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Statistical screening of process variables for improving bioethanol production using alkali-pretreated of some environmental wastes

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ABSTRACT

Bioethanol is considered the most proposed next automotive fuel generation. For the bioethanol production, the microbial conversion from some local environmental wastes (rice straw and manure) and the indigenous strains, namely *Streptomyces aegyptia* and *Candida tropicalis* were used in this study. Improving the conditions that may affect the process such as alkali pretreatment, enzymatic hydrolysis, and fermentation were investigated using the Plackett-Burman design (PBD) via submerged fermentation. X-ray diffraction (XRD) analysis revealed that the crystallinity index of pretreated rice straw (52.2%) was significantly (p-value) lower as compared to the untreated one (63.1%). The results showed that the optimization of the microbial conversion reached 6.6 folds in comparison to the unoptimized process. An improvement in enzymatic activities of *Streptomyces aegyptia* was achieved in the design that the maximal lignin peroxidase, Mn-dependent peroxidase, total cellulase, endocellulase, and xylanase activities were 120 U/min.l, 1020 U/min.l, 3.7 U/min.l, 57.3 U/min.l and 78.1 U/min.l, respectively. Maximum ethanol concentration and bioconversion process efficiency were 0.8 g/l and 13.3%, respectively.

Key Words:

Alkali pretreatment, bioethanol, microbial conversion, manure, rice straw, Plackett-Burman design.

1. INTRODUCTION

The most available and inexpensive feedstock for bioethanol production is the agricultural residues, which are the lignocellulosic materials generated as wastes from crops and animals **[1]**. One of the most abundant crop residues in the world is the rice straw. Its annual production is about 731 million tons which could potentially produce 205 billion liters of bioethanol per year **[2].** The second available source of agricultural residues is manure. Animals produced about 10 million tons of organic matter through their manure daily **[3]**. Manure degradation could effectively bridge manure management and energy production

[4]. A large number of studies performed on the microbial conversion of rice straw and manure wastes into fermentable sugars for bioethanol production **[5–9].**

The biological conversion of a mixture of rice straw and manure into ethanol requires three main steps: (1) pretreatment to liberate cellulose and hemicellulose from their complex with lignin, (2) hydrolysis to depolymerize the carbohydrate polymers (cellulose and hemicellulose) and produce free sugars, (3) fermentation of mixed hexose and pentose sugars to produce ethanol **[10, 11].** Calcium hydroxide is one of the chemical pretreatments that has been recommended as a potential alkali for straw pretreatment since it is cheap and readily available **[12].** It causes swelling, leading to decreased crystallinity, and the separation of structural linkages between lignin and carbohydrates while retaining cellulose and a significant portion of hemicellulose in the recovered solids **[13].** Previous studies showed that mild calcium hydroxide treatment followed by microbial hydrolysis led to higher yields for sugar **[14, 15].** Microbial hydrolysis of lignocelluloses is a substantially very complex process, due to the high complexity of the raw materials. Actinomycetes and particularly *Streptomyces* species are capable of playing a significant role in the decomposition of lignocellulosic plant materials in nature **[16].** The enzymatic system of *Streptomyces* species includes two types of enzymes: hydrolytic, responsible for polysaccharide degradation; and oxidative, which degrade lignin **[17].** The ability to ferment pentoses along with hexoses is not widespread among microorganisms. The most promising yeasts that have the ability to use both C_5 and C_6 sugars are *Candida tropicalis* and *Pichia stipites* **[18]**.

Variations in the concentration of bioethanol were observed with respect to several cultural factors such as substrate concentration, mineral salts, surfactant (tween 80), pH, and inoculum size of isolates **[19].** Screening of the factors that affect the bioconversion of bioethanol production can be applied using a statistical model, Plackett–Burman design, which is an empirical modeling matrix performed for evaluating the relationship between a set of factors and the observed results in several runs. It can determine the significant factors in the process for further optimization techniques **[20].**

In this study, the evaluation of using calcium hydroxide pretreatment coupled with the co-culture of *Streptomyces aegyptia* and *Candida tropicalis* for bioethanol production from rice straw and manure was applied. Factors that could improve the steps of the bioconversion process (pretreatment, hydrolysis, and fermentation) were studied through the Plackett-Burman design.

2. MATERIAL AND METHODS

2.1. Microorganisms and environmental wastes

Strains of Actinomycetes and yeast used in this study were previously isolated and identified by the molecular method as *Streptomyces aegyptia* (Genebank accession number MT534590) and *Candida tropicalis* (Genebank accession number MT874505), respectively **[21]**. *Streptomyces aegyptia* was maintained on starch casein agar medium that containing (g L^{-1}): soluble starch, 10.0; casein (vitamin-free), 0.3; KNO_3, 2.0; NaCl, 2.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02; $FeSO_4.7H_2O$, 0.01 **[22]**. *Candida tropicalis* was maintained on yeast-peptone glucose (YPG) agar medium which containing (g L^{-1}): yeast extract, 10; peptone, 20; glucose, 20 **[23].**

Rice straw and manure used in this study were supplied from the farm of the Faculty of Veterinary Medicine of Suez Canal University (Ismailia, Egypt). Rice straw was stored in plastic pages and manure was immediately autoclaved after collection at 121º C for 30 min, dried in an oven at 70º C to constant weight then stored in a refrigerator until use. The nitrogen and organic carbon contents for the rice straw were 1.12% and 32.2% and for the manure were 2.1% and 30%, respectively. Thus, the C:N ratios for the rice straw and manure were 28.7:1 and 14.3:1, respectively.

2.2. Pretreatment of environmental wastes

Pretreatment of a mixture wastes used was conducted in a 250-ml capacity bottle containing 2.0 g of the rice straw and manure mixture (1:1 W/W). Pretreatment with $Ca(OH)_2$ was done at 100° C, with a water loading of 6 ml H_2O/g air-dried wastes and an alkaline loading of 2% wt of oven-dried wastes.

2.3. Submerged hydrolysis and fermentation

Pretreated wastes (2g) were supplied with the mineral salt medium (50 ml) containing (g/l): Yeast extract, 1.0; KH_2PO_4, 0.3; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $ZnSO_4$, 0.001 **[24]**, and autoclaved at 121º C for 20 minutes. Afterward, $1x10^7$ equivalent CFU/ml of *Streptomyces aegyptia* spores were added and grown at 30º C in an orbital incubation shaker (120 rpm) for 72 hours. Subsequently, the fermentation process was conducted in the same flasks by adding 5 ml of *Candida tropicalis* culture (OD_{660} = 0.5, that equal to 1×10^8 CFU ml^{-1}). After 72 hours of static incubation, ethanol was determined.

2.4. Parameters screening based on the Plackett-Burman design

Screening of the significant parameters affecting bioethanol production was performed by the Plackett-Burman design **[25].** It is a two-level experimental matrix that allows the investigation of 'n-1' variables with at least 'n' experiments. Each generated response was calculated according to the first-order linear equation:

$$Y = B_0 + \Sigma B_i X_i$$

Where Y is the response, B_0 is the model intercept, B_i is the linear coefficient, and X_i is the level of the independent variable. The main effect was calculated as the difference between the average of measurements made at the high level (+) and the average of measurements observed at the low level (−) of each factor [20], as shown in the following Equation:

$$E(X_i) = 2\ (\sum P_i^{+} + P_i^{-}) / N$$

$P_i^{+} + P_i^{-}$ are the response measured from the trials where the variable (X_i) measured was present at high and low concentrations, respectively; and N is the number of trials. This design requires that the frequency of each level of a variable should be equal and that in each test, the number of high and low variables should be equal **[26]**.

A total number of 19 independent variables were selected for this process, with each being represented at two levels, high (+1) and low (-1). Center points were also performed at the medium level (0) as shown in Table 1. Matrix design was performed using Minitab (version 19.1) as shown in Table 2. The 19 independent variables were chosen based on the literature survey that may affect and improve pretreatment, hydrolysis, and fermentation processes as follows:

- Three parameters were chosen that may improve the pretreatment step namely $Ca(OH)_2$ loading, time, and temperature of pretreatment. The ranges considered for pretreatment conditions were determined based on the results of previous studies **[14, 27–29].**
- Eleven parameters were chosen that may improve the hydrolysis step namely size of rice straw, inoculum size of *Streptomyces*, pH of hydrolysis medium, and medium components of rice straw concentration, manure concentration, KH_2PO_4, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$ and Tween 80. Hydrolysis conditions were determined based on the results of previous studies **[27, 30–38].**
- Five parameters were chosen that may improve the fermentation step namely inoculum size of *Candida*, pH of fermentation medium and medium components of manure concentration, rice straw concentration, and KH_2PO_4. Fermentation conditions were determined based on the results of previous studies **[19, 26, 39].**

The results were analyzed using Minitab (version 19.1) software to obtain the analysis of variance (ANOVA), regression coefficients, and polynomial regression equation from the data of the experiment. The behavior of the model in the experimental area was graphically investigated. The significant terms in the model equation were determined in terms of P-value and the accuracy of the model was evaluated from its regression analysis r^2 **[40].**

Table 1. Experimental variables at different levels used in the Plackett-Burman design for improving bioethanol production

Factor code	Examined Factors	Unit	Low level (-1)	Medium level (0)	High level (+1)
A	$Ca(OH)_2$ loading	% (wt/wt)	**2**	**4**	**6**
B	Time	h	**1**	**12**	**24**
C	Temperature	°C	**25**	**50**	**100**
D	Size of rice straw	cm	**0.2**	**0.6**	**1**
E	Rice straw	g/l	**4**	**10**	**16**
F	Manure	g/l	**4**	**10**	**16**
G	KH_2PO_4	g/l	**0.5**	**1.25**	**2**
H	$MgSO_4.7H_2O$	g/l	**0.3**	**0.65**	**1**
J	$ZnSO_4.7H_2O$	g/l	**0.0015**	**0.00575**	**0.01**
K	$FeSO_4.7H_2O$	g/l	**0.001**	**0.0055**	**0.01**
L	$MnCl_2.4H_2O$	g/l	**0.0016**	**0.0058**	**0.01**
M	Tween 80	ml/l	**0.2**	**1.1**	**2**
N	*Streptomyces* Inoculum	CFU/ml spores	**$1x10^6$**	**$5x10^6$**	**$1x10^7$**
O	pH	-	**5.7**	**6.8**	**7.9**
P	Manure	g/l	**4**	**10**	**16**
Q	Rice straw	g/l	**4**	**10**	**16**
R	KH_2PO_4	g/l	**0.5**	**1.25**	**2**
S	pH	-	**4**	**5.5**	**7**
T	Inoculum size of *Candida*	% (v/v)	**1**	**2.5**	**4**

Selection of low and high levels of the factors was based on the literature survey of related researches.

Table 2. Plackett–Burman screening design for 19 variables with coded values

Run	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q	R	S	T
1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1
2	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
4	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1
5	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1
6	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1
7	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1
8	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1
9	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1
10	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1
11	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1
12	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1
15	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1
16	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1
17	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1
18	-1	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1
19	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1
20	+1	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1

21	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1

A, B and **C** represent $Ca(OH)_2$ loading, time and temperature of pretreatment stage.
D, E, F, G, H, J, K, L, M, N and **O** represent size of rice straw, rice straw, manure, KH_2PO_4, $MgSO_4$, $ZnSO_4$, $FeSO_4$, $MnCl_2$, tween 80, *Streptomyces* inoculum and pH in hydrolysis stag.
P, Q, R, S and **T** represent manure, rice straw, KH_2PO_4, pH and *Candida* inoculum in fermentation stage.

2.5. Analytical methods

2.5.1. X-ray diffraction analysis

The dried rice straw samples were powdered and analyzed using X-ray diffraction analysis (XRD) to identify the crystallinities of the samples. This analysis was carried out using a Philips PW1370 X-ray generator fitted with a PW 1390 channel control, a PW1050 vertical goniometer, and a digitizer. X-rays were obtained by applying a potential of 40 KV and a current of 30 mA on a PW2273/20 copper anode tube. The crystallinity index (CrI) of the sample was calculated by the following equation **[41]:**

$$\textbf{Crystallinity index (CrI) \%} = \frac{(I_{002} - I_{am})}{I_{002}} \times 100$$

Where I_{002} is the intensity of crystalline area of (002) plane at $2\Theta = 22.4^{\circ}$, it means both crystalline and amorphous intensity (background).
I_{am} is the intensity of amorphous region at $2\Theta = 18.7^{\circ}$, it represents background intensity only.

2.5.2. Reducing sugar and ethanol determination

Reducing sugars ware determined by the 3,5-dinitro salicylic acid DNS method **[42]** and glucose was used as the standard. Ethanol was measured using tri-n-butyl phosphate (TBP) solvent extraction **[43],** and its content was estimated by the spectrophotometric method **[44].** The ethanol concentration in the sample was quantified based on the values of its abundance to a calibrated standard ethanol curve while the bioethanol yield and bioconversion efficiency were calculated based on the following formula **[45]**:

$$\textbf{Bioethanol yield (mg/g)} = \frac{\textbf{Bioethanol concentration obtained (mg)}}{\textbf{Initial sugar concentration in the biomass sample (g)}}$$

$$\textbf{Bioconversion efficiency (\%)} = \frac{\textbf{Bioethanol yield (g/g)} \times 100}{0.51}$$

2.5.3. Enzymatic assay

Endocellulase and xylanase activities were assayed according to the analytical procedure recommended by the International Union of Pure and Applied Chemistry (IUPAC) using 1 % (w/v) carboxymethylcellulose and 1% (w/v) birchwood xylan**,** respectively **[46].** The reaction mixture ،containing suitably diluted fermentation solution as a source of enzyme crude solution (0.25 ml) and 0.25 ml of substrate solution prepared in 50 mM citrate buffer (pH 5.0), was incubated at 30^{o} C for 30 minutes. The filter paper assay (FPA) published by the International Union of Pure and Applied Chemistry (IUPAC) is

widely used to determine total cellulase activity **[46].** The reaction mixture (850 µl) contained 500 µl of 50 mM citrate buffer (pH 5.0), 350 µl appropriately diluted samples, and a 30×6 mm stripe of Whatman No.1 filter paper. The reaction was incubated at 30º C for 60 min. In all the cases, after incubation, the released reducing sugar was estimated by the DNS method **[42].** The reducing sugar was estimated from the absorbance measured at 540 nm using glucose as a standard for endocellulase and total cellulase by the equation of Y = 0.25X - 0.26, r^2=0.99 and xylose as standard for xylanase by the equation of Y = 0.5X - 0.69, r^2=0.98. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugar/min.l **[47, 48].**

Lignin peroxidase assay was determined in a reaction mixture containing (final concentration) sodium tartrate buffer (125 mM, pH 3.0), Azure B (160 µM), and 1.0 ml of fermentation solution as a crude enzyme. The reaction is initiated by adding 0.5 ml of H_2O_2 (200 µM) **[49]**. One unit of enzyme activity is equivalent to an absorbance decrease of 0.1 units/l/minute **[50]**. Mn-dependent peroxidase assay was determined in a reaction mixture (1 ml) consisting of phenol red (0.0l %), lactate (25 mM), $MnSO_4$ (l00µM), egg albumin (0.l %), and H_2O_2 (100µM) in 1.0 ml of 20 mM Na-succinate buffer (pH 4.5). Reactions were carried out at 30° C for 5 min and terminated with the addition of 2 N NaOH (40µ1). Absorbance was read at 610 nm using UV spectrophotometer. Control was routinely included in which H_2O_2 and $MnSO_4$ were omitted **[51].** One unit of enzymatic activity is equivalent to an absorbance increase of 0.1 units/min/l **[52].**

2.5.4. Scanning electron microscopy analysis

The rice straw samples were coated with gold-palladium membranes and examined by scanning electron microscopy (Scanning Electron Microscope; Jeol JSM-6510 L.V, Mansoura University, Egypt) at 30 kV.

3. RESULTS

3.1. Ethanol production from some environmental wastes

A mixture of rice straw and manure pretreated with calcium hydroxide was used as a cultivation media for bioethanol production using a co-culture of local strains namely, *Streptomyces aegyptia*, and *Candida tropicalis*. An ethanol concentration of 0.28 g/l was obtained at the end of the fermentation process with a bioconversion efficiency of 2%.

3.2. Plackett–Burman screening design

Through twenty-one runs design matrix, a set of analyzes was carried out to study the effects of process variables on the model's responses and the results were as follows:

3.2.1. X-ray diffraction (XRD) of the pretreated substrates

The experiment started with substrate pretreatment with $Ca(OH)_2$ at different conditions. XRD analyses were applied to determine the degree of crystallinity in the substrate after pretreatment. The degree of crystallinity is represented by the crystal index, CrI. The crystallinity index of treated rice straw and manure with $Ca(OH)_2$ were all lower (52.2 - 61.6%) than the untreated sample crystallinity index (63.1%), depending on pretreatment conditions shown in Table 3. The highest chemical loading (6%) had a high effect on decreasing the crystallinity degree even at the low time and temperature of the pretreatment. However, the low chemical loading (2%) required both high temperature (100º C) and longer time treatment (24 h) to get an effective decreasing the crystallinity degree in comparison with the untreated

one. Thus, a chemical loading of $Ca(OH)_2$ (6%), a period of 1 h, and at room temperature (25° C) could be considered as the best suitable conditions for removal of the crystalline region of the substrate fibers.

Table 3. Crystallinity index (CrI) of substrate samples pretreatment with $Ca(OH)_2$ at different conditions

Samples	$Ca(OH)_2$ loading (%)	Time (h)	Temperature (°C)	CrI (%)
Untreated substrate	_	_	_	63.1
Substrate pretreated with $Ca(OH)_2$	2	1	25	59.5
	2	24	25	61.5
	2	1	100	61.6
	2	24	100	54.1
	6	1	25	52.9
	6	24	25	52.2
	6	1	100	52.7
	6	24	100	53.5

3.2.2. Enzymatic activities and reducing sugars concentrations

The results illustrated in Table 4 showed lignocellulosic enzymatic activities and sugar production in the hydrolysis step. The highest sugar concentration (0.6 g/l) and enzymatic activities of *Streptomyces aegyptia*, lignin peroxidase (0-120 U/min.l), Mn-dependent peroxidase (0-1020 U/min.l), total cellulase (0-3.7 U/min.l), endocellulase (0.3-57.3 U/min.l) and xylanase (0-78 U/min.l) were obtained in Plackett-Burman design used in this study. The minimum and maximum values of enzymatic activities indicated the effect of the design in improving the enzymatic activities of the organism.

Table 4. Enzymatic activities, sugars and ethanol concentrations, and bioconversion efficiency as responses in the Plackett-Burman design

Run No.	Response							
	1	2	3	4	5	6	7	8
	Lignin peroxidase (U/min.l)	**Mn-dependent peroxidase** (U/min.l)	**FPU ase** (U/min.l)	**CMCase** (U/min.l)	**Xylanase** (U/min.l)	**Sugar conc.** (g/l)	**Ethanol conc.** (g/l)	**Bioconversion efficiency** (%)
1	85	50	0.0	3.4	0.0	0.07	0.31	3.6
2	0	195	1.6	4.9	7.6	0.37	0.32	3.2
3	15	70	0.4	0.7	0.0	0.10	0.16	5.6
4	25	690	0.0	8.4	8.5	0.15	0.22	3.3
5	0	380	1.5	13.1	6.3	0.34	0.28	3.8
6	60	397.5	0.0	4.0	6.4	0.11	0.25	8.9
7	70	435	0.4	5.4	0.0	0.11	0.41	7.3
8	0	1020	3.1	13.1	1.9	0.52	0.32	2.9
9	0	0	0.0	2.0	0.4	0.30	0.45	7.7
10	20	212.5	2.6	5.8	1.3	0.23	0.31	4.5

11	0	250	3.7	25.2	31.5	0.59	0.46	5.6
12	70	0	1.8	15.7	78.1	0.25	0.65	7.8
13	0	785	1.7	7.4	5.8	0.31	0.34	4.9
14	0	500	1.9	3.9	6.1	0.31	0.46	5.4
15	40	0	0.3	0.3	3.2	0.13	0.54	13.3
16	60	410	1.4	11.5	6.3	0.19	0.82	11.7
17	0	285	1.1	7.1	15.1	0.34	0.67	9.9
18	0	60	1.4	2.0	7.3	0.16	0.47	5.9
19	0	202.5	1.6	57.3	36.3	0.50	0.64	6.7
20	120	0	0.6	9.2	9.2	0.25	0.35	8.1
21	0	500	2.2	54.1	32.4	0.60	0.76	11.5

Obtained results in Table 5 showed the statistical analyses of the factors in response to lignocellulosic enzymatic activities (Lignin peroxidase, Mn-dependent peroxidase, total cellulase, endocellulase, and xylanase) and sugars concentration. All models demonstrated very low p-values ($p < 0.0001$). These low values indicated that most of the variation in these models can be explained by the regression model equations. The models presented good determination coefficients ($r^2 = 92 – 99.9\%$) and adjusted coefficients ($r^{2(adj)} = 86 – 99.9\%$), showing a close agreement between experimental and the theoretical values predicted by the first-order polynomial results. Based on the analyses of the regression coefficients of the tested variables and their p-values, it was evident that the most significant variables affecting the responses were $MnCl_2$ in lignin peroxidase activity, manure in Mn-dependent peroxidase and endocellulase activities, rice straw in total cellulase activity, $FeSO_4$ in xylanase activity, manure in sugar concentration

Table 5. ANOVA values and estimated regression coefficients for enzymatic activities and sugars concentrations in the hydrolysis process from the Plackett-Burman design

Term	**Lignin peroxidase** (U/min.l)		**Mn-dependent peroxidase** (U/min.l)		**FPU ase** (U/min.l)		**CMCase** (U/min.**l**)		**Xylanase** (U/min.l)		**Sugar** **(g/l)**	
	Coef	**P-Value**	**Coef**	**P-Value**	**Coef**	**P-Value**	**Coef**	**P-Value**	**Coef**	**P-Value**	**Coef**	**P-Value**
Model	25.83	0.000	337.9	0.000	1.15	0.000	10.2	0.000	7.86	0.00	0.259	0.000
$Ca(OH)_2$ loading (P)	2.17	0.354	-37.3	0.064	0.36	0.000	7.95	0.000	7.27	0.00	0.079	0.000
Time (P)	-9.33	0.002	71.0	0.003	0.28	0.000	1.26	0.000	2.44	0.00	0.079	0.000
Temperature (P)	-0.83	0.748	-37.9	0.090	0.44	0.000	8.34	0.000	4.39	0.00	0.096	0.000
Size of rice straw (H)	1.17	0.609	76.4	0.001	0.33	0.000	-0.65	0.000	2.01	0.00	0.030	0.000
Rice straw (H)	-4.33	0.105	-37.2	0.096	0.49	0.000	4.80	0.000	9.59	0.00	0.084	0.000
Manure (H)	-23.83	0.000	162.2	0.000	0.85	0.000	9.78	0.000	9.14	0.00	0.147	0.000
KH_2PO_4 (H)	3.17	0.183	25.0	0.206	0.33	0.000	7.94	0.000	5.54	0.00	0.049	0.000
$MgSO_4$ (H)	-10.83	0.000	42.9	0.049	-0.10	0.000	2.01	0.000	-2.82	0.00	0.050	0.000
$ZnSO_4$ (H)	-14.33	0.000	99.5	0.000	0.09	0.090	2.57	0.000	-2.78	0.00	0.060	0.000
$FeSO_4$ (H)	8.17	0.003	-72.4	0.002	0.14	0.117	3.83	0.000	11.17	0.00	0.042	0.000
$MnCl_2$ (H)	14.17	0.000	-46.8	0.035	-0.22	0.022	2.25	0.000	7.77	0.00	0.002	0.165
Tween 80 (H)	-17.83	0.000	-0.5	0.983	0.28	0.001	1.42	0.000	-2.06	0.00	0.053	0.000
Strept. *Aegytia* inoculum (H)	-8.33	0.003	127.5	0.000	0.48	0.000	0.06	0.429	2.79	0.00	0.052	0.000
pH (H)	-0.33	0.895	-53.5	0.018	0.08	0.000	1.56	0.000	7.56	0.00	-0.007	0.002
Manure (F)	-7.33	0.007	207.4	0.000	0.17	0.182	6.37	0.000	-2.65	0.00	0.051	0.000
Rice straw (F)	-5.83	0.027	134.4	0.000	0.36	0.007	-2.78	0.000	-7.38	0.00	0.047	0.000
KH_2PO_4 (F)	-12.33	0.000	84.7	0.001	0.28	0.000	1.99	0.000	0.64	0.00	0.051	0.000
pH (F)	12.17	0.000	-32.3	0.136	0.24	0.000	-1.73	0.000	-0.21	0.00	0.003	0.112
Cand. *Tropicalis* inoculum (F)	8.67	0.002	-129.2	0.000	-0.02	0.000	0.81	0.000	6.58	0.00	-0.0008	0.677
Model Summary	r^2 = 93.15%, $r^{2(adj)}$ =87.23%		r^2 = 92.45%, $r^{2(adj)}$ =85.92%		r^2 = 95.61%, $r^{2(adj)}$ =91.81 %		r^2 = 99.96%, $r^{2(adj)}$ =99.93%		r^2 = 99.99%, $r^{2(adj)}$ =99.99 %		r^2 = 99.78%, $r^{2(adj)}$ = 99.59%	

The main effects of the examined factors on the enzymatic activities, and sugars concentration were calculated and presented graphically in Figure 1. High values of all tested factors had positive effects on almost responses with different degrees. However, high values of pretreatment temperature, rice straw, tween 80, and pH of the fermentation medium had negative effects on ligninase enzymes (lignin peroxidase and Mn-dependent peroxidase activities).

3.2.3. Ethanol concentration and bioconversion efficiency

Obtained results in Table 4 showed ethanol concentration and bioconversion efficiency as responses at the end of the fermentation stage. The highest ethanol concentration (0.8 g/l) and bioconversion efficiency (13%) were obtained. Table 6 showed the analysis of variance (ANOVA) for ethanol concentration (g/l) demonstrated that the model was significant with F-value (29.8) and a very low p-value ($p < 0.0001$). The model presented a good determination coefficient ($r^2 = 96.3\%$) and adjusted coefficient ($r^{2(adj)} = 93\%$). The statistical analysis demonstrated that the variation of the ethanol concentration was strongly dependent on $Ca(OH)_2$ loading and *Candida tropicalis* inoculum with high F-values of 151.8 and 123.3, respectively.

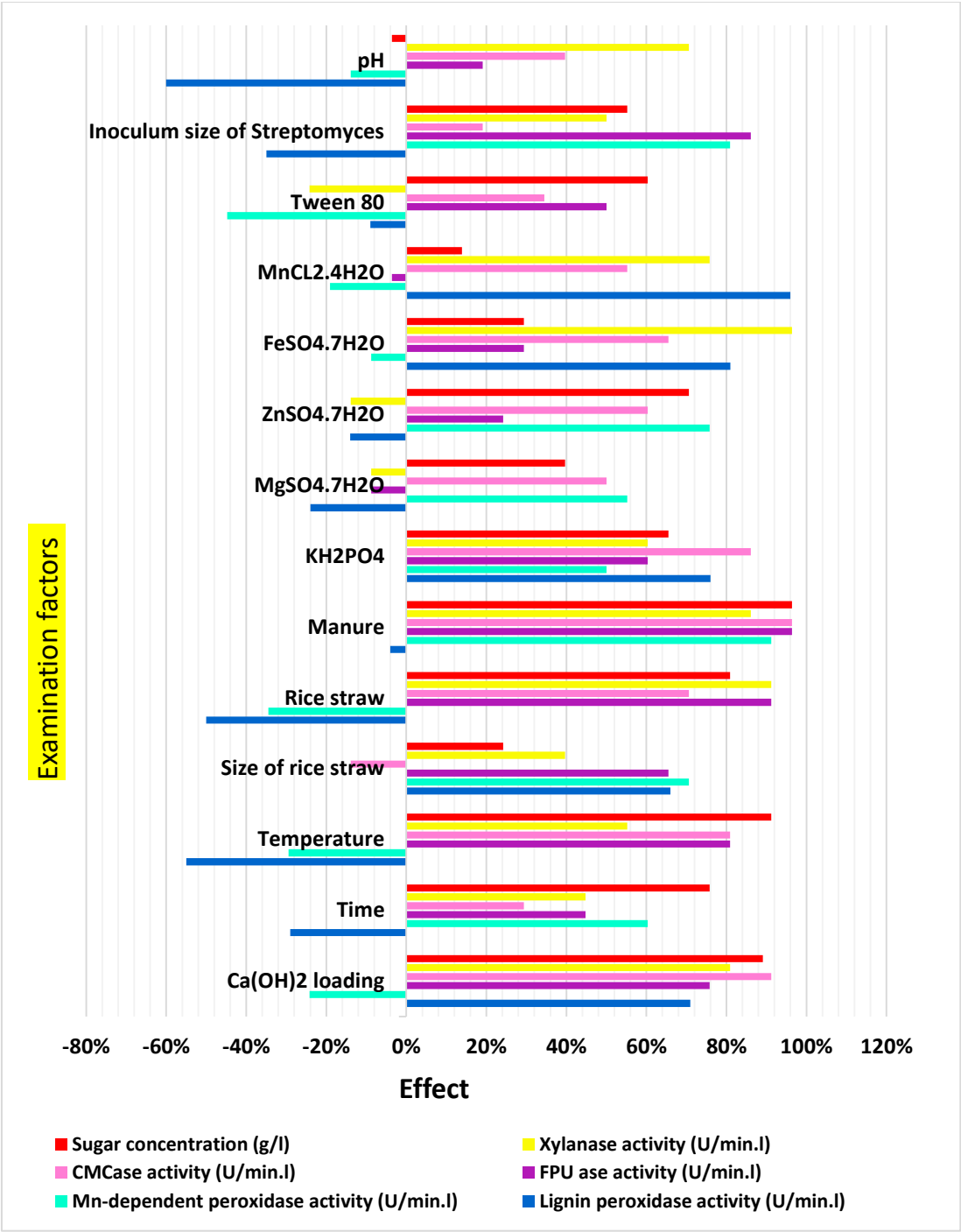

Figure 1. Chart for the percentage effects of process factors on the enzymatic activities of *Streptomyces aegyptia* (U/min.l) and sugars concentration (g/l) in the Plackett-Burman design

Table 7 showed the analysis of variance (ANOVA) for the bioconversion efficiency (%) demonstrating that the model was significant with an F-value (45.8) and a very low p-value ($p < 0.0001$). The model presented a good calculated coefficient (r^2 = 97.5%) and adjusted coefficient ($r^{2(adj)}$ = 95.4%). The statistical analysis demonstrated that the variation of the bioconversion efficiency was strongly dependent on rice straw and *Candida tropicalis* inoculum with high F-values of 172.5 and 130.5, respectively.

Table 6. Analysis of variance (ANOVA) for bioethanol concentration (g/l) from the Plackett-Burman design

Source	DF	Sum of Squares	Mean Square	F-Value	P-Value
Model	19	1.29937	0.068388	29.79	0.000
$Ca(OH)_2$ loading (P)	1	0.34856	0.348564	151.83	0.000
Time (P)	1	0.00828	0.008276	3.60	0.071
Temperature (P)	1	0.02573	0.025735	11.21	0.003
Size of rice straw (H)	1	0.00361	0.003606	1.57	0.223
Rice straw (H)	1	0.06430	0.064304	28.01	0.000
Manure (H)	1	0.02740	0.027400	11.93	0.002
KH_2PO_4 (H)	1	0.06660	0.066600	29.01	0.000
$MgSO_4$ (H)	1	0.02090	0.020900	9.10	0.006
$ZnSO_4$ (H)	1	0.05413	0.054127	23.58	0.000
$FeSO_4$ (H)	1	0.06495	0.064949	28.29	0.000
$MnCl_2$ (H)	1	0.00819	0.008186	3.57	0.072
Tween 80 (H)	1	0.00978	0.009783	4.26	0.051
Streptomyces inoculum (H)	1	0.06512	0.065116	28.36	0.000
pH (H)	1	0.00007	0.000072	0.03	0.862
Manure (F)	1	0.05785	0.057849	25.20	0.000
Rice straw (F)	1	0.09473	0.094730	41.26	0.000
KH_2PO_4 (F)	1	0.05298	0.052982	23.08	0.000
pH (F)	1	0.00930	0.009299	4.05	0.057
Candida inoculum (F)	1	0.28305	0.283054	123.29	0.000
Error	22	0.05051	0.002296		
Total	41	1.34988			
Model Summary	r^2 = 96.26%, $r^{2(adj)}$ = 93.03%, $r^{2(pred)}$ = 93.35%				

The process step in which the parameter was applied, was given in parentheses. (P) denotes the pretreatment step, (H) denotes the hydrolysis, and (F) denotes the fermentation step.

Source	DF	Sum of Squares	Mean Square	F-Value	P-Value
Model	19	350.808	18.4636	45.85	0.000
$Ca(OH)_2$ loading (P)	1	32.874	32.8738	81.64	0.000
Time (P)	1	1.440	1.4396	3.58	0.072
Temperature (P)	1	6.417	6.4167	15.94	0.001
Size of rice straw (H)	1	3.936	3.9360	9.77	0.005
Rice straw (H)	1	16.262	16.2620	40.38	0.000
Manure (H)	1	22.865	22.8647	56.78	0.000
KH_2PO_4 (H)	1	0.025	0.0246	0.06	0.807
$MgSO_4$ (H)	1	2.343	2.3432	5.82	0.025
$ZnSO_4$ (H)	1	33.631	33.6305	83.52	0.000
$FeSO_4$ (H)	1	8.801	8.8014	21.86	0.000
$MnCl_2$ (H)	1	0.821	0.8213	2.04	0.167
Tween 80 (H)	1	0.140	0.1403	0.35	0.561
Streptomyces inoculum (H)	1	5.911	5.9109	14.68	0.001
pH (H)	1	3.581	3.5810	8.89	0.007
Manure (F)	1	0.206	0.2060	0.51	0.482
Rice straw (F)	1	69.465	69.4652	172.51	0.000
KH_2PO_4 (F)	1	0.665	0.6645	1.65	0.212
pH (F)	1	2.178	2.1782	5.41	0.030
Candida inoculum (F)	1	52.561	52.5610	130.53	0.000
Error	22	8.859	0.4027		
Total	41	359.666			
Model Summary	r^2 = 97.54%, $r^{2(adj)}$ = 95.41%, $r^{2(pred)}$ = 95.62%				

Table 7. Analysis of variance (ANOVA) for bioconversion efficiency (%) from the first Plackett-Burman design

The process step in which the parameter was applied, was given in parentheses. (P) denotes the pretreatment step, (H) denotes the hydrolysis step, and (F) denotes the fermentation step

The standardized effects of the examined variables on ethanol concentration and bioconversion efficiency were calculated and presented in the normal plot graphs shown in Figure 2. The graphs illustrated the order of significance of the variables affecting the ethanol concentration and bioconversion efficiency. The positive significant factors affecting the ethanol concentration and bioconversion efficiency were pretreatment temperature, $FeSO_4$, $MgSO_4$, and *Streptomyces aegyptia* inoculum in the hydrolysis process. The highest positive significant factors as indicated by normal plots were $Ca(OH)_2$ loading and *Candida tropicalis* inoculum.

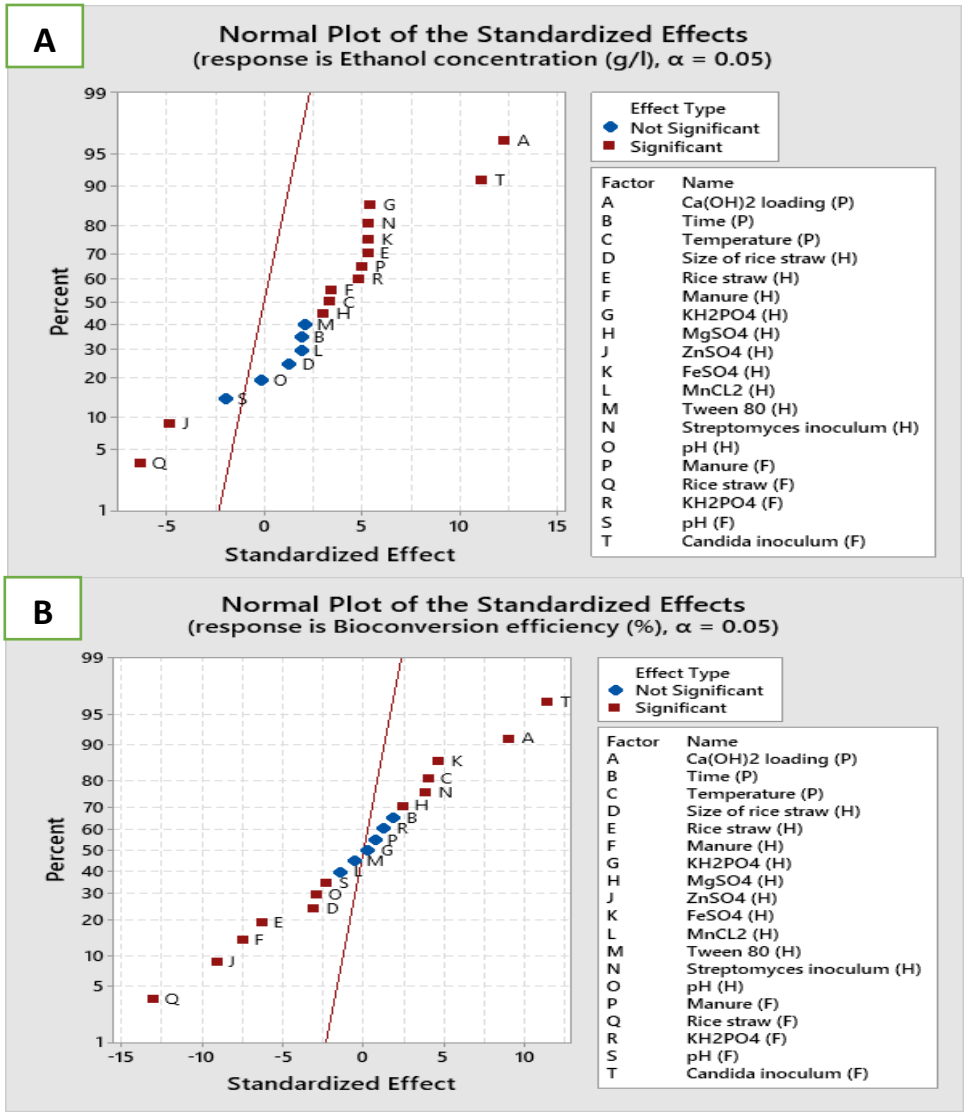

Figure 2. Normal plots of standardized effects of the tested factors in the first Plackett-Burman design on ethanol concentration (g/l) and bioconversion efficiency (%), response in (A) was ethanol concentration (g/l) and in (B) bioconversion efficiency (%). The process step in which the parameter was applied, was given in parentheses. (P) denotes the pretreatment step, (H) denotes the hydrolysis, and (F) denotes the fermentation step.

The first-order polynomial equations in Table 8 described the correlation between the examined nineteen factors and the model's responses.

Table 8. Regression equations of the fitted models

Lignin peroxidase (U/min.l) = 25.83 + 2.17 A - 9.33 B - 0.83 C + 1.17 D - 4.33 E - 23.83 F + 3.17 G - 10.83 H - 14.33 I + 8.17 K + 14.17 L - 17.83 M - 8.33 N - 0.33 O - 7.33 P - 5.83 Q - 12.33 R + 12.17 S + 8.67 T
Mn-dependent peroxidase (U/min.l) = 337.9 - 37.3 A + 71.0 B - 37.9 C + 76.4 D - 37.2 E + 162.2 F + 25.0 G + 42.9 H + 99.5 I - 72.4 K - 46.8 L - 0.5 M + 127.5 N - 53.5 O + 207.4 P + 134.4 Q + 84.7 R - 32.3 S - 129.2 T
FPU ase (U/min.l) = 1.1539 + 0.3620 A + 0.2893 B + 0.4476 C + 0.3333 D + 0.4959 E + 0.8533 F + 0.3342 G - 0.1020 H + 0.0958 I + 0.1435 K - 0.2253 L + 0.2824 M + 0.4810 N + 0.0804 O + 0.1709 P + 0.3661 Q + 0.2878 R + 0.2411 S - 0.0258 T
CMCase (U/min.l) = 10.2081 + 7.9591 A + 1.2670 B + 8.3403 C -0.6532 D + 4.8024 E + 9.7847 F + 7.9433 G + 2.0106 H + 2.5770 J + 3.8366 K + 2.2574 L + 1.4292 M + 0.0666 N + 1.5686 O + 6.3727 P - 2.7816 Q + 1.9981 R - 1.7311 S + 0.8132 T
Xylanase (U/min.l) = 7.8681 + 7.2714 A + 2.4452 B + 4.3884 C + 2.0082 D + 9.5887 E + 9.1461 F + 5.5436 G - 2.8255 H - 2.7801 J + 11.1686 K + 7.7764 L - 2.0622 M + 2.7949 N + 7.5628 O - 2.6506 P - 7.3815 Q + 0.6450 R - 0.2130 S + 6.5786 T
Sugar concentration (g/l) = 0.25971 + 0.07921 A + 0.07936 B + 0.09687 C + 0.03002 D + 0.08431 E + 0.14742 F + 0.04949 G + 0.05032 H + 0.06072 I + 0.04293 K + 0.00286 L + 0.05387 M + 0.05235 N - 0.00706 O + 0.05115 P + 0.04771 Q + 0.05193 R + 0.00329 S - 0.00084 T
Ethanol concentration (g/l) = 0.42052 + 0.10551 A + 0.01835 B + 0.03211 C + 0.01056 D + 0.05076 E + 0.03279 F + 0.04642 G + 0.02786 H - 0.04571 I + 0.04967 K + 0.01768 L + 0.01943 M + 0.05014 N - 0.00165 O + 0.04636 P - 0.05932 Q + 0.04498 R - 0.01884 S + 0.10369 T
Bioconversion efficiency (%) = 6.650 + 1.025 A + 0.242 B + 0.507 C - 0.349 D - 0.807 E - 0.947 F + 0.028 G + 0.295 H - 1.139 I + 0.578 K - 0.177 L - 0.074 M + 0.478 N - 0.370 O + 0.087 P - 1.606 Q + 0.159 R - 0.288 S + 1.413 T

A, B and **C** represent $Ca(OH)_2$ loading, time, and temperature of pretreatment stage

D, E, F, G, H, J, K, L, M, N, and **O** represent size of rice straw, rice straw, manure, KH_2PO_4, $MgSO_4$, $ZnSO_4$, $FeSO_4$, $MnCl_2$, tween 80, *Streptomyces aegyptia* inoculum and pH in hydrolysis stage

P, Q, R, S, and **T** represent manure, rice straw, KH_2PO_4, pH, and *Candida tropicalis* inoculum in fermentation stag

3.2.4. Surface morphology observation by scanning electron microscope (SEM)

For untreated samples, an ordered structure of matrix with whole cells was observed in Photo (1A). The rice straw surface after sequential processes of pretreatment, hydrolysis, and fermentation processes, showed fine voids, rugged, unsmooth, and broken face along with *Streptomyces aegyptia* mycelium (Photo 1B). At the same time, there were more substances attached to the surface of the hydrolyzed fiber in comparison with the untreated sample. The transverse view in Photo (1C) showed the ordered shape of uninoculated sample in comparison with the compact structure of the transverse section of rice straw sample in Photo (1D). The inoculated sample in Photo (1D) also showed *Streptomyces aegyptia* mycelium penetration through the straw structure.

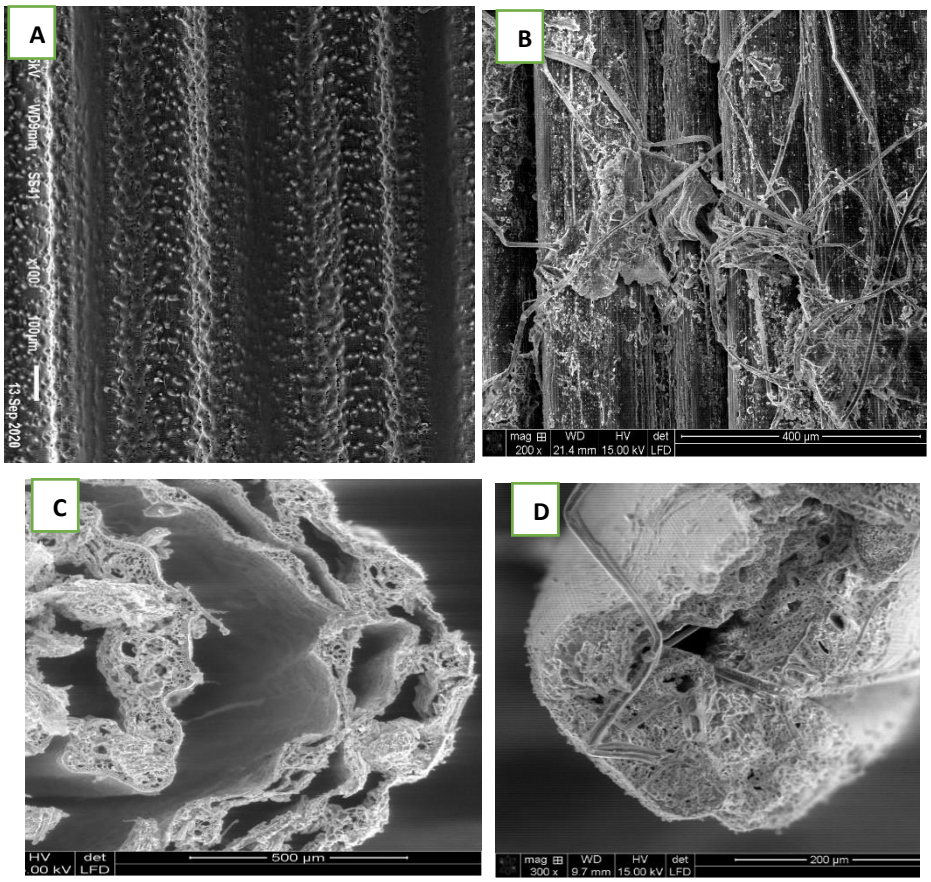

Photo 1. Scanning electron microscope (SEM) images of (A) The surface of untreated rice straw sample. (B) The surface of rice straw sample pretreated with $Ca(OH)_2$ followed by hydrolyzed using *Streptomyces aegyptia* and fermented by *Candida tropicalis.* (C) Transverse view of uninoculated sample (D) Transverse view of rice straw sample showed *Streptomyces* penetration in rice straw sample. The sample was taken from the center point run (No. 13).

4. DISCUSSION

In this study, calcium hydroxide was applied for the pretreatment of rice straw and manure blending. *Streptomyces aegyptia* was inoculated for three days before adding *Candida tropicalis* in the same bottle. As the growth rate of actinomycete, *Streptomyces aegyptia* and yeast, *Candida tropicalis* are different and the time taken by *Streptomyces aegyptia* for lignocellulosic enzyme secretion was longer than sugar to be available at a sufficient level once the yeast, *Candida tropicalis* was grown in the fermentation medium. At the end of the process, a bioethanol concentration of 0.28 g/l was obtained with a bioconversion efficiency of 2%. These results demonstrated the possibility of using calcium hydroxide pretreatment and co-culture of *Streptomyces aegyptia* and *Candida tropicalis* for bioethanol production. A similar combination was applied previously in the solid-state fermentation of rice straw by *Aspergillus fumigatus* G-13 **[53]** and the cultivation of the *Bacillus cereus, B. paranthracis* and *B. anthracis* on the pretreated rice straw with calcium hydroxide **[54].** During the bioconversion process, screening of various factors that may improve the process were carried out using the Plackett-Burman statistical design.

The pretreatment step is the most important rate-limiting step in the overall bioethanol production process **[55].** To evaluate the factors influencing the calcium hydroxide pretreatment step, three variables,

chemical loading, time, and temperature of pretreatment were studied. The XRD analysis demonstrated decreasing in the crystallinity index of all rice straw pretreated with calcium hydroxide in comparison with untreated samples. In lignocellulose biomass, hemicellulose and lignin are amorphous in nature, while cellulose is crystalline. A lower crystallinity index would indicate a broken cellulose crystallization zone and that means more reduction in the degree of polymerization and crystallinity and that the amorphous area of the materials were increased **[56]**. Results of XRD analysis also indicated that high calcium hydroxide loading (6% wt/wt) at all levels of pretreatment time and temperature had the most effect on the crystallinity reduction of rice straw and manure (11%) from 63 to 52 %. However, the low loading (2%wt/wt) showed a certain dependency on pretreatment time and temperature for effective reduction of the crystallinity (9%) in which short reaction times (1 hour) generally required high temperatures (100º C). A similar trend was also observed in the statistical analyses of calcium hydroxide effects on enzymatic activities, sugar, and ethanol concentrations in used design. High chemical loading (6%wt/wt), temperature (100° C) and time (24 h) increased the sugars and ethanol concentrations by 86%, 91%, 76% and 96%, 50%, and 35%, respectively. Enzymatic activities and sugar concentration can be improved by removing acetate groups from hemicellulose when enough calcium hydroxide is added **[57].** High calcium hydroxide loading caused the disintegration of cellulose structures and allowed the enzyme to degrade the cellulose microfibrils, thereby sugars and ethanol were significantly enhanced **[58].** The temperature of alkali pretreatment governs the dissociation constant and ion concentration. Due to higher dissociation at the higher temperature (100° C), weak alkalis can affect with high efficiency, similar to strong alkalis **[59].** The longer an alkali is incubated with its substrate, the greater the removal of lignin will be formed. Thus, the XRD analysis and fiber data of the hydrolyzed substrates revealed that a maximum effect of alkali pretreatment can be obtained in high chemical loading and in two regions: low temperature and high pretreatment time or high temperature and low pretreatment time. The severity of the pretreatment conditions can be at a mild level that caused the disintegration of fiber structures without negative effects on enzymatic activities, especially ligninase enzymes **[27].** Similar results were shown in calcium hydroxide pretreatment of bagasse residues and wheat straw that suggested short pretreatment times (1–3 h), high temperatures (85-135°C) to achieve high sugar yields, whereas, for long pretreatment times (24 h), low temperatures (50–65°C) were effective [**57],** delignification of rice straw from 13.1% to 27.0% for calcium hydroxide pretreatment at 95°C and chemical loading 10%(w/w) **[58],** and calcium hydroxide for delignification of kikuyo grass (33.25%) and sugarcane bagasse (32.75%) that was achieved using the lowest level of temperature (40° C), while the high alkali concentration (50% W/W) had the highest delignification effect **[27]**.

In general, the genes Streptomyces had strong abilities to degrade rice straw and release cellulase, xylanase, and ligninase. In this study, the highest enzymes activities of *Streptomyces aegyptia*, xylanase (78.1 U/min.l), endocellulase (57.3 U/min.l), lignin peroxidase (120 U/min.l), Mn-dependent peroxidase (1020 U/min.l) and total cellulase (3.7 U/min.l) activities were detected in the Plackett-Burman design. *Streptomyces aegyptia* had a higher capability of producing lignin-degrading enzymes in this study in comparison with the highest lignin peroxidase activity results of *Streptomyces chromofuscus* A2 (160 U/min.l) and *Streptomyces viridosporus* T7A (150 U/min.l) obtained in a previous study **[60]**. The lignin-degrading enzymatic activities in this study were also markedly higher than the results of anothers **[52]** in which the lignin peroxidase and Mn-dependent peroxidase activities were 16.13 U/min.l and 13.23 U/min.l, respectively. The highest carbohydrate-degrading enzymes activities of *Streptomyces aegyptia*, endocellulase (57 U/min.l), and xylanase (78 U/min.l) in this study were similar to the results obtained previously **[61]** in obtaining the maximum endocellulase activity of 80 U/min.l using *Streptomyces fimicarius* AE73P.

Streptomyces aegyptia secreted more lignocellulolytic enzymes and the sugar concentration increased when the particle size of rice straw was 1 cm and in the case of powdered manure (2 mm). The adherence and penetration of *Streptomyces aegyptia* as well as enzyme action on the substrate depend on the particle size of the substrate **[62]**. When a mixed substrate which contained different particle sizes was used, the enzyme production was better than that was obtained with lower and higher particle size substrates. This was probably due to the reason that lower particle size results in substrate agglomeration and decreased heat transfer while larger particles reduce the production due to limited surface area for the microbial attack **[63].** A similar particle size of these substrates was found in a previous study **[64]** to be promising for rice straw degradation and produced a sugars concentration of 85 mg/l.

When the effects of the concentration of the rice straw (as lignocellulose substrates) and manure were investigated, the highest activities of lignocellulolytic enzymes of *Streptomyces aegyptia* were detected on low rice straw concentration (4 g/l) blended with a high concentration of manure (16 g/l) which was corresponded to a C:N ratio of around 15:1. Degradation of residues is dependent upon the carbon to nitrogen (C:N) ratio, a C:N ratio less than 20 allows reside to decompose effectively while a C:N ratio greater than 20 requires additional nitrogen and slow down decomposition **[65]**. A high concentration of manure (22 g/l) is required for *Streptomyces aegyptia* to reduce the high C:N ratio of rice straw (28:1) as well as being a source for various minerals which are helpful for the initiation of growth and replication of microorganisms. That was similar to the results of optimum C:N ratio for several degrading microorganisms in rice straw hydrolysis **[30].** The same C:N ratio (15:1) was favored by *Candida tropicalis* in the fermentation step. That trend was different than illustrated in a study **[66]** that evaluated the effect of the C:N ratio on the fermentation process using *Sachormyces cerevisiae* and obtained maximum ethanol yield at a high C:N ratio (35:1). However, the optimum C/N ratio for ethanol production by the recombinant *Saccharomyces cerevisiae* YKU 131 was 7.9 **[67].**

The main effects of the concentration of KH_2PO_4 and $MgSO_4$ on lignocellulolytic enzymes activity showed that the high concentration of KH_2PO_4 (3 g/l) with the low concentration of $MgSO_4$ (0.3 g/l) increased the production of the lignocellulolytic enzymes. The reason may be that KH_2PO_4 acts as a role of buffering capacity, changing the concentration of phosphate can strongly affect cell growth as well as enzyme production. Although Mg^{2+} is an essential factor for *Streptomyces aegyptia* cultivation in the activation of several enzymes involved in metabolic pathways, it is argued that the efficiency of microbial conversion of the substrate may be improved by altering Mg concentration ratios in a way that makes more magnesium available to the microbial cells **[68].** This result was the same as results reported before **[31]** in which the high concentration of KH_2PO_4 (1.5 g/l) with the low concentration of $MgSO_4$ (0.1 g/l) increased the cellulase production of *Streptomyces ruber* on rice straw.

Some divalent metal salts, Mn^{++} and Fe^{++} Zn^{++} were added in different concentrations to the basal cultivation medium supplemented with rice straw and manure as agricultural byproducts after sterilization. Their highest concentration (0.01 g/l) gave high stimulated enzymes formation by *Streptomyces aegyptia*. Positive effects of Mn^{++} and Fe^{++} on xylanase enzymatic activity were also previously observed **[32]**.

It was found that the addition of tween-80 enhanced the carbohydrates degrading enzyme production but not ligninase enzymes, suggesting the beneficial effects of this surfactant on enzymatic hydrolysis **[5].** The addition of surfactant up to 2 ml/l stimulated the enzyme production by improving the permeability of the cell membrane so that enzymes synthesized intracellularly can be secreted outside the cell more easily [**69**]. The hydrophobic interaction of tween 80 with lignin occurs at the substrate surface forming non-specific bonds and that reduced the cellulase adsorption to the lignin and at the same time decreases

the ligninase enzymatic activity **[70].** Xylanase activity of *Streptomyces chartreusis* L1105 was found to greatly enhanced by Tween 80 **[35].**

Actinomycetes cellulases and xylanases generally act optimally at pH values close to neutrality **[71]** and the optimum pH (7.9) reported here for straw-saccharifying activity conformed to this pattern. These were similar to those reported for *Streptomyces* sp. F2621 **[33]** and *Streptomyces* sp. strain 11AG8 **[34].** Thus, *Streptomyces* saccharifying enzymes may offer advantages for that processes use lignocellulose as the substrate **[72].** However, ligninase enzymatic activities and sugar concentration increased at the pH of 5.7. In the fermentation step, the acidic conditions were favored by *Candida tropicalis* (4.0- 5.0). A slightly acidic pH of around 4.5 favors the reproduction and growth of yeast. The permeability of some essential nutrients into the cells as well as the stability of produced enzymes are influenced by the concentration of H^+ in the fermentation broth. Thus, a pH of around 5 was more suitable for the bioconversion process.

The amount of microbial inoculum is one of the essential factors that influence bioethanol fermentation. The optimum inoculum amount for maximum enzymatic activities of *Streptomyces aegyptia* and sugars concentration was 1×10^7 CFU/ml. Low inoculum size may require a longer time for microbial growth and substrate utilization. Similar results were obtained with the effect of inoculum size of *Streptomyces viridiochromogenes* for rice straw decomposition **[8]**. The optimal inoculum size of *Streptomyces* sp. ESRAA-301097 **[35]** and *Streptomyces psammoticus* that yielded maximum ligninase activity (33.4 U/g) was 1.5 × 107 CFU/ml **[73].** In the fermentation step, increasing of inoculum size of *Candida tropicalis* increased the ethanol concentration by 91%. Increased cell microbial concentration within a certain range allowed the rapid growth of cells in the fermentation media that immediately consumes fed sugars producing ethanol **[74, 75]**. That was similar to a study that showed positive effects on fermentation from 25% sucrose when the inoculum size of *Saccharomyces cerevisiae* was increased from 3% to 6% **[39].**

Rice straw samples before and after the bioconversion process were observed using scanning electron microscopic analysis to know what occurred in the structure of the fiber. The fine voids in the surface of rice straw fiber may be produced as a result of calcium hydroxide pretreatment. That results from the fact that calcium ions extensively crosslinked lignin molecules under alkaline conditions which results in increased biomass porosity **[27, 76].** Degradation of framework structure combined with fiber data of the hydrolyzed substrates confirmed the phenomenon caused by the degradation of the substrate by *Streptomyces*. These results demonstrated the synergistic effect of calcium hydroxide pretreatment and the microbial conversion using co-culture of *Streptomyces aegyptia* and *Candida tropicalis* for ethanol production cost-effectively.

5. CONCLUSION

The present study evaluated calcium hydroxide as an inexpensive alkali used in the pretreatment of rice straw and manure blending for bioethanol production. The results of x-ray diffraction (XRD) analysis combined with fiber data of the hydrolyzed substrates revealed that a chemical loading of $Ca(OH)_2$ (6%), a period of 1 h at a room temperature (25°C) were the best conditions for removal of a crystalline region of the fiber in such a way that the fiber obtain a less crystalline nature by 11%.

The suitable cultivation conditions appeared to be an efficient for sugar production (0.6 g/l) under submerged hydrolysis by *Streptomyces aegyptia* were rice straw (4 g/l) with particle size of 1 cm, manure (16 g/l), KH_2PO_4 (2 g/l), $MgSO_4$ (1 g/l), $ZnSO_4$ (0.01 g/l), $FeSO_4$ (0.01 g/l), $MnCl_2$ (0.01 g/l), Tween 80 (2 ml/l), with (1×10^7 CFU/ml) at pH 5.7. The enzymatic activities of *Streptomyces aegyptia*, lignin peroxidase (0-120 U/min.l), Mn-dependent peroxidase (0-1020 U/min.l), total cellulase (0-3.7 U/min.l), endocellulase (0.3-57.3 U/min.l) and xylanase (0-78 U/min.l) were obtained in the Plackett-Burman design used in this study. However, the fermentation conditions for *Candida tropicalis*, manure (16 g/l), rice straw (4 g/l), KH_2PO_4 (2 g/l) with inoculum size of 4% at pH 4 were required for ethanol

concentration (0.8 g/l), and bioconversion efficiency (13%). The analysis demonstrated the synergistic effect of this combination (calcium hydroxide pretreatment and co-culture of *Streptomyces aegyptia* and *Candida tropicalis*) which led to ethanol production in a cost-effective manner and satisfying level. The three most significant positive factors in the process were calcium hydroxide loading in the pretreatment step, KH_2PO_4 in the hydrolysis step, and *Candida* inoculum size in the fermentation step. These selected factors by the Plackett-Burman design were recommended for further optimization by central composite design to maximize ethanol yield.

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