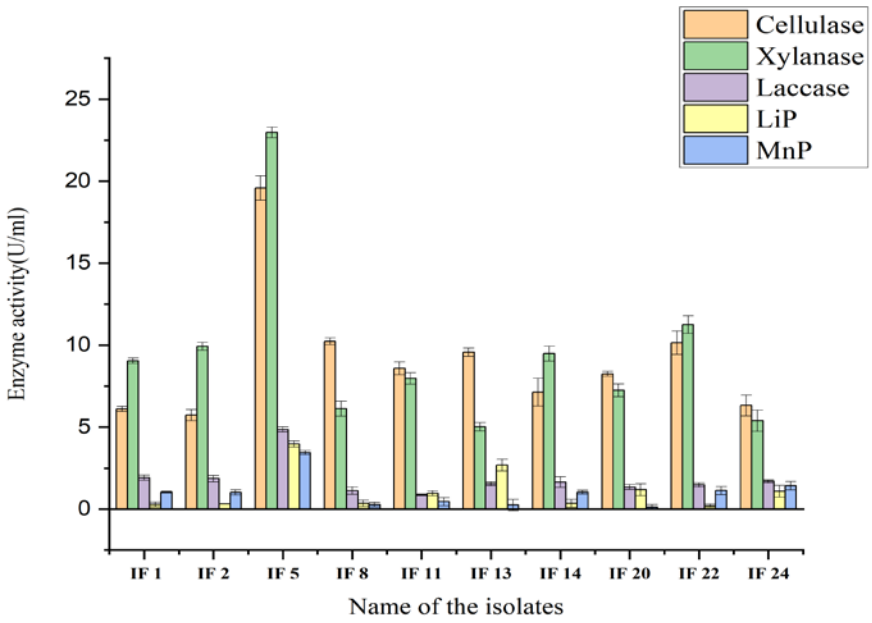
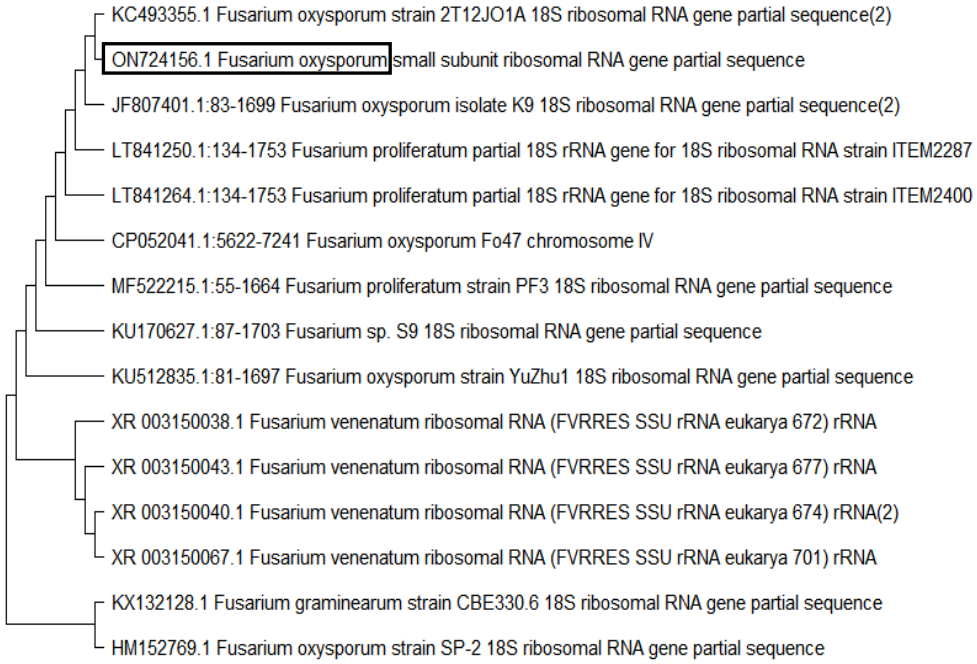


Figure 3.4 The Neighbor-Joining Tree Method illustrates the evolutionary position of IF5



3.3 Assessing substrate for ligninolytic enzyme synthesis by solid-state fermentation

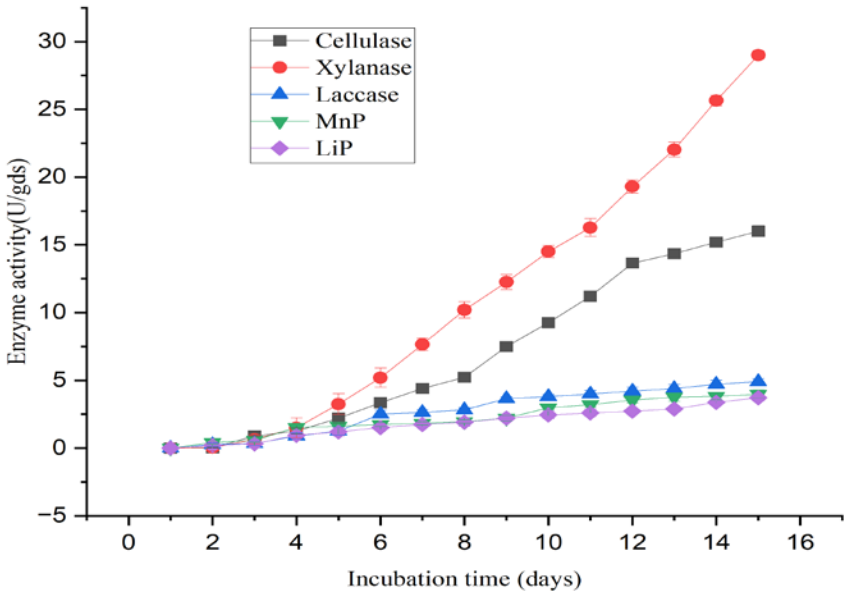
Diverse lignocellulosic substrates were tested to determine their appropriateness as substrates for enzyme complex synthesis. Corn stover was the most effective substrate for lignocellulolytic enzyme production, with enzyme activities of cellulase 16 ± 0.76 U/gds, xylanase 29 ± 1.25 U/gds, laccase 4.91 ±1.02 U/gds, Lignin peroxidase 3.98 ± 0.87 U/gds, and Manganese peroxidase 3.21 ± 0.98 U/gds. The next most effective substrate was corn cob (Cellulase 13 ± 1.76 U/gds, xylanase 15 ± 1.82 U/gds, laccase 3.85 ± 0.75 U/ gds, Lignin peroxidase 3.72 ± 0.68 U/gds, and Manganese peroxidase 3.10 ± 0.54 U/gds). Without any pretreatment, lignocellulosic substrates were employed for solid-state fermentation to create ligninolytic enzymes.

Depending on the structure of the lignocellulose in each substrate, the fungus may or may not be able to create ligninolytic enzymes on that substrate. Despite this, this fungus is capable of utilizing all substrates and exhibits complex enzyme production. Patents on the manufacturing and application of cellulase are still being developed, albeit less often since the emphasis has switched to enhancing the microbiological capabilities of production

processes. SK Chemicals stated that the new strain of *Pholiata adiposa* SKU 714 has a cellulase activity of 15 U/ml in this context. The *F. oxysporum* cellulase activity was higher than reported in this work; however, this is due to the substrate, which was rice straw (Siqueira et al., 2020). During fermentation, the xylanase activity of *F. oxysporum* was greater than other enzyme activities in the present study. According to some evidence, this fungus produces xylanase in large amounts (Gomez- Gomez et al., 2001). The peroxidase enzyme activity in *Myrothecium verrucaria*, which was used for the bioconversion of corn stover, was higher than that noted by Su et al. (2018).

Compared to bacteria and yeast, filamentous fungi produce an abundance of ligninolytic extracellular enzymes (Haltrich et al., 1996). Panagiotou et al. (2003) reported that *F.oxysporum* produces cellulase and xylanase enzymes from corn stover. There are reports that *F. oxysporum* is capable of simultaneously hydrolyzing and fermenting lignocellulosic material. This fungus can use both hexoses and pentoses to produce enzymes that degrade biomass. The presence of inhibitors in lignocellulosic materials determines the fermentation efficiency of *F. oxysporum (*Panagiotou et al., 2011).

Figure 3.3 Asssssss study of the time course of enzyme production by *Fusarium oxysporum* grown on corn stover at 28 ℃in SSF.



3.6 Response Surface Methodology for Lignocellulase Growth Condition Synthesis

Response surface methodology employing BBD has been used to assess the interaction between five response functions (cellulase, xylanase, laccase, lignin peroxidase

(LiP), and manganese peroxidase (MnP) and four selectable factors (moisture, initial pH, biomass size, and inoculum volume) designated A, B, C, and D. The findings of a BBD experiment were examined using the F-test, analysis of variance (ANOVA), and an independent variable design matrix. The table displays the experimental response data, and the quadratic model represented cellulase by equation (A.1), xylanase by equation (A.2), laccase, lignin peroxidase, and manganese peroxidase by equations (A.3), (A.4), and (A.5), respectively.

$$P = 24.2 - 0.041A + 0.70B + 0.20C + 0.5D - 9.16A^2 - 10.03B^2 - 9.66C^2 - 9.28D^2 - 1AC - 0.62AD + 0.37BC + 0.5BD + 0.75CD \quad \text{…… Eq A.1}$$

$$P = 36.15 - 0.37A - 0.20B + 0.16C + 0.083D - 15.22A^2 - 15.72B^2 - 15.90C^2 - 15.78D^2 + 1AB - 0.62AC - 0.5AD + 0.37BC + 0.5BD - 0.5CD \quad \text{…….. Eq A.2}$$

$$P = 16.25 + 0.20A - 0.41B - 0.41C - 0.41D + 0.062AB - 0.5AC + 0.18AD - 0.06BC - 0.12BD + 0.18CD - 7.31A^2 - 7.25B^2 - 7.37C^2 - 6.81D^2 \quad \text{…… Eq A.3}$$

$$P = 10.25 + 0.015A - 0.12B + 0.22C + 0.065D - 4.64A^2 - 4.71B^2 - 4.67C^2 - 4.66D^2\ 0.23AB - 0.12AC + 0.06\ AD - 0.12BC + 0.015BD + 0.024CD \quad \text{……. Eq A.4}$$

$$P = 6.12 - 0.04A + 0.068B + 0.43C - 1.34A^2 - 1.49B^2 - 1.47C^2 - 1.37D^2 - 0.046AB + 0.045AC - 0.068AD + 0.064BC - 0.044BD - 0.029CD \quad \text{……. EqA.5}$$

The model projected that the ideal enzyme concentrations would be greater than those reported by earlier research. The findings obtained from BBD were 24 U/g for cellulase, 36.23 U/gds for xylanase, 10.16 U/gds for laccase, 6.78 U/gds for lignin peroxidase, and 6.15 U/gds for manganese peroxidase. This illustrates the importance of improving processing settings. Three-dimensional response surface plots illustrating the individual and interacting impacts of the specified variables were generated using the established models (Figure 3. 6a, 3.6b, 3.6c, 3.6d and 3.6 e). The adequacy and fitness models were built using ANOVA, and it was discovered that the model was very significant at the P = 0.0001 level. Fisher's value (F-Value) was calculated to be (31.59 for cellulase, 151.29 for xylanase, 123.33 for laccase, 128.15 for lignin peroxidase, and 169.52 for manganese peroxidase) with a low probability value (P = 0.000), indicating a high degree of efficiency and polynomial models.

The R^2 values derived from the regression equation were 0.9736 for cellulase, 0.9944 for xylanase, 0.9931 for laccase, 0.9934 for lignin peroxidase, and 0.9950 for manganese peroxidase. These results suggested that the quadratic model was very significant and could explain 97% of the variance in enzyme production. Multiple authors have stated that a variance analysis with a high coefficient of determination R^2 and a significance threshold of P 0.05 is

statistically significant (Comen and Bahrim, 2011; Khusro et al., 2016; Perince and Duran, 2016).

Table 3.6. a Analysis of Variance test for (ANOVA) for Box - Behnken design Cellulase

Source	Sum of Squares	DF	Mean Square	*F* Value	*P* Value	
Model	**991.99**	**14**	**70.86**	**31.59**	**<0.0001**	**Significant**
A - Moisture content	**0.0208**	**1**	**0.0208**	**0.0093**	**0.9248**	
B -Time	**6.02**	**1**	**6.02**	**2.68**	**0.1273**	
C- Biomass size	**0.5208**	**1**	**0.5208**	**0.2322**	**0.6386**	
D -Inoculum volume	**1.02**	**1**	**1.02**	**0.4551**	**0.5127**	
A*B	**1**	**1**	**1**	**0.4458**	**0.517**	
A*C	**4**	**1**	**4**	**1.78**	**0.2065**	
A*D	**1.56**	**1**	**1.56**	**0.6966**	**0.4202**	
B*C	**0.5625**	**1**	**0.5625**	**0.2508**	**0.6256**	
B*D	**1**	**1**	**1**	**0.4458**	**0.517**	
C*D	**2.25**	**1**	**2.25**	**1**	**0.3363**	
A^2	**447.74**	**1**	**447.74**	**1**	**<0.0001**	
B^2	**537.34**	**1**	**537.34**	**199.61**	**<0.0001**	
C^2	**497.94**	**1**	**497.94**	**239.56**	**<0.0001**	
D^2	**460.04**	**1**	**460.04**	**221.99**	**<0.0001**	
Residual	**26.92**	**12**	**2.24**	**205.1**		
Lack of fit	**26.92**	**10**	**2.69**			
Pure error	**0**	**2**	**0**			
Total	**1018.91**	**26**				

Model Summary

Std.Dev	Mean	C. V %	R^2	Adjusted R^2	Predicted R^2	Adequate Precision
1.5	**7.24**	**20.67**	**0.9736**	**0.9428**	**0.8478**	**18.6553**

Table 3.6 b Analysis of Variance test for (ANOVA) for Box - Behnken design Xylanase

Source	Sum of Squares		Mean Square	*F* Value	*P* Value	
Model	**2629.23**	**14**	**187.8**	**151.29**	**<0.0001**	**Significant**
A - Moisture content	**1.69**	**1**	**1.69**	**1.36**	**0.2663**	
B -Time	**0.5208**	**1**	**0.5208**	**0.4196**	**0.5293**	
D- Biomass size	**0.3333**	**1**	**0.3333**	**0.2685**	**0.6137**	
D -Inoculum volume	**0.0833**	**1**	**0.0833**	**0.0671**	**0.7999**	
A*B	**4**	**1**	**4**	**3.22**	**0.0978**	
A*C	**1.56**	**1**	**1.56**	**1.26**	**0.2838**	
A*D	**1**	**1**	**1**	**0.8056**	**0.3871**	
B*C	**0.5625**	**1**	**0.5625**	**0.4531**	**0.5136**	
B*D	**1..00**	**1**	**1..00**	**0.8056**	**0.3871**	
C*D	**1**	**1**	**1**	**0.8056**	**0.3871**	
A^2	**1235.59**	**1**	**1235.59**	**995.39**	**<0.0001**	
B^2	**1318.1**	**1**	**1318.1**	**1061.86**	**<0.0001**	
C^2	**1349.73**	**1**	**1349.73**	**1087.34**	**<0.0001**	
D^2	**1328.61**	**1**	**1328.61**	**1070.32**	**<0.0001**	
Residual	**14.9**	**12**	**1.24**			
Lack of fit	**14.9**	**10**	**1.49**			
Pure error	**0.0000**	**2**	**0.0000**			
Total	**2644.12**	**26**				

Model Summary

Std.Dev	Mean	C. V %	R2	Adjusted R2	Predicted R2	Adeq Precision
1.11	**8.31**	**13.4**	**0.9944**	**0.9878**	**0.9676**	**39.1662**

Table 3.6 c Analysis of Variance test for (ANOVA) for Box - Behnken design Laccase

Source	Sum of Squares	DF	Mean Square	*F* Value	*P* Value	
Model	**554.56**	**14**	**39.61**	**123.33**	**<0.0001**	**Significant**
A - Moisture content	**0.5208**	**1**	**0.5208**	**1.62**	**0.227**	
B -Time	**0.0208**	**1**	**0.0208**	**0.0649**	**0.8033**	
C- Biomass size	**0.0208**	**1**	**0.0208**	**0.0649**	**0.8033**	
D -Inoculum volume	**0.0208**	**1**	**0.0208**	**0.0649**	**0.8033**	
A*B	**0.0156**	**1**	**0.0156**	**0.0486**	**0.8291**	
A*C	**1**	**1**	**1**	**3.11**	**0.1031**	
A*D	**0.1406**	**1**	**0.1406**	**0.4378**	**0.5207**	
B*C	**0.0156**	**1**	**0.0156**	**0.0486**	**0.8291**	
B*D	**0.0625**	**1**	**0.0625**	**0.1946**	**0.667**	
C*D	**0.1406**	**1**	**0.1406**	**0.4378**	**0.5207**	
A^2	**285.19**	**1**	**285.19**	**887.94**	**<0.0001**	
B^2	**280.33**	**1**	**280.33**	**872.82**	**<0.0001**	
C^2	**290.08**	**1**	**290.08**	**903.18**	**<0.0001**	
D^2	**247.52**	**1**	**247.52**	**770.66**	**<0.0001**	
Residual	**3.85**	**12**	**0.3212**			
Lack of fit	**3.85**	**10**	**0.3854**			
Pure error	**0**	**2**	**0**			
Total	**558.42**	**26**				

Model Summary

Std.Dev	Mean	C. V %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
0.5667	**3.47**	**16.32**	**0.9931**	**0.985**	**0.9602**	**36.3486**

Table 3.6.d Analysis of Variance test for (ANOVA) for Box - Behnken design Lignin peroxidase

Source	Sum of Squares	DF	Mean Square	*F* Value	*P* Value	
Model	**234.39**	**14**	**16.74**	**128.15**	**<0.0001**	**Significant**
A -Moisture content	**0.003**	**1**	**0.003**	**0.023**	**0.8819**	
B -Time	**0.1875**	**1**	**0.1875**	**1.44**	**0.254**	
C-Biomass size	**0.6165**	**1**	**0.6165**	**4.72**	**0.0506**	
D -Inoculum volume	**0.052**	**1**	**0.052**	**0.3981**	**0.5399**	
A*B	**0.2209**	**1**	**0.2209**	**1.69**	**0.2179**	
A*C	**0.0625**	**1**	**0.0625**	**0.4784**	**0.5023**	
A*D	**0.0156**	**1**	**0.0156**	**0.1196**	**0.7355**	
B*C	**0.576**	**1**	**0.576**	**0.4409**	**0.5193**	
B*D	**0.0009**	**1**	**0.0009**	**0.0069**	**0.9352**	
C*D	**0.0025**	**1**	**0.0025**	**0.0191**	**0.8923**	
A^2	**115.24**	**1**	**115.24**	**882.05**	**<0.0001**	
B^2	**118.8**	**1**	**118.8**	**909.3**	**<0.0001**	
C^2	**116.42**	**1**	**116.42**	**891.09**	**<0.0001**	
D^2	**115.86**	**1**	**115.86**	**886.8**	**<0.0001**	
Residual	**1.57**	**12**	**0.1306**			
Lack of fit	**1.57**	**10**	**0.1568**			
Pure error	**0**	**2**	**0**			
Total	**235.96**	**26**				

Model Summary

Std. Dev	Mean	C. V %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
0.3615	**1.94**	**18.65**	**0.9934**	**0.9856**	**0.9617**	**36.0495**

Table 3. 6.e Analysis of Variance test for (ANOVA) for Box - Behnken design Manganese peroxidase

Source	Sum of Squares	DF	Mean Square	*F* Value	*P* Value	
Model	221.21	14	15.8	169.52	<0.0001	Significant
A - Moisture content	0.0008	1	0.0008	0.0089	0.9262	
B -Time	0.0102	1	0.102	0.1095	0.7462	
C-Biomass size	0.2852	1	0.2852	3.06	1.58	
D -Inoculum volume	0.0033	1	0.0033	0.358	0.8532	
A*B	0.04	1	0.04	0.4291	0.5248	
A*C	0.0506	1	0.0506	0.5431	0.4753	
A*D	0.1406	1	0.1406	1.51	0.2429	
B*C	0.09	1	0.09	0.9655	0.3452	
B*D	0.0756	1	0.0756	0.8113	0.3854	
A^2	104.61	1	104.61	1122.25	<0.0001	
B^2	112.12	1	112.12	2202.84	<0.0001	
C^2	111.51	1	111.51	1196.29	<0.0001	
D^2	112.42	1	112.42	1206.12	<0.0001	
Residual	1.12	12	0.0932			
Lack of fit	1.12	10	0.1119			
Pure error	0	2	0			
Total	222.33	26				

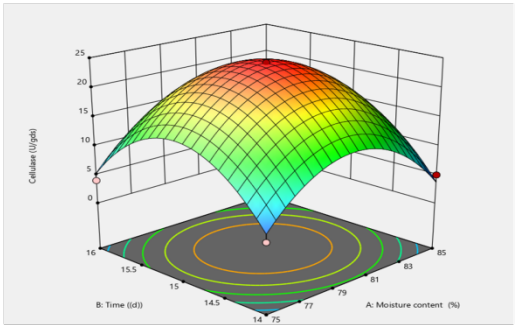
Model Summary

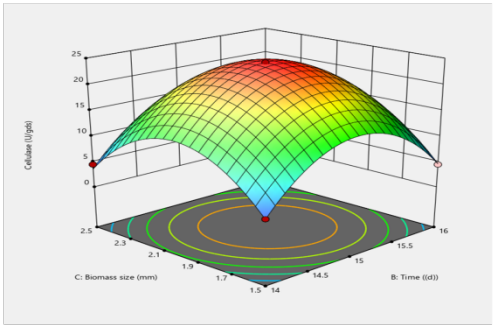
Std.Dev	Mean	C. V %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
0.3053	2.04	14.96	0.995	0.9891	0.971	41.6792

Figure C.1 Response surface plots of effects of different variables on enzyme production a)

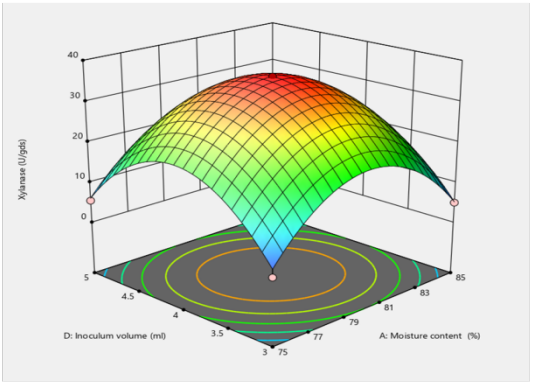
Cellulase b) Xylanase c) Laccase d) Lignin peroxidase e) Manganese peroxidase by *F. oxysporum*

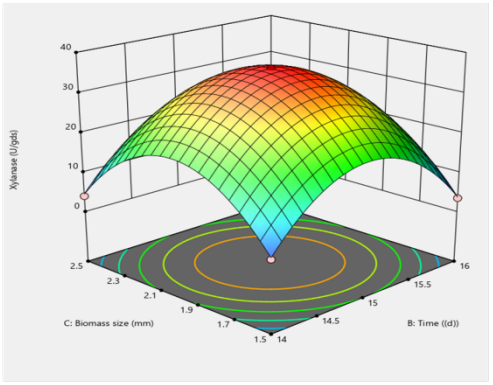
a





b





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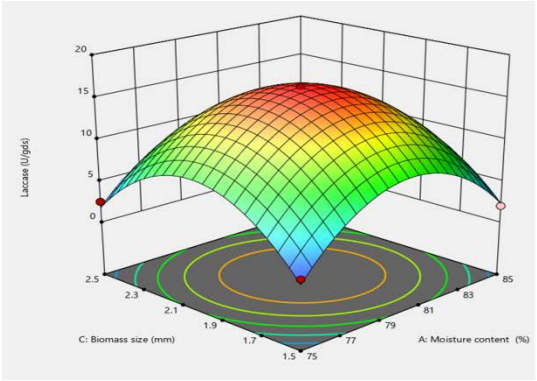
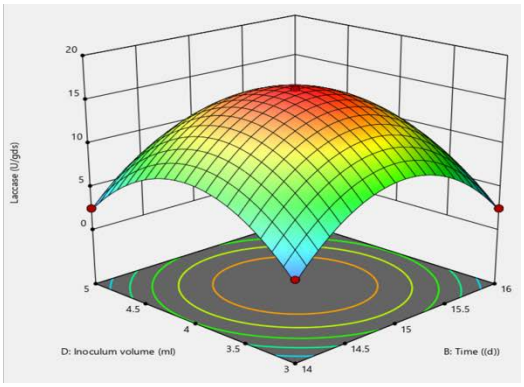
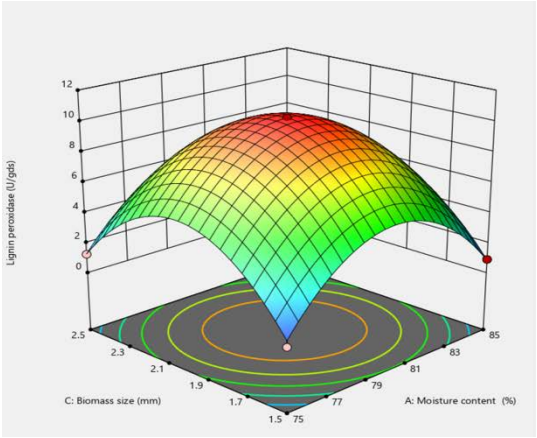
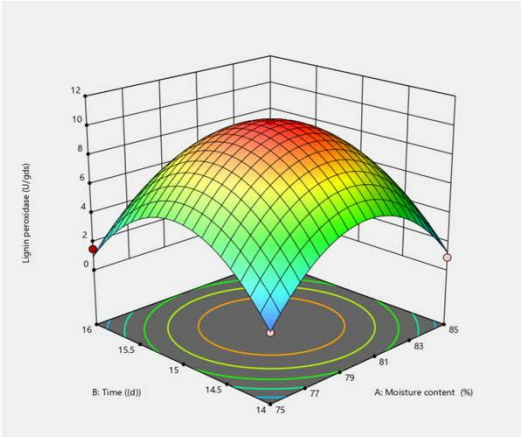


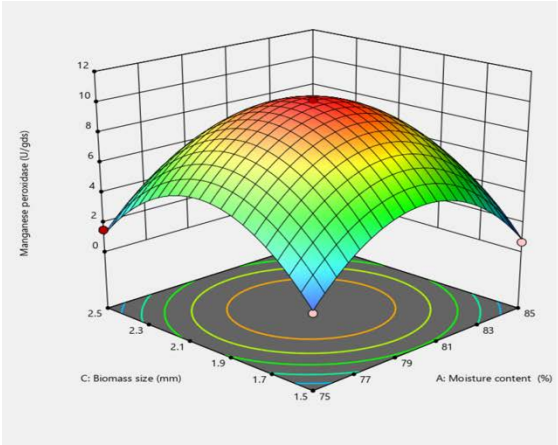


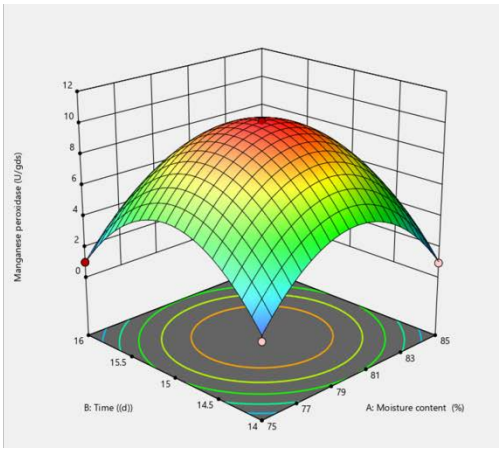
d





e





4. Conclusions

In several industrial applications, it is essential to investigate novel microbial strains for the manufacture of lignocellulolytic enzyme complexes. The isolated fungus, *Fusarium*

oxysporum (IF5), was able to simultaneously synthesize the enzyme complex from corn stover, a low-cost renewable biomass, without any prior treatment. The fermentation conditions were successfully optimised using Box Behnken's Response Surface Methodology (RSM) to generate the highest levels of cellulase, xylanase, laccase, lignin peroxidase, and manganese peroxidase activity. *Fusarium oxysporum* (IF5) is a promising strain for the biodegradation of lignocellulosic substrates and a variety of industrial purposes, according to the findings of this study.

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