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Optimization of Cellulolytic enzymes production from local potent fungi using full factorial design for bioethanol production from MFEX-treated rice straw using *Klebsiella oxytoca* P2.

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Rice straw was applied to physiochemical pretreatment using Multipurpose Fiber Explosion technology (MFEX) then enzymatic hydrolysis carried out. Cellulases and hemicellulases producing fungi were isolated, purified, identified and screened for their enzymatic activity. After isolation, purification and identification, five fungal isolates were selected for cellulase and hemicellulses enzymes production. The cellulase enzymes production process was statistically optimized by full factorial design method, which indicated that five interesting fungal isolates were found to have potent cellulolytic and hemicellulolytic activities demonstrated by FP-ase, CMC-ase, β-glucosidase, xylanase and β-xylosidase assay. Maximum FP-ase, CMC-ase and β-glucsidase, production was found to be 0.367, 0.912 and 0.612 IU/mL, respectively with *Aspergillus sp.* under shaked conditions for 11.8 days. It could be concluded that the studied factors (microorganism, aeration and time) at optimum conditions strongly support the enzyme production. The Simultaneous Saccharification and Fermentation (SSF) was performed in pH-controlled, stirred fermenters. A total reducing sugars of about 440 mg/g dry MFEX-treated RS was achieved within 24 hours' hydrolysis using 10 IU of laboratory prepared enzymes. Of this total, about 260 mg/g RS (59.091 %) was glucose, and about 90 mg/g RS (20.455 %) was xylose. Glucose was rapidly fermented within 24 hours by genetically-engineered *Klebsiella oxytoca* P_2 (5% V/V, OD=1) leading to an ethanol yield of about 125mg/g dry MFEX-treated RS. Glucose utilization was rapid and complete, whereas xylose utilization was slow and incomplete. In order to develop an economically bioethanol production process, improved co-fermentation technologies appear necessary to utilize all the fermentable sugars derived from RS.

Keywords: Rice straw, Cellulolytic enzymes, Fermentable sugars, Bioethanol, Full Factorial Design

INTRODUCTION

In an increasingly competitive and expanding global economy, the demands for cheap, clean, and efficient energy sources are growing. The world population has grown, more countries have industrialized, energy consumption has increased steadily, increase in demand of energy, fossil fuel resources limitation, increasing costs, and the associated environmental issues (Balat and Balat, 2009; Bozell, 2010; Shafiee and Topal, 2009). Therefore, there is great interest in exploring alternative energy sources, which fulfills the

criteria of sustainable development (Aditiya et al., 2016; Chinnici et al., 2018; Gebresemati and Gebregergs, 2015; Mohd Azhar et al., 2017; Saini et al., 2015). A potential method for solving this problem is to utilize lignocellulosic materials such as agricultural wastes (Domínguez-Bocanegra et al., 2015; Margeot et al., 2009; Sarkar et al., 2012; Tan and Lee, 2014). A variety of lignocellulosic agricultural wastes are available for bioethanol production. Among all the lignocellulosic substances, cereal straws are most abundant, cheap, renewable and easily available (Sun and Ren, 2010). These materials are accumulated in bulks every year without much usage in the form of agro-residues, etc. Large amounts of theses residues are burned in the fields causing pollution. Typical example is the burning of the most abundant residues such as rice straw. Unsustainable use and open burning of rice straw in the field not only produces threat to the environment by producing large amount of greenhouse gas (GHG) emission, but also make farmer's loose a very viable by-product. More sustainable technologies for bioethanol production from lignocellulosic biomass have been investigated (Aditiya et al., 2016; Battista et al., 2016; Nair et al., 2017). Rice straw is a promising alternative for bioethanol production (Hafid et al., 2017; Liguori et al., 2013). One of the alternative solutions is producing biofuels and biomaterial building blocks from biomass waste, e.g. rice straw can be used in bioethanol production and bring additional income and sustainable utilization. It will also provide clean energy solution to ever increasing energy demand. This technology of waste to energy includes the pretreatment of biomass, subsequently converted to fermentable sugars by enzymatic hydrolysis, which thereafter fermented into bioethanol by recombinant microorganisms.

Lignocelluloses are composed of cellulose, hemicelluloses and lignin (Zhang et al., 2015).The celluloses and hemicelluloses from the agro-residues can be acted up on by microbes using solid-state as well as submerged fermentation and produce value-added compounds such as enzymes, bioethanol, etc. The bioconversion of lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, and actinomycetes are considered as a subject of intensive research as a contribution to the development of a large scale conversion process beneficial to humanity (Sun et al., 2016). Hydrolytic enzymes mainly cellulases and hemicellulases are the key element in biodegradation process of lignocellulosics to useful products (Hu et al., 2015; Shawky et al., 2011). In order to convert lignocellulosic biomass into fuels effectively, it is necessary to improve the efficiency of enzyme production. Formation of enzymatic cocktail is one of the best methods to improve the efficiency. Since the cost of the enzymatic cocktails influences the viability of the process, utilization of enzyme produced by the microorganisms could be more significant (Shawky et al., 1984). Cellulases is a multi-enzyme system composed of several enzymes, exoglucanase, endoglucanase, and β-glucosidase, which act synergistically (Liu and Cao, 2014). Exoglucanases are mainly active on crystalline regions of cellulose. Endogluconases are highly active against the amorphous regions of cellulose and β-glucosidase are needed to cleave cellobiose to glucose (Rani et al., 2014).

Lignocellulosic ethanol opens up the new possibility for bioethanol production on a sustainable basis because nonfood biomass resources are utilized, and does not compete with using food crops. Biotechnological conversion of lignocellulosic biomass into liquid fuel bioethanol is a challenge that, sooner or later will have to be taken up. This investigation aims to study the production of cellulases and hemicellulases by a locally potent fungal strains, to evaluate the application of these in-house prepared enzymes in enzymatic hydrolysis of MFEX-treated RS, besides bioethanol fermentation with genetically engineered *Klebsiella oxytoca* P_2.

MATERIALS AND METHODS

Isolation of cellulases and hemicellulases producing Fungi

Broad variety of samples rich in decayed cellulosic materials such as: biodegraded plant residues, rotten hay, humus, peat, compost, soil, etc. were collected from different localities. Continually and after samples collection 1:10 sample suspension in sterile tap water was prepared, vigorously shaken and serial dilutions were used for surface inoculation of enrichment medium (Shawky and Hickisch, 1984), which consist of (g/L distilled water) NaCl, 3.0; $(NH4)_2SO4$, 1.0; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $MgSO_4$. $7H_2O$, 0.1;$CaCl_2.2H_2O$, 0.1; yeast extract, 0.25; Cellulose, 5. For solid medium, 2% agar was added. The pH was adjusted to 5.5 before autoclaving at 121^{o}C for 20 minutes. After incubation at 30 ^{O}C for 3 - 7 days, developing colonies differing in morphological characteristics

were picked up and transferred to the previous medium following Shawky and Hickisch protocol (Shawky and Hickisch, 1984).

Purification of isolated fungi

Streak inoculation method was applied, developing colonies were picked up after 1-3 days of incubation and purified at least five times. The fungal cultures were considered pure when microscopically examination revealed no contamination appears with any other undesirable microorganisms. The fungal isolates were maintained on both Shawky and Hickisch medium (Shawky and Hickisch, 1984) and Potato Dextrose Agar (PDA) slants and preserved at 5^{o}C until needed.

Identification of purified isolated fungi

The morphological, cultural and physiological characteristics of the pure isolated fungi were determined using the methods giving by (Sharma, 1989).

Enzyme Production

250 mL specific medium (Under Publication) for each fungal isolate was prepared containing (g/L distilled water): KH_2PO_4, 6.0; $CaCl_2$. $2H_2O$, 1.0; $MgSO_4.7H_2O$, 1.0; $(NH_4)_2SO_4$, 4; yeast extract, 0.2; peptone, 0.6; rice bran, 20; Tween 80 solution, 2; cellulose, 40 and trace elements (mg/L distilled water) $FeSO_4.7H_2O$, 20; $MnSO_4.H_2O$, 6; $ZnSO_4.7H_2O$, 10; $CoCl_2$, 6; $NaMO_4.2H_2O$, 0.2; $CuSO_4.5H_2O$, 0.6; Citric acid.H_2O, 6; H_3BO_3, 0.2. The components were mixed and the pH was adjusted to 5.5 before autoclaving at 121 ºC for 30 minutes. After autoclaving the medium supplemented by 0.5 mL filter sterilized urea then inoculated with 5.0 mL of fungal spore suspension and incubated at 30^{o}C on both static and shacked (100rpm) culture conditions for 3, 6 and 9 days. Cultures were harvested by centrifugation at 5000 rpm for 15 minutes and the cell-free culture supernatants were used for the determination of cellulases and hemicellulases activity (FP-ase, CMC-ase, β-Glucosidase, xylanase and β-xylosidase).

Optimization of enzyme production

Enzyme production experiments optimized by full factorial design (FFD) using Design Expert Software (version 7.0). The lower and upper levels of optimized factors were selected on the basis of the suitable conditions for efficient enzyme production. (Table 1).

The levels of the factors studied and the layout of the FFD were presented in Table 1. Each of the 30 enzyme production experiments denoted by "runs" was carried out as per the defined values of three different parameters in different levels (Table 1). All experiments were carried out in 500 mL Erlenmeyer flasks.

Cultures were harvested by centrifugation at 5000 rpm for 15 minutes and the cell-free culture supernatants were used for the determination of cellulase activities, namely FP-ase, CMC-ase, β-Glucosidase, xylanase and β-xylosidase as well.

Cellulolytic enzymes assay

Cellulase assay was done following dinitrosalicylic acid (DNS) method (Miller, 1959) and the enzyme activity was expressed in IU.

Exo β-1,4- glucanase (FP-ase) assay

Filter paper assay method (FP-ase) for total cellulase enzyme activity in the culture filtrate was applied according to (Mandels et al., 1976) by mixing 0.2 mL of the cultural filtrate as enzyme source was added to Whitman No. 1 filter paper strip (1 x 6 cm; 50 mg) dissolved in 1.8 mL of 0.05 M sodium citrate buffer of pH 4.8. After incubation at 50^{o}C for 1h, the reducing sugar released was estimated by DNS method (Miller, 1959) by adding 1.0 mL of DNS reagent and immersing in boiling water bath for 15 min. After adding 3 mL of distilled water and stirring using vortex mixer, the color intensity was measured in terms of optical density (OD) at 540 nm using spectrophotometer (Hitachi, model U2000). The reducing sugar thus liberated and quantified by following the same method on glucose as standard. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per mL per min.

Endo β-1,4- glucanase (CMC-ase) assay

CMC-ase enzyme activity in the culture filtrate was determined according to (Mandels et al., 1976) by mixing 0.1 mL of culture filtrate as enzyme source was added to 0.5mL of 1% CMC dissolved in 0.4 mL of 0.05M sodium citrate buffer pH 4.8. After incubation at 50^{o}C for 1h, the reducing sugar released was estimated by DNS method of (Miller, 1959).One unit of CMC-ase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per mL per min.

Table 1: Matrix Layout of the full factorial design.

Run	Factor A	Factor B	Factor C	Run	Factor A	Factor B	Factor C
1	1	Static	3.00	**16**	1	Shaked	3.00
2	1	Static	6.00	**17**	1	Shaked	6.00
3	1	Static	9.00	**18**	1	Shaked	9.00
4	3	Static	3.00	**19**	3	Shaked	3.00
5	3	Static	6.00	**20**	3	Shaked	6.00
6	3	Static	9.00	**21**	3	Shaked	9.00
7	10	Static	3.00	**22**	10	Shaked	3.00
8	10	Static	6.00	**23**	10	Shaked	6.00
9	10	Static	9.00	**24**	10	Shaked	9.00
10	17	Static	3.00	**25**	17	Shaked	3.00
11	17	Static	6.00	**26**	17	Shaked	6.00
12	17	Static	9.00	**27**	17	Shaked	9.00
13	23	Static	3.00	**28**	23	Shaked	3.00
14	23	Static	6.00	**29**	23	Shaked	6.00
15	23	Static	9.00	**30**	23	Shaked	9.00

β-Glucosidase assay (Salicinase assay)

β-Glucosidase enzyme activity in the culture filtrate was determined according to (Mandels et al., 1976) by mixing 0.1 mL of culture filtrate as enzyme source was added to 0.5mL of 1% salicin dissolved in 0.4 mL of 0.05M sodium citrate buffer pH 4.8. After incubation at 50 ^{o}C for 1h, the reducing sugar released was estimated by DNS method of (Miller, 1959). One unit of β-glucosidase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per mL per min.

Xylanase assay

Xylanase activity in the culture filtrate was determined according to (Bailey et al., 1992) by mixing 0.025 mL of culture filtrate as enzyme source was added to 0.5 mL of 1% xylan dissolved in 0.45 mL of 0.05M sodium citrate buffer pH 4.8. After incubation at 50^{o}C for 15 min, the reducing sugar released was estimated by DNS of (Miller, 1959) The reducing sugar thus liberated was quantified by following the same method on xylose (Sigma) as standard. One unit of Xylanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per mL per min.

β-xylosidase

β-xylosidase activity was measured by a spectrophotometric method with p-nitro phenyl-β-xylopyranoside as the substrate. The assay mixture contained 200 µL of substrate solution (2.0 mM with p-nitro phenyl-β-xylopyranoside in 50 mM citrate phosphate buffer, pH 5.5), and 200 µL of appropriately diluted enzyme solution. After incubation at 50 °C for 30 min, the reaction was stopped by the addition of 1.6 mL of 0.5 M Na_2CO_3. The absorbance at 405 nm due to the release of p-nitrophenol in the mixture was measured. Blank received buffer solution instead of enzyme. One unit of β-xylosidase activity was defined as the amount of enzyme which produced 1 µmol of p-nitrophenol per min at 50 °C. The assay was repeated three times.(Chang et al., 2005)

Analysis of the full factorial experiments (Runs).

The statistical software package (Design Expert, version 7.0) was used to determine the outcomes of the fractionation runs. The responses values in terms of FP-ase, CMC-ase, β -glucsidase, Xylanase, β-xylosidase activities and S/N ratios of FFD in 30 runs were analyzed to extract independently the main effects of the factors; the analysis of various conditions were then applied to determine which factors were statistically significant. The controlling factors were identified, with the magnitude of the effects qualified and the statistically significant effects determined. Accordingly, the optimal conditions were estimated by combining the levels of factors that had the highest main effect value. The analysis of variance (ANOVA) for the responses of FP-ase, CMC-ase, β -glucsidase, Xylanase and β-xylosidase activities were carried out according to the factors contribution by the FFD. The factors in

the experimental design considered to be statistically significant at more than 94% confidence limit were used to determine the ratio (F) and the p-value ($p < 0.05$).

Validation of the Experimental Model.
The model was validated by performing the enzyme production trial employing full factorial optimized production process parameters. The validation of the experimental model was executed by determining the enzyme activity.

Biomass and MFEX–Pretreatment:
Rice straw (RS) was sun-dried, ground to about 5 mm. The biomass was MFEX-treated in a 20-L stainless steel 316 reactor according to the procedure described by Shawky (2009) [Patent ASRT No. 24507], using steam. The MFEX-treated biomass was then air-dried and stored in sealed heavy plastic bags and refrigerated at 5^{o}C until required.

Hydrolysis:
Hydrolysis was done in capped 500-mL flasks agitated with a magnetic stirrer. 15g of substrate in 300 mL 0.05 M citrate buffer, pH 4.8, i.e., 5% (w/v, solid/liquid) was sterilized at 121^{o}C for 30 minutes. The pH was aseptically readjusted to 4.8 after autoclaving. MFEX-treated and untreated RS was enzymatically hydrolyzed by 10 IU commercial cellulases/g dry substrate at 48^{o}C for 24 hours. During hydrolysis the pH was maintained constant at 4.8 by addition of 1N potassium hydroxide solution to the flasks when required. One mL liquid samples were taken at 24 hours, boiled in capped test tubes for 15 minutes to stop the hydrolysis and then filtered through a 0.22 micron nylon membrane for total reducing sugars determination by DNS assay (Miller, 1959) as well as individual sugars (glucose and xylose) determination using HPLC

Fermentation:
The Simultaneous Saccharification and Fermentation (SSF) was performed in pH-controlled with stirring. After 24 hours of enzymatic hydrolysis of the substrate, the temperature was readjusted to 35^{o}C and the pH to 5.5. *Klebsiella oxytoca* P_2 was kindly provided by Prof. Dr. Lonnie O. Ingram (University of Florida, Gainesville, FL 32611). The flasks were inoculated aseptically by a standardized volume of *Klebsiella oxytoca* P_2 suspension to give an optical density of 1.0 at 550 nm to start the fermentation. The fermentation temperature and the pH were maintained at 35^{o}C and 5.5, respectively. Samples were taken in sterile vials at various times, placed immediately in an ice bath, and then centrifuged at 10.000g and 4^{o}C for 20 min. Supernatants were stored at –20^{o}C until required for analysis using HPLC.

RESULTS AND DISCUSSION

Isolation of cellulases and hemicellulases producing fungi
Table 2 shows the growth, pigmentation and source of the main five fungi that isolated from different decayed cellulosic materials.

Optimization of process parameters of cellulolytic enzymes production by FFD method.
The influence of 3 factors on the cellulolytic enzymes production was tested by FFD design in 30 runs (Table 1). The response values in terms of enzyme activities (IU) chosen for optimization of enzyme production. Figure 1 shows the efficiency of fungal cellulases production ranging from 0.01 to 0.39%, corresponding to the combined effect of the three factors. It could be concluded that the fungal enzyme production mainly effected by the strain specificity as well as the culture condition, which runs parallel with the findings of (Devi and Kumar, 2012; Sethi and Gupta, 2014).

ANOVA Analysis of the FFD runs.
The F-value models of FP-ase, 9.60; CMC-ase, 39.15; β -glucsidase, 18.65; Xylanase, 3.12; β-xylosidase, 3.46 which indicate that all models are significant concerning the statistical methods implementation. Values of "Prob > F" less than 0.0500 indicate model terms are significant, i.e. the terms A, B and C are significant model terms for the FP-ase, CMC-ase, β-glucsidase activity responses, while A and B are the only significant factors for Xylanase activity response. Values greater than 0.1000 indicate the model terms are not significant. (Table 3). For all response models, the "Pred. R-Squared" is in reasonable agreement with the "Adj. R-Squared". Predicted vs actual plot illustrate how the model predicts successfully. (Fig 2).

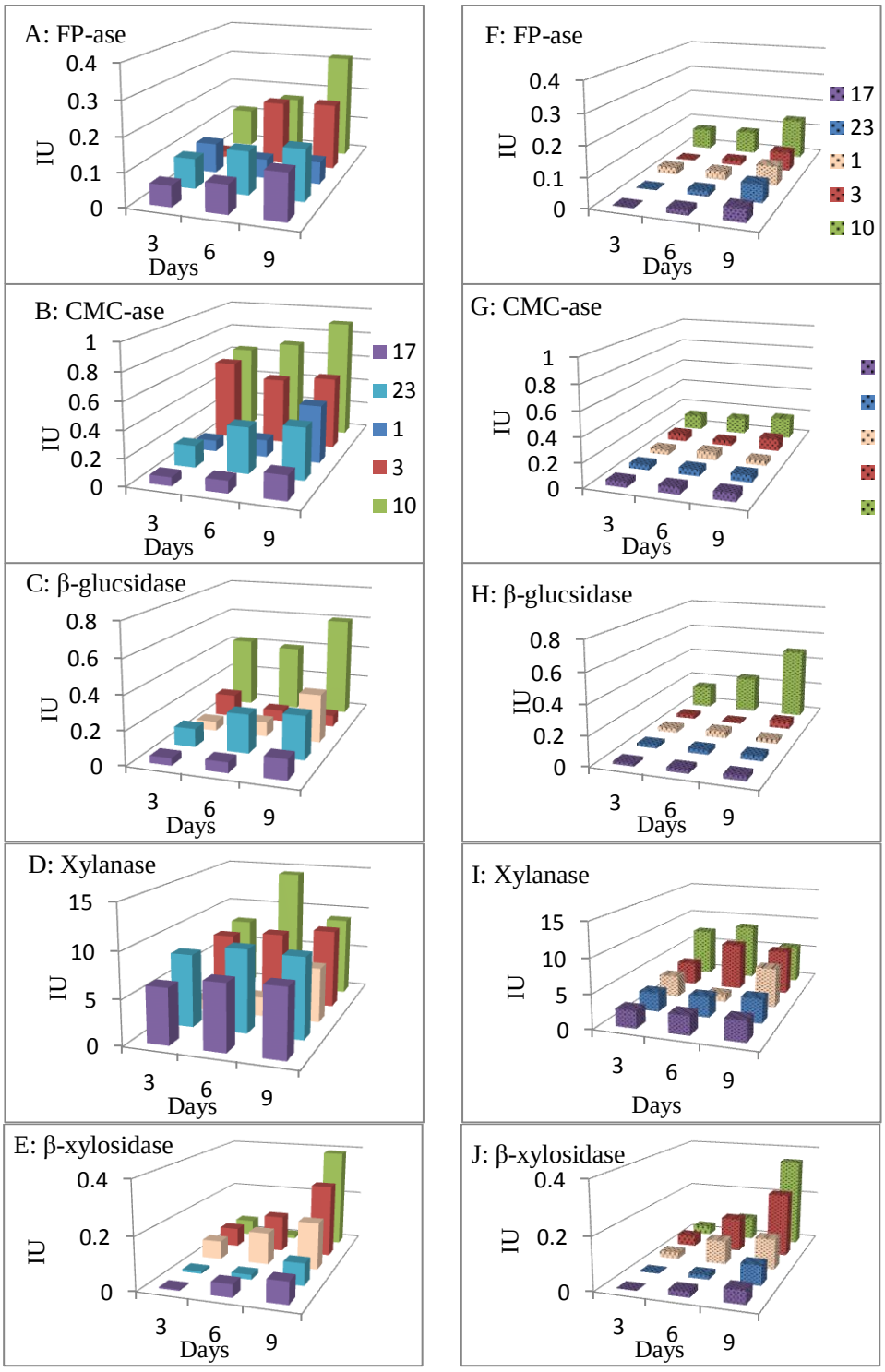

Figure 1: Response values of Full Factorial Design. (cellulolytic enzymes production from different fungal isolates grown under shaked (A, B, C, D and E) and static (F, G, H, I and J) culture conditions.

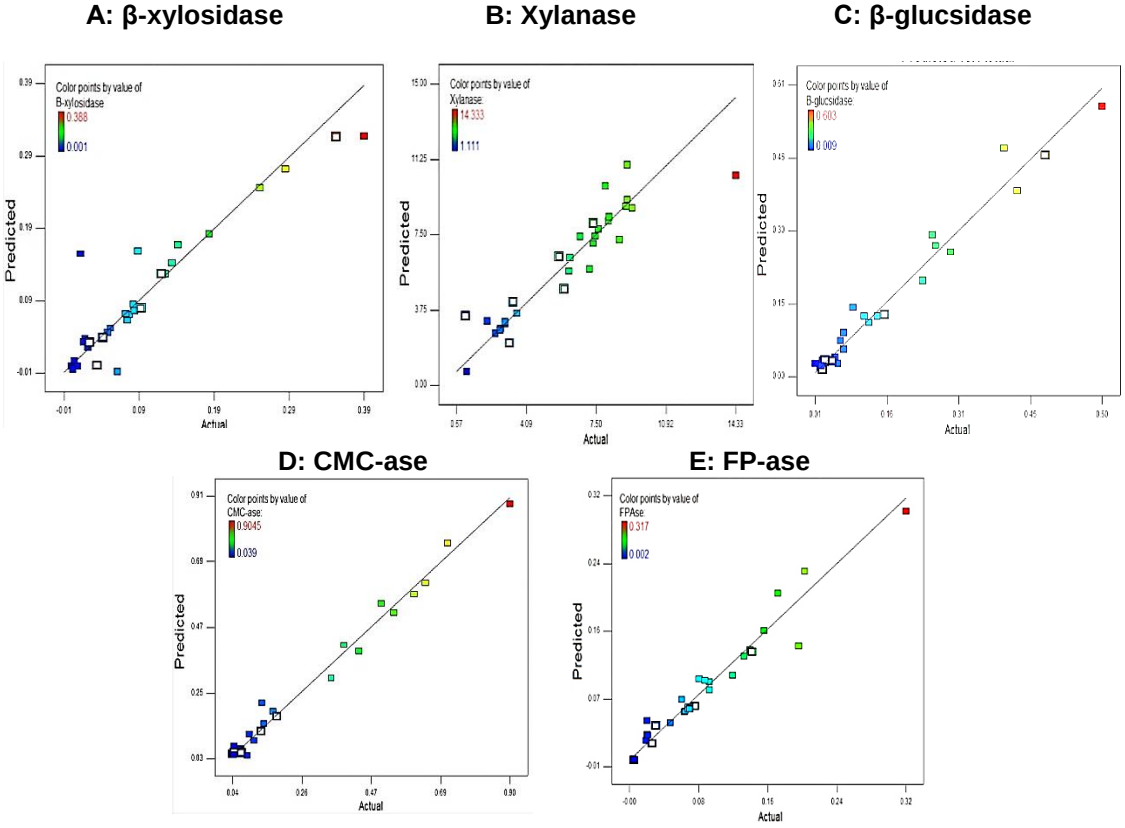

Figure. 2 : predicted vs actual values of cellulolytic enzymes activity (IU)

Table 2: **Cellulolytic fungal isolates from decayed cellulosic materials grown on enrichment solid agar medium Shawky and Hickisch (1984 a).**

Isolate No.	Code No.	Source	Growth & pigmentation		Expected organism
			24-48 Hours	7-10 Days	
1	H 86	Deteriorated palm stem	+ White	++ White lime	*Rhizopus sp.*
3	H 13	Tree rotten roots	++ Green	+++ Deep green	*Trichoderma sp.*
10	H 71	Rotten rice straw	++ Black	+++ Black	*Aspergillus sp.*
17	B 04	Decayed wood peel	+ Light green	++ Gray green	*Penicillium sp.*
23	B 44	Rotten corn stalks	+ rose red	++ purple red	*Fusarium sp.*

Table 3: **ANOVA for Response Surface Cubic Model**

Source	Prob > Fp-Value				
	FP-ase	CMC-ase	β-glucsidase	Xylanase	β-gxylosidase
Model	0.0004	< 0.0001	< 0.0001	0.0348	0.0245
A-Microorganism	0.0011	< 0.0001	< 0.0001	0.0068	0.0132
B-Aeration-Agitation	< 0.0001	< 0.0001	< 0.0001	0.0017	0.4345
C-Time	0.0001	0.0008	0.0008	0.1104	0.0003
AB	0.1827	< 0.0001	0.0934	0.1883	0.9586
AC	0.0569	0.1108	0.0151	0.6331	0.0715
BC	0.1008	0.0048	0.3765	0.6393	0.8513
ABC	0.1385	0.0633	0.0576	0.9252	0.9999

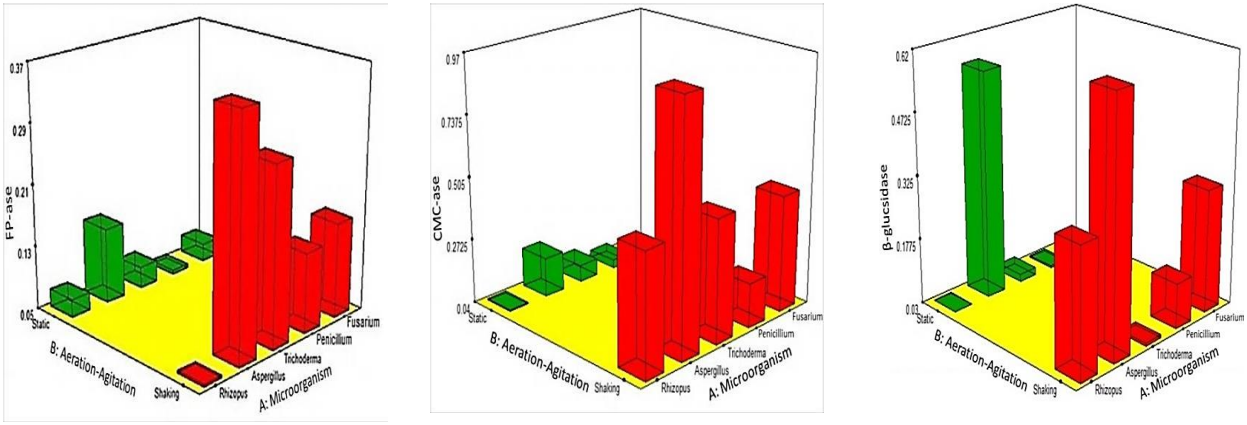

Figure. 3: 3D response surface graphs display the characteristic effects of key process variables on different cellulolytic enzymes production (IU)

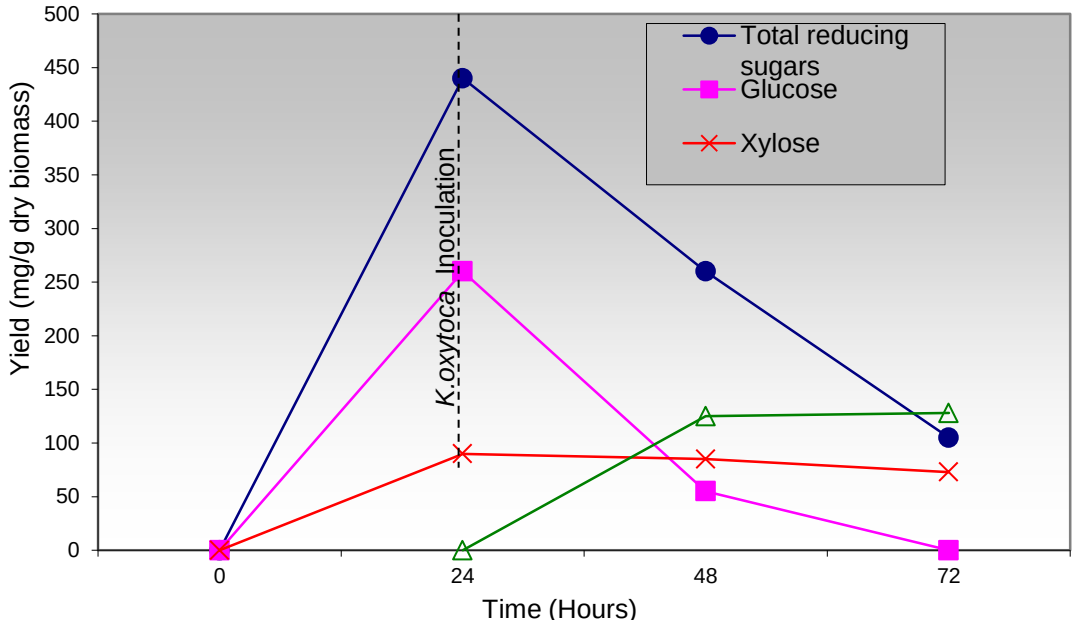

Figure. 4. Total reducing sugars, glucose, xylose, and bioethanol profiles of MFEX - treated rice straw hydrolyzed by laboratory prepared cellulolytic enzymes and fermented by *Klebsiella oxytoca P2*.

Response surface methodology (RSM).

The experimental results suggest that these factors at optimum level strongly support the fungal enzymes production. 3D response surface graphs display the characteristic effects of key process variables on enzyme production (Fig. 3). Maximum FP-ase, CMC-ase and β-glucsidase, production predicted by the model was found to be 0.394, 1.006 and 0.647 IU/mL, respectively with *Aspergillus sp.* under shaked condition for 11.8 days. The data was further validated, where the experiment was carried out under optimized condition.

Validation of the experimental model.

The model was validated by performing the best predicted cellulolytic enzymes production parameters. It was clear that the validation of the experimental model was executed by determining the enzyme activity (IU), which was almost equal to predicted (Table 4).

Table 4: Actual vs. Predicted values

	FP-ase	CMC-ase	β-Glucsidase
Actual	0.367	0.912	0.612
Predicted	0.394	1.006	0.647

Hydrolysis and fermentation.

Figure 4 shows the hydrolysis and fermentation profile of MFEX-treated rice straw. Within 24 hours, a total reducing sugar yield of 440 mg/g dry substrate was obtained. The glucose yield was 260 mg/g dry substrate at 24 hours. The hydrolyzate was inoculated with *Klebsiella oxytoca* P_2 (5% V/V, OD=1) at 24hours of hydrolysis, and the fermentation conducted at pH 5.5 and 35^{o}C. For MFEX-treated rice straw, bioethanol production reached 125 mg/g dry substrate within 24 hours of inoculation. It was clear that glucose was almost completely depleted within 12 hours of the ethanologenic organism inoculation with corresponding rapid bioethanol production. Xylose was utilized slowly, and bioethanol production increased steadily.

This agrees with the findings of (De La Rosa et al., 1994; Lawford and Rousseau, 2003; Reshamwala et al., 1995; Shawky, 2017; Szczodrak, 1988), who used different pretreated lignocellulosic biomass [Switch grass, Coastal Bermuda grass, Bagass, Rice straw, and Cotton stalks].

It is concluded that the MFEX-pretreatment method significantly increased the total reducing sugars as well as the experimental bioethanol yields from RS as compared to the untreated substrate. There is an increased interest in producing bioethanol as an octane booster or as a liquid fuel. Lignocellulosic biomass from different crop residues has been used for conversion to bioethanol (Rabinovich, 2006). Using SSF for conversion of lignocellulosic biomass to bioethanol is technically and economically feasible (Hinman et al., 1992; Santoso, 2012) . The SSF increases the yields of bioethanol by minimizing product inhibition as well as eliminates the need for separate reactors for saccharification and fermentation (Hari Krishna et al., 2001) .Efforts are being made to improve the feasibility of converting lignocellulosics to fuel bioethanol. Improvements are required in the hydrolysis and fermentation of the lignocellulosic biomass especially the co-fermentation of both hexoses and pentoses during Simultaneous Saccharification and Fermentation (SSF) appear necessary (Hinman et al., 1989; Reshamwala et al., 1995).

CONCLUSION

Cellulolytic enzymes production in shaked culture condition were extremely greater, compared to static culture condition. The fungal enzyme production efficiency mainly affected by both strain specificity and culture condition. The present work highlights the significant progress that has been achieved in development of optimized processes and microbial strains for cellulosic bioethanol production using rice straw as agricultural residues. Conversion of lignocellulosic biomass to useful products is difficult technically and economically mainly because of its recalcitrance. Therefore, biomass must be pretreated before it can be used in bioconversion. Generally, all the known pretreatment methods have limitations. Multipurpose Fiber Explosion [MFEX] (Reshamwala et al., 1995) provide an effective, economic, and clean pretreatment process for lignocellulosic materials, which enhances subsequent enzymatic hydrolysis of such substrates to prduce fermentable sugars and fuel bioethanol there from (Shawky, 2017). It is optimistic that the production efficiency of bioethanol from cellulosic wastes can be further improved by implementation of more optimized conditions and using novel technologies including synthetic biology and genome editing to develop superior microbes particularly fungi. On the other hand, it is necessary to consider that MFEX pretreatment, statistically optimized cellulolytic

enzymes production using FFD and mixed-sugar fermentation with recombinant *Klebsiella oxytoca* P_2 are units in a whole process during consideration of process optimization. Strain specificity had significant role in fungal cellulases production, hence more efforts should be made for strain development to adapt to authentic industrial production conditions. It is concluded that the MFEX-pretreatment method significantly increased the total reducing sugars as well as the experimental bioethanol yields from RS as compared to the untreated substrate. A total reducing sugar yield of 440 mg/g dry RS was obtained. The glucose yield was 260 mg/g dry substrate at 24 hours. Bioethanol production reached 125 mg/g dry substrate within 24 hours of *Klebsiella oxytoca* P_2 inoculation. Glucose was almost completely depleted within 12 hours of the ethanologenic organism inoculation with corresponding rapid bioethanol production. Xylose was utilized slowly, and bioethanol production increased steadily. Improved co-fermentation technologies appear necessary to utilize all the fermentable sugars derived from RS.

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CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

BTS and HMH designed the experiments. BTS and HMH isolated, purified, and identified celluletic organisms in addition to optimize enzymatic hydrolysis. MFEX treatment as well as fermentation carried out by BTS and HMH. TIMR, BTS, HMH and MKZ contribute in the hydrolysis step. HMH analyzed the results statistically. Manuscript was reviewed by MKZ and TIMR. All authors read and approved the final version.

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