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Statistical optimization of rapid production of cellulases from *Aspergillus niger* MA1 and its application in bioethanol production from rice hulls

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The aim of this study was the utilization of rice hulls (RH) as a clean, cost effective, renewable and abundant agro-waste in bioethanol production by simple and applicable methods; for this purpose *Aspergillus niger* MA1 was isolated from RH and selected from many other isolates. This isolate produced 15.0, 3.38 and 49.1 U/g RH for CMCase, FPase and β-glucosidase, respectively, by solid state fermentation of RH after 36 h. The cellulases of the fungus showed good thermal and pH stability with maximum activity at 50°C and pH 5. An increase in cellulases productivity by statistical optimization and multi-response of SSF medium was revealed. Addition of 0.25% brej 35 to tap water or saline is very effective in elution of *A. niger* MA1. Cellulase which was successfully used in saccharification of steam explosion pretreated RH, revealed 16.36 g/L reducing sugars and subsequently fermentation by *Saccharomyces cerevisiae* which produced 9.42 g/L ethanol after 24 h.

Key words: Rice hulls, bioethanol, cellulases, solid state fermentation, *Aspergillus niger* MA1, statistical optimization, multi-response.

INTRODUCTION

Trade-off between food and energy in most African countries make it difficult to use agricultural crops to produce bioethanol. The alternative option is the use of lingocellulosic agro-wastes. One of these abundant agro-wastes in Egypt is rice hulls (RH) which represents 20% (mass fraction) of the harvested rice. Its composition comprises around 20 to 25% lignin, 35 to 40% cellulose and 15 to 20% hemicelluloses (Saha and Cotta, 2008).

The essential key in this bioconversion is cellulases which are multi-enzyme system, composed of several enzymes with numerous isozymes which act synergistically and catalyze the hydrolysis of cellulose and cello-oligosaccharide derivatives (Chinedu et al., 2008), but the contribution of the commercial enzyme cost to bioethanol production cost is quite significant (Klein-Marcuschamer et al., 2012). For economic

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production of cellulases from wastes, solid state fermentation (SSF) is highly an effective method, in which cellulosic substrate acts as the carbon source in sufficient moisture without free water. Optimal design of the culture medium by application of statistical experimental design techniques or response surface methodology (RSM) in fermentation process can result in improved product yields, reduced process variability, closer confirmation of the output response to nominal target requirements and reduced development time and overall costs.

In this work RH is the substrate from which *A. niger* MA1 was isolated and upon which rapid production of cellulases was statistically optimized which was subsequently used in the saccharification of pretreated RH and used successfully in bioethanol production.

MATERIALS AND METHODS

Solid state fermentation

In 100 ml glass flask, one gram of dried RH moisten with 1 ml of basal salt solution contains (g/L) KH_2PO_4, 15; $(NH_4)_2SO_4$, 5; $MgSO_4 \cdot 7H_2O$, 0.6; $ZnSO_4 \cdot 7H_2O$, 0.14; $MnSO_4 \cdot 6H_2O$, 0.16; $CaCl_2 \cdot 6H_2O$, 0.37; and peptone, 2, pH 4.8; autoclaving was done at 120°C for 20 min. Inoculated was done with 0.5 ml of fungal spore suspension (10^6 spore /ml) of the tested species and incubated at 28 ±2°C for 10 days.

Elution of cellulases enzymes with different eluents

After fermentation, elution of cellulase enzymes were carried out by different eluents such as tap water, saline solution and buffer solution alone (1:10 w/v) or mixed with different surfactant Triton, Berj 35 and Tween 80 (0.25%). After shaking at 200 rpm for 30 min, the supernatant was separated by filtration and used for cellulases assay.

Carboxy methyl cellulose (1.4 - β - D - glucan glucano hydrolase EC 3.2.1.4)

A mixture of 0.5 ml of substrate (0.5 w/v of carboxymethyl cellulose, sigma) in 0.2 M acetate buffer, pH 4.8 plus 0.5 ml of enzyme was incubated for 30 min at 50°C (Magnelli and Forchiassin, 1999). One unit of endoglucanase activity is the amount of enzyme required to release reducing sugars equivalent to 1 µmole glucose per min under the above experimental conditions.

Filter paper activity (FPase)

According to Ghose (1987), 1 ml of supernatant of culture is incubated with 50 mg filter paper Whatman No. 1 (1.0 × 6.0 cm) in 1 ml of 0.2 mol acetate buffer (pH 4.8) at 50°C for 60 min. One unit of FPase activity corresponds to 1 µmole of glucose equivalent released per minute under the experimental assay conditions.

β-Glucosidase (cellabiase or β D-glucoside glucohydrolase E C 3.2.1.21)

β-glucosidase activity against cellobiose was determined by 0.1 ml of culture supernatant to 0.5 ml of cellibiose in 0.2 mol acetate buffer (pH 4.8). The reaction mixture was incubated at 50°C for 30 min. One β-glucosidase activity unit is equivalent to 1 µmole of glucose per minute under the above experimental conditions.

The released reducing sugars of three enzymes were measured by glucose oxidase Kit using glucose as standard (Spinreact Company, Spain).

Characterization of CMCase and FPase and β-glucosidase produced by *A. niger* MA1

Determination of optimum incubation temperature

The influence of temperature on cellulase activities was determined by assaying the cellulase activities in the standard reaction mixture at various degrees (20, 30, 40, 50 and 60°C).

Thermal stability

Thermostability were determined by incubating the enzyme for different times (60, 120, 180 and 240 min) at various temperatures (30, 40, 50, 60, 70, 80 and 90°C); the remaining cellulase activities were then assayed in its optimum conditions.

Determination of optimum pH

The pH of moistening agent was adjusted using 0.05 M acetate buffers to different pH levels (4.6, 4.8, 5, 5.4, 5.7 and 6). Then the flasks were incubated for 42 h at 30°C. The flasks were harvested for extraction and determination of cellulases activity.

pH stability

Mixture of the enzyme solution and 0.5 acetate buffers were adjusted to cover the pH range from 3.6 to 5.6 and were incubated at 4°C for 6, 12, and 18 and 24 h, the remaining cellulase activities were then assayed in its optimum conditions.

Pretreatment of RH

Alkali pretreatment: NaOH at the concentrations of 10% (w/v), was mixed with RH in 1:7 weight of RH to volume of NaOH, autoclaved at 121°C for 20 min, washed with distilled water thoroughly and then dried at 50°C.

Steam explosion: Ten grams of RH was placed in 100 ml glass flask, treated with 2 par of saturated steam in autoclave. After 5 min, the pressure is suddenly reduced to atmospheric pressure.

Acidic pretreatment: Ten grams of RH was treated with diluted H_2SO_4 (2%)then autoclaved at 121°C for 20 min, washed with distilled water thoroughly and then dried at 50°C.

Statistical optimization of cellulases enzyme production in solid-state fermentation

Plackett-Burman design

For each variable, a high (+1) and low (-1) level was tested. All trials were performed in triplicate, and each experiment was repeated twice, with the mean considered for the response. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for the determination of variable significance.

Table 1. The levels of variables chosen for the Box-Behnken optimization experiment.

Variable	Code	(-1)	0	(+1)
Yeast extract (mg/gm)	X_1	2	4	6
Peptone (mg/gm)	X_2	2	4	6
Corn steep liquor (µl/gm)	X_3	20	40	60

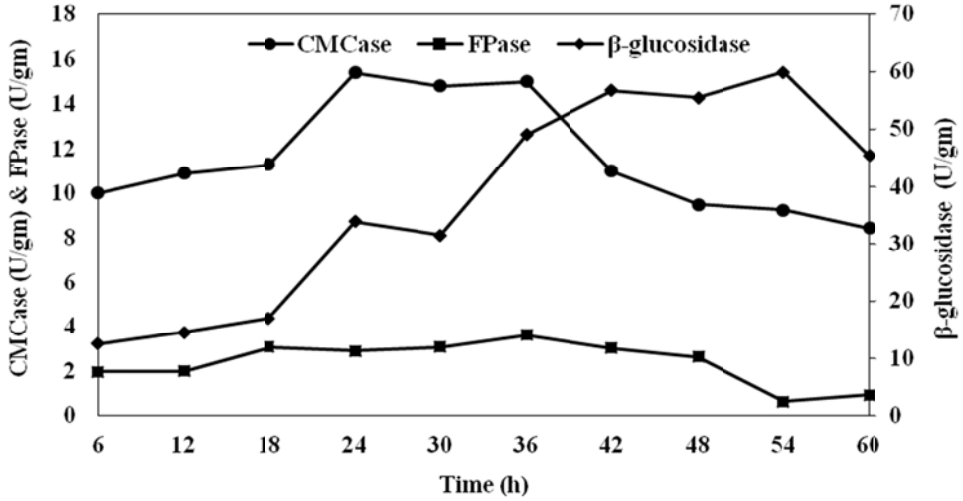


Figure 1. Time course of CMCase, FPase and β-glucosidase production by *A. niger* MA1 on SSF of RH.

Box-Behnken design

Once critical factors were identified via screening, a Box-Behnken design for three independent variables (Table 1), each at three levels with three replicates at the center points was done (Box and Behnken, 1960).

Fermentation for bioethanol production

At the beginning of the experiment the fermentor was sterilized. The tested sugary material was estimated for total fermentable sugars by the Anthrone method (Hedge and Hofreiter, 1962) and supplemented with nitrogen and phosphorus as follow; KH_2PO_4 0.1%, $(NH_4)_2SO_4$ 0.5%, $MgSO_4.7H_2O$ 0.05%, and Yeast extract 0.1%. The pH of the medium was adjusted to 5.0. Then, the prepared sugary syrup was loaded in the fermentor followed by the yeast, *S. cerevisae* inoculums and incubated at 30°C. The appropriate air flow was provided through port that entered through the top of the fermentor (for about 2 h) to allow the yeast to begin growth and reproduction. Samples were extracted from the fermentor every 1 h therefore to measure the changes in temperature, pH and density of the solution. When two similar results were obtained the fermentation process was stopped. The fermentation broth was obtained in order to measure the ethanol (Caputi et al., 1968) and remaining sugar concentrations.

Fermentation efficiency

Fermentation efficiency = (Actual ethanol recovery / Theoretical recovery) × 100
Theoretical recovery = Total sugars × 0.64

Actual ethanol recovery = Actual ethanol obtained

RESULTS

Fungal isolate

One of the best source of fungal strains capable of hydrolyzing RH is RH itself and we achieved that by plating RH samples from different localities in Egypt over sterilized and moisten filter paper. *A. niger* MA1 was selected from many mesophilic fungal isolates which were tested for their cellulolytic activity.

Time course of cellulases productivity of *A. niger* MA1 in SSF of RH

The highest production of CMCase (Unit/gm RH) was observed in the time range from 24 to 36 h of fermentation (around 15 U/g), then decreased gradually to 8.43 U/g after 60 h of fermentation period. The maximum production of FPase is released after 36 h (3.63 U/g), and decreased gradually to 0.63 and 0.93 U/g after 54 and 60 h. The activity of β-glucosidase take relatively more time, it gave its maximum activity after 54 h of fermentation (60 U/g) then decreased to 45.4 U/g after 60 h (Figure 1).

Table 2. Box-Behnken factorial experimental design, representing the response of cellulases activity as influenced by yeast extract, peptone and corn steep liquor by *A. niger* MA1 on RH after 42h of incubation.

Trial	Yeast extract	Peptone	Corn steep	CMCase (U/g)		FPase (U/g)		β-glucosidase (U/g)	
				Response	Predicted	Response	Predicted	Response	Predicted
1	-1	-1	0	16.81	16.71	3.70	3.76	64.60	63.87
2	1	-1	0	13.04	12.86	3.44	3.32	60.80	61.44
3	-1	1	0	13.33	13.43	4.20	4.31	58.01	57.36
4	1	1	0	20.07	20.08	4.10	4.03	48.90	49.62
5	-1	0	-1	13.04	13.16	3.10	2.97	47.70	49.25
6	1	0	-1	18.01	18.21	3.60	3.66	48.20	48.38
7	-1	0	1	17.30	17.08	4.80	4.74	66.00	65.81
8	1	0	1	15.02	14.83	3.21	3.33	58.07	56.51
9	0	-1	-1	10.21	10.12	1.88	1.93	60.09	59.25
10	0	1	-1	17.52	17.20	2.31	2.31	35.06	34.15
11	0	-1	1	15.23	15.50	2.42	2.41	54.76	55.66
12	0	1	1	12.30	12.32	3.33	3.27	61.60	62.43
13	0	0	0	18.01	17.30	3.90	3.97	86.10	85.98
14	0	0	0	17.28	17.30	4.05	3.97	87.05	85.98
15	0	0	0	16.72	17.30	3.98	3.97	84.80	85.98

Statistical optimization of medium using response surface methodology for cellulase production by *A. niger* MA1 under SSF of RH

Placket Burman experimental design

The analysis of randomized Plackett-Burman experimental of 15 independent variables (data not shown) namely; weight, moisture content, pH, inoculum size, sand particles, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$, peptone, yeast extract, corn steep liquor, wheat bran, $MgSO_4$, $CaCl_2$, and Tween 80, showed that, yeast extract, corn step liquor and peptone had significantly influence on the productivity of CMCase, FPase and β-glucosidase of *A. niger* MA1.

Box-Behnken design (BBD)

The result of 15 runs in BBD of yeast extract (X_1), peptone (X_2), and corn steep liquor (X_3) chosen for optimization of *A. niger* MA1 cellulases are shown in Table 2. All the three activities varied distinctly with the conditions tested. CMCase showed divergence from 10.21 to 20.07 U/g, FPase activity between 1.88 and 4.80 U/g while β -glucosidase varied from 35.06 to 87.05 U/g.

The experimental results suggest that these variables strongly affected the fermentation process. The analysis of variance (ANOVA) of the model for CMCase, FPase and β-glucosidase (Table 3) showed validity of predictions in terms of p-value (<0.05) and the coefficients of determination (R^2) of their models were 98.78, 98.97 and 99.60%, where the R^2(adj) were 96.57, 97.13 and 98.89% respectively. The R^2 and adj R^2 value provides a measure of variability in the observed response values that can be explained by the experimental factors and their interactions. The closer the R^2 and adj R^2 value to 1, along with non-significant lack of fit, the stronger the model is, the better it predicts the response.

CMCase productivity optimization

The interactive effects of the studied variables on cellulases activity were studied by plotting 3D surface curves against any two independent variables, while keeping other variables at its central (0) level. The response surface of CMCase is shown in Figure 2a to c. It shows the increase in CMCase at high level of any of the corn steep liquor and yeast extract, interaction between high levels of peptone and corn steep liquor and low levels of peptone and yeast extract; it was also enhanced by high levels of yeast extract which interacted with moderate level of corn steep liquor. The final response function to predict CMCase activity was

$$YCMCase = 17.30 + 0.70X_1 + 0.98X_2 + 0.13X_3 + 2.62\ X_1X_2 - 1.82X_1X_3 - 2.55\ X_2X_3 + 0.25\ X_1^2 - 1.77\ X_2^2 - 1.72X_3^2$$

FPase productivity optimization

As shown in Figure 3a-c, the interaction of moderate levels of yeast extract, corn steep liquor and peptone supported high FPase activity. For predicting the optimal point; within experimental constrains, the following second-order polynomial function was fitted to the experimental results of FPase activity:

Table 3. ANOVA of CMCase, FPase and β-glucosidase production of *A. niger* MA1 under SSF of RH.

Enzyme	Source	DF	Adj SS	Adj MS	F	P
[1]CMCase	Regression	9	100.71	11.19	44.85	0
	Linear	3	11.873	3.9575	15.86	0.005
	Square	3	21.938	7.3128	29.31	0.001
	Interaction	3	66.895	22.298	89.37	0
	Residual error	5	1.248	0.2495		
	Lack-of-fit	3	0.388	0.1292	0.3	0.827
	Pure error	2	0.86	0.43		
[2]FPase	Regression	9	8.6613	0.9624	53.62	0
	Linear	3	2.0737	0.6912	38.51	0.001
	Square	3	5.4316	1.8105	100.87	0
	Interaction	3	1.156	0.3853	21.47	0.003
	Residual Error	5	0.0897	0.018		
	Lack-of-Fit	3	0.0785	0.0262	4.64	0.182
	Pure Error	2	0.0113	0.0056		
[3]β-Glucosidase	Regression	9	3115.3	346.15	139.89	0
	Linear	3	524.69	174.9	70.68	0
	Square	3	2311.9	770.63	311.44	0
	Interaction	3	278.74	92.91	37.55	0.001
	Residual error	5	12.37	2.47		
	Lack-of-fit	3	9.82	3.27	2.57	0.293
	Pure error	2	2.55	1.28		

[1]S = 0.4995, PRESS = 8.135, R^2 = 98.78%, R^2 (pred) = 92.02%, R^2 (adj) = 96.57%; [2]S = 0.1339, PRESS = 1.280, R^2= 98.97%, R^2 (pred) = 85.36%, R^2 (adj) = 97.13%; [3]S = 1.5730, PRESS = 162.86, R^2= 99.60%, R^2 (pred) = 94.79%, R^2 (adj) = 98.89%; DF: degree of freedom; SS: sum of squares; MS: mean of squares; F:F-value; P: significance level of P-value (at significance level<0.05).

$$YFPase= 3.97 - 0.18X_1 + 0.31X_2 + 0.35X_3 + 0.04\ X_1\ X_2\ - 0.52\ X_1\ X_3 + 0.\ 0.12\ X_2X_3\ + 0.53\ X_1^2 - 0.65\ X_2^2 - 0.83X_3^2$$

β-Glucosidase productivity optimization

For β-glucosidase (Figure 4a to c), the interaction between the moderate concentration of the three variable give maximum production of the enzyme. Elliptical contour plots obtained from the data of the present study clearly show significance of the mutual interactions between the variables. The following second-order polynomial function was fitted to the experimental results of β-glucosidase activity:

$$Y\ \beta\text{-glucosidase} = 85.98 - 2.54X_1 - 4.58X_2 + 6.17X_3 - 1.32\ X_1\ X_2 - 2.10\ X_1X_3 + 7.96X_2X_3\ - 12.89\ X_1^2\ -15.01\ X_2^2 - 18.09X_3^2$$

Multi-response optimization

Based on actual values of the multi-response optimization of the three enzymes, the data in Table 4 shows that the amounts of the tested nutrient could be added to RH for maximizing the cellulases production simultaneously, in this respect, the desirability function recorded 0.846; this value is somehow accepted.

Scaling up via solid-state tray fermentation

Taking into consideration the multi-response optimization, *A. niger* MA1 cellulases production process was scaled-up in the laboratory using simple and cheap solid-state tray fermentation. The total productivity of the three enzymes increased with increasing in the size of fermentation container in contrast to productivity per gram of RH. As shown in Table 5 the maximum productivity of CMCase, FPase and β-glucosidase are noticed when *A. niger* MA1 was cultivated in Petri dishes (20 g in 20 cm diameter Petri dish) giving 16.7, 3.02 and 55.9 U/g and in glass flask (10 g in 120 ml) giving 10.02, 3.17 and 52.32 U/g respectively. In trays the three enzymes productivity decreased gradually with increasing amount of RH parallel to tray size.

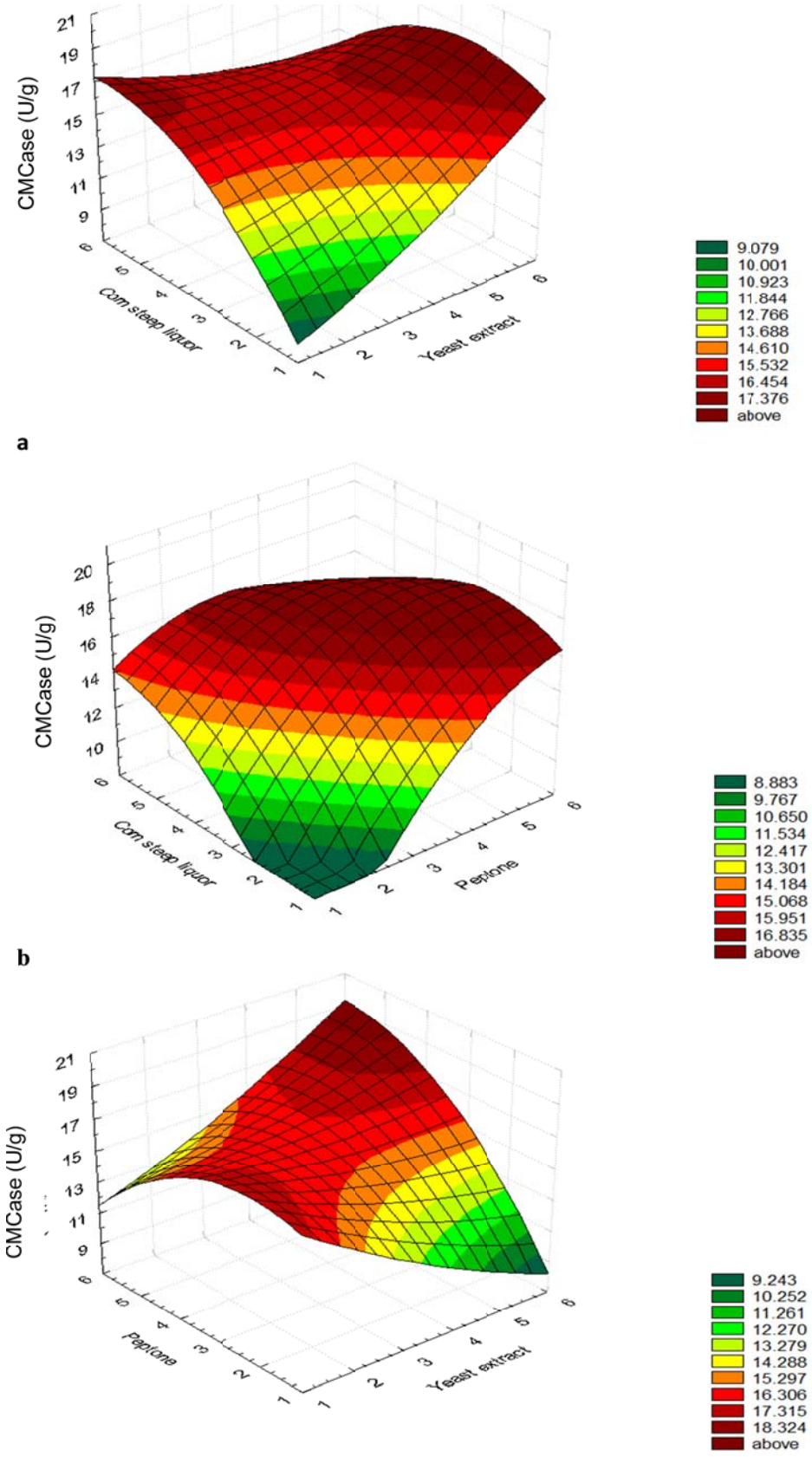


Figure 2. a. Response surface graphs illustrating the effect of yeast extract and corn step liquor. **b.** Peptone and corn step liquor. **c.** Yeast extract and peptone on CMCase production.

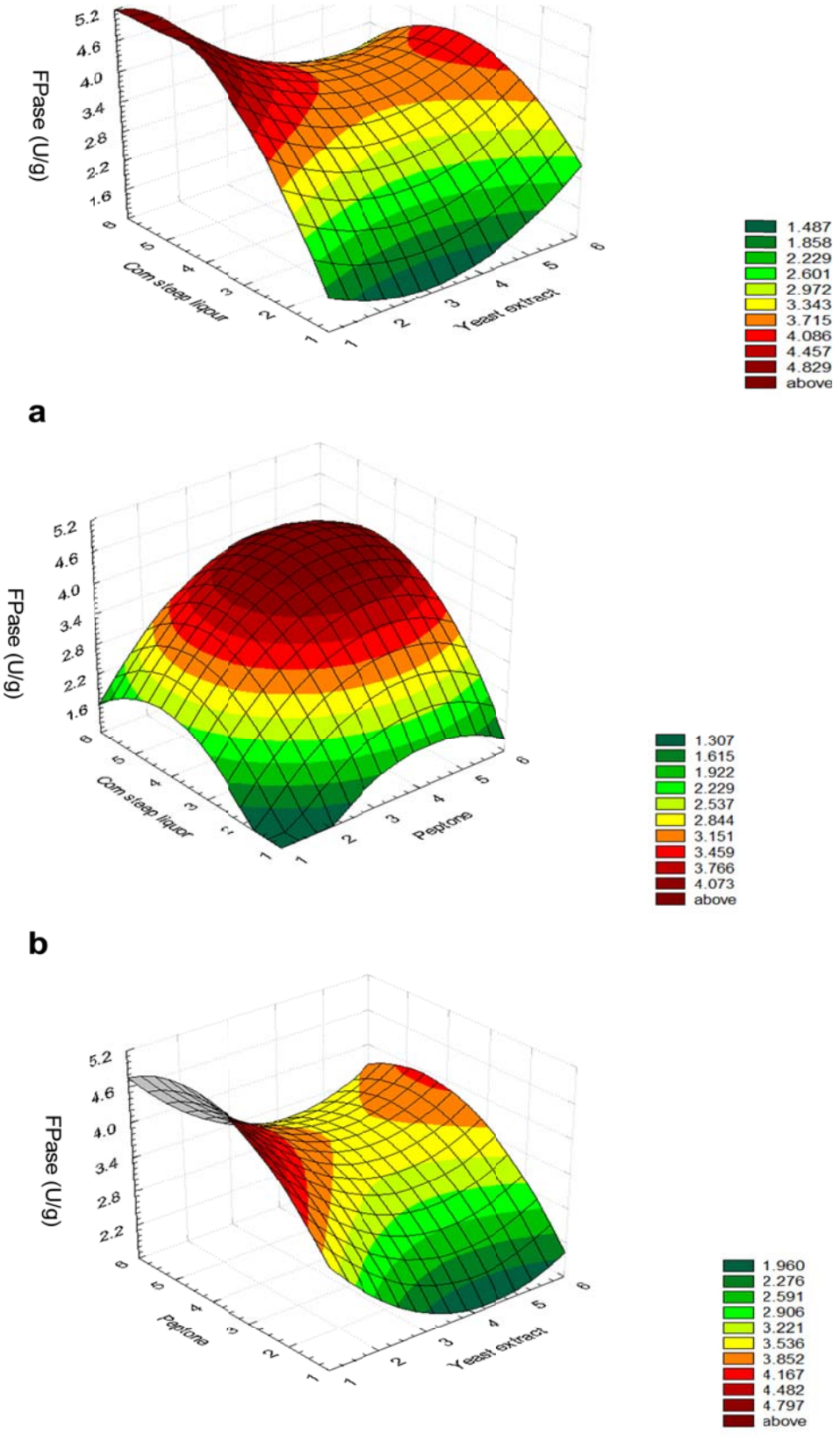
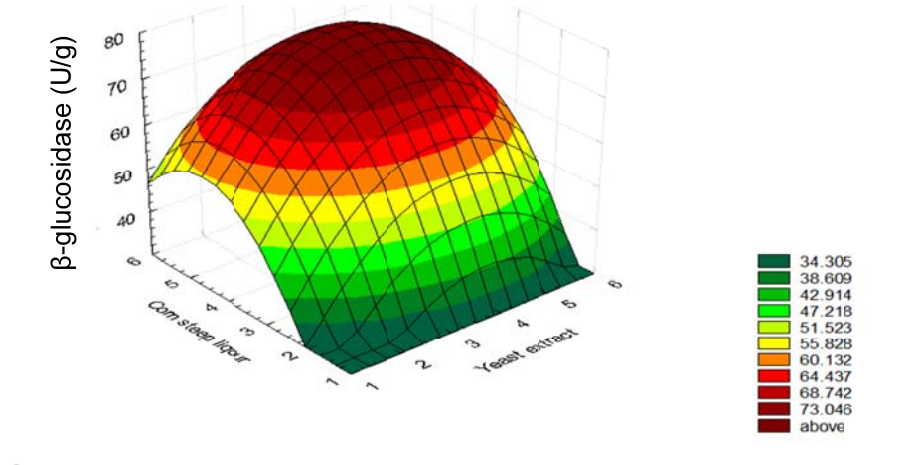
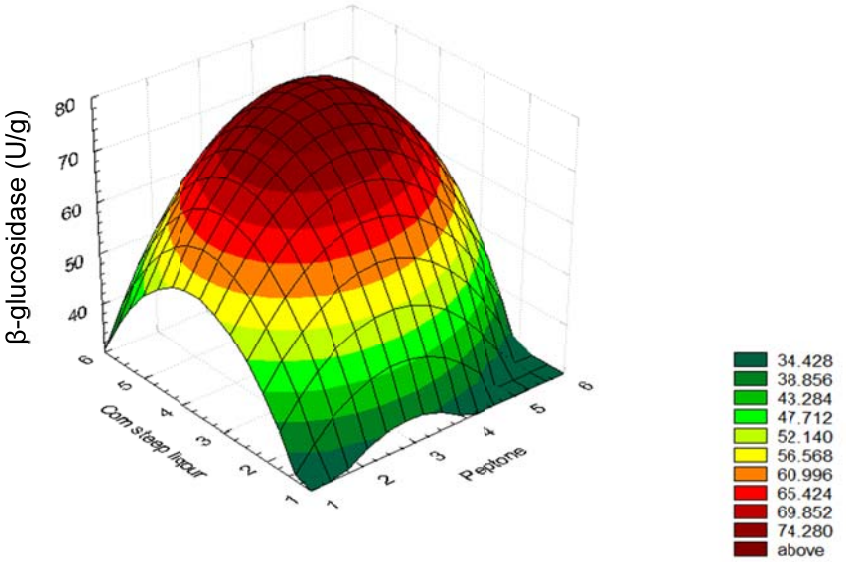


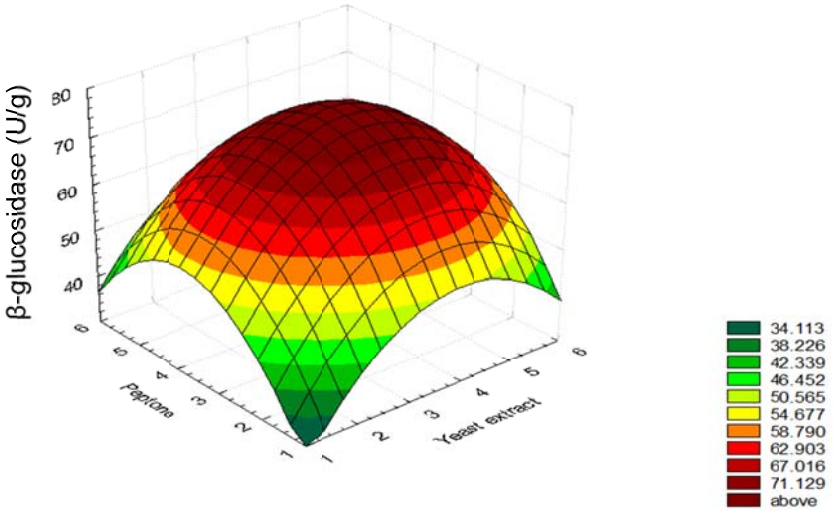
Figure 3a. Response surface graphs illustrating the effect of yeast extract and corn step liquor; 3b**.** Peptone and corn step liquor; **3c** Yeast extract and peptone on Fpase production.



a



b



c

Figure 4a. Response surface graphs illustrating the effect of yeast extract and corn step liquor; 4b**.** Peptone and corn step liquor; **4c.** Yeast extract and peptone on β-glucosidase production.

Table 4. Multi-response optimization of CMCase, FPase and β-glucosidase production of *A. niger* MA1 under SSF of RH after 42 h of fermentation

Yeast extract (mg/gm)	Peptone (mg/g)	Corn steep (µl/gm)	CMCase (U/g)		FPase (U/g)		β-glucosidase (U/g)		*Desirability
			Response	Predicted	Response	Predicted	Response	Predicted	
2.06	3.68	47.87	16.41	17.73	4.28	4.80	78.60	76.39	0.846

*Desirability function is used to test the possibility of optimizing more than one response at the same time, the closer to 1, the more accuracy of desirability function.

Table 5. Scaling up of cellulases production by *A. niger* MA1 after 42 h of SSF.

RH (g)	Container	CMCase		FPase		β-glucosidase	
		u/g	Total	u/g	Total	u/g	Total
10	Glass flask (120 ml)	10.02	100	3.17	32	52.32	523
20	Petri dishes (20 cm)	16.70	334	3.02	60	55.90	1118
Tray							
100	(2494 cm^3)	8.20	820	2.70	270	47.60	4760
150	(3438 cm^3)	6.60	990	2.04	306	45.02	6753
200	(4582 cm^3)	6.20	1240	1.80	360	42.13	8426

Elution of *A. niger* MA1 cellulases by different eluents

After scaling up, elution process is very critical for this purpose. Many eluents were tested and as shown in Figure 5, maximum activity of CMCase wer released with Tap water + 0.25% Brej 35 which gave 16.87 U/g with 149.37% efficiency as well as FPase; 4.65 U/g with 184.40% efficiency, for β-glucosidase Brej 35 was also more efficient but this time with saline solution.

Characterization of CMCase, FPase and β-glucosidase of *A. niger* MA1

Temperature of incubation

In different incubation temperature from 20 to 60°C, cellulases activity was increased with increase in temperature with maximum activity of CMCase, FPase and β-glucosidase at 50°C which recorded 16.01, 4.90 and 62.94 U/g respectively, while further increase in temperature showed decrease in cellulases activity.

Thermal stability

CMCase enzyme showed good thermal stability in temperatures below 70°C as 81.69, 74.65 and 73.71% of the activity remained after 12, 18 and 24 h respectively at 50°C and 68.54 and 58.22% of the activity remained after 12 and 18 h respectively at 60°C with nearly half life time reaching to 24 h at 60°C; similar results were obtained for FPase activity where β-glucosidase is more resistance to thermal denaturation as its activity was 93.3% after 12 h of exposure to 50°C and retain 60% of its activity after 24 h of exposure to 70°C and reached 32.31% of its activity after 6 h at 80°C, where it denatured and lost all of its activity after 24 h of exposure to 90°C.

Effect of pH

CMCase have high activity in a pH range from 4.6 to 6; the highest CMCase produced by *A. niger* MA1 was recorded at pH 5, where FPase and β-glucosidase showed high activity at pH range from 4.6 to 5.6 with optimum at 4.8.

pH stability

CMCase have a high pH stability, it retained 99.00, 96.25, 95.25, 99.00, 97.25, 98.25 and 95.75% of (3.6 to 5.6) respectively and showed 95.75 and

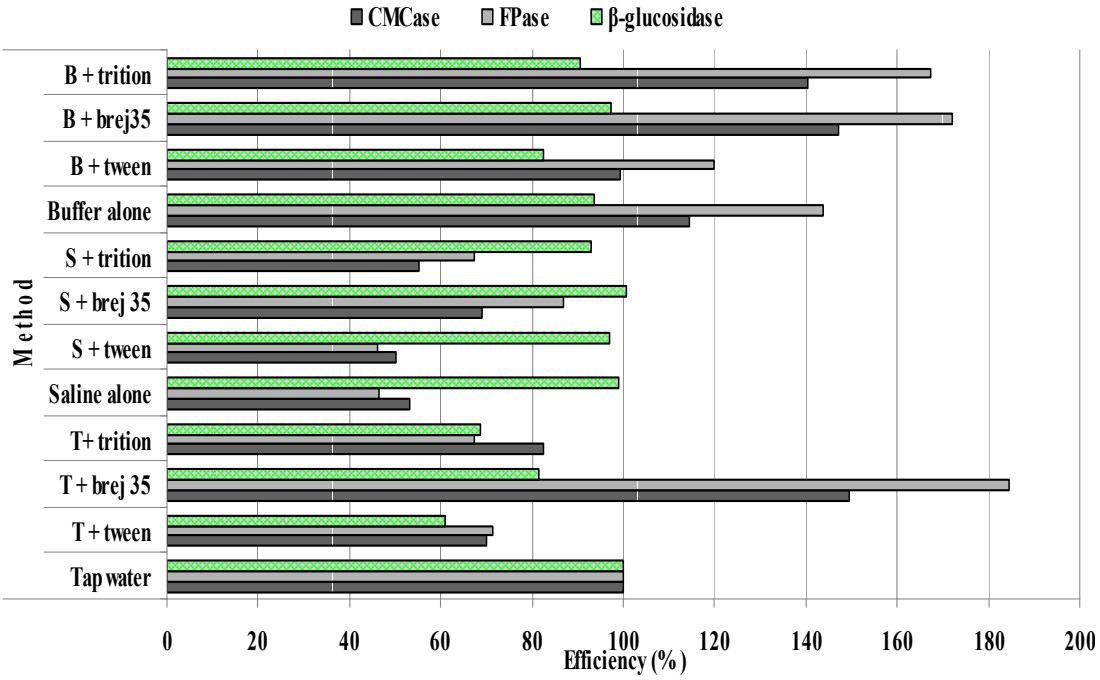


Figure 5. Effect of different eluent solutions in *A. niger* MA1 cellulases recovery from SSF of RH.

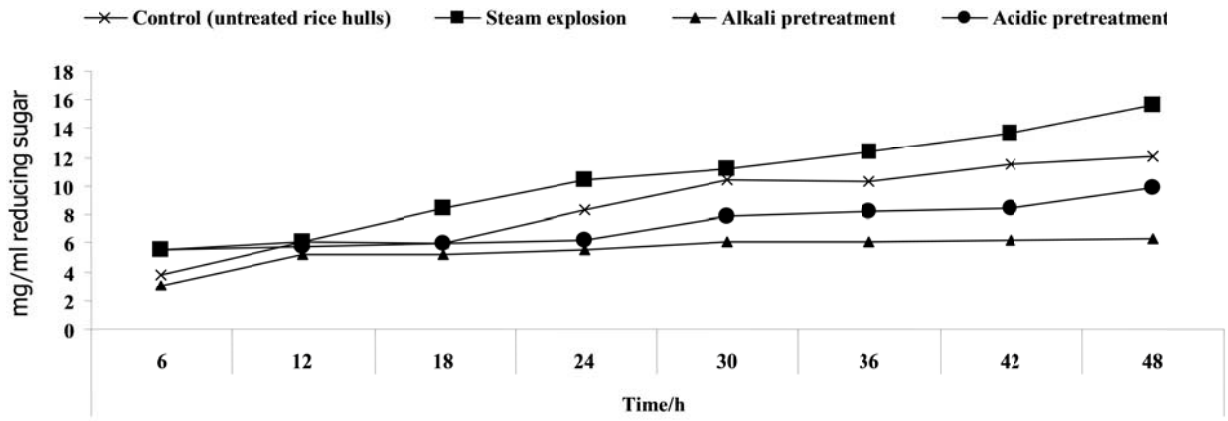


Figure 6. Effect of different treatment of RH in its saccarification by *A. niger* MA1 cellulases.

95.00% activity after 24 h at 3.6 and 4 pH values respectively. FPase was stable in pH range 3.6-5.6 with high stability at pH 4.8 and the enzyme lost only 7% of its activity after 24 h at pH 5. β-glucosidase was more stable, as it lost only 0.62% in activity after 24 h in pH value of 4.8. In more acidic pH of 3.6, 4 and 5.6 the enzyme lost only 3.4, 2.8 and 3.1% of its activity respectively after 24 h of incubation.

Hydrolysis of RH by *A. niger* MA1 cellulases

In enzymatic hydrolysis process cellulase enzymes of *A. niger* MA1 containing 2, 0.42 and 8.6 U/ml of CMCase, FPase and β-glucosidase respectively were used to convert cellulose of RH into sugars which then fermented into ethanol.

As shown in Figure 6, untreated, steam explosion, alkaline and acid treated RH were used in hydrolysis trials. Generally the liberated reducing sugar is produced with different values according to type of pretreatment of RH and increases gradually till its maximum activity after 48 h. It is clear that, steam explosion pretreatment is the most efficient method as it recorded 16.36 mg/ml of reducing sugars after 48 h of enzymatic hydrolysis, followed by untreated RH which release 12.10 mg/ml.

Table 6. Bioconversion of enzymatically hydrolyzed RH sugars to bio-ethanol by *Saccharomyces cerevisae*.

Analyses (%)	Steam explosion
Total reducing sugars (g/L)	16.36 (±0.03)
Ethanol content (g/L)	9.42 (±0.08)
Fermentation efficiency (%)	89.93 (±4.9)
Remaining reducing sugars (g/L)	0.40 (±0.03)

Contrarily, amount of reducing sugar are lower in alkali and acidic pretreatment of RH which recorded 6.33 and 9.90 mg/ml.

Bioconversion of enzymatically hydrolyzed RH sugars into bioethanol

As shown in Table 6, the resultant yield of total sugars after enzymatic saccharification of steam exploited RH using cellulases enzymes of *A. niger* MA1 to final concentration of 16.36 mg/ml; this amount of sugar was fermented by *S. cerevisae* for 24 h to 9.42 g/L ethanol.

DISCUSSION

In fungal bioconversion, the selection of active isolate is essential. In this work the RH is the target of hydrolysis and in the same time the source of fungal isolates; by this strategy the selected *A. niger* MA1 is more adapted to produce cellulases upon RH and showed maximum activity after only 36 h of incubation. This relatively short incubation time is shorter than 3 to 8 days reported as optimum incubation period of cellulolytic enzymes during SSF of lignocellulosic residues (Abo-State et al., 2010) and shorter than 80 h recorded by *A. niger* in production of cellulases from potato peel (Santos et al., 2012) also shorter than 96 h recorded by *A. niger* in saw dust (Acharya et al., 2008) and in rice straw (Pericin et al., 2008) whereas the results obtained by Panagiotou et al. (2003) and Narasmish et al. (2006) are in agreement with our results. Generally the optimum incubation time of cellulase production depends on the substrate and the tested fungus (Alam et al., 2005).

Solid state fermentation (SSF) is an attractive and economic process to produce cellulases due to lower capital investment, lower operating expenses (Yang et al., 2004), ease of use, superior productivity, use of simpler fermentation media, reduced production of waste water and easier control of bacterial contamination (Pandey et al., 1994). Among the advantages of SSF, it is often cited that enzyme titer is higher in SSF than in submerged fermentation (SMF) (Gonzalez et al., 2002). In this study using RH as a substrate for cellulases production is based on reduction in the production cost which is a critical target in cellulase research. *A. niger* MA1 cellulases productivity using RH as a sole carbon source is more than that produced by *Trichoderma* sp. FETL on RH or on mixture of sugar cane baggase and palm kernel cake via SSF (Pang and Ibrahim, 2006). More recently, it was also better than that produced by *A. fumigatus* in SSF of alkali treated rice straw (Sherief et al., 2010). Our result confirms the high β-glucosidase productivity of *A. niger* similar to records of Wen et al. (2005) and Kang et al. (2004).

In screening the factors affecting production of cellulases enzymes, it's very important to test as many factors as possible to identify the significances of each of them. Plackett- Burman designs offers good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component. Among fifteen independent variables, yeast extract, peptone and corn steep liquor have significant effect on production of *A. niger* MA1 cellulases which further optimized in BBD and revealed considerable increases in the three enzymes. In this connection Gao et al. (2008) and Ng et al. (2010) reported that the organic nitrogen sources favored cellulases production by *A. terreus* M11 and β-glucosidase production by *P. citrinum* YS40-5. On the contrary, Sasi et al. (2012) found that *A. flavus* showed the highest production of cellulase enzyme utilizing ammonium sulfate as nitrogen source than yeast extract. In scaling up trials, the cellulases enzyme production per gram decreases with increase in quantity of RH and size of tray, due to low aeration and heat transfer with increase in the depth of substrate (Gowthaman et al., 2001).

The effectiveness of elution is necessary to recovery of enzyme from the fermented biomass, to best of our Knowledge; Brej 35 is not used before in extraction of cellulases, the addition of this surfactant gives maximum elution of CMCase and FPase activity with tap water. Tap water is commonly available, save and low cost extraction and used by other workers (Ahmed, 2008). The eluted CMCase and FPase enzymes of *A. niger* MA1 in the present investigation, showed good thermal stability with half-life time reached to 24 hours at 60°C, similar to (CMCase and Aviclase) of *A. fumigatus* (Parry et al., 1983), *T. reesei* (Busto et al.,, 1996) and more than that of *A. oryzae* KBN616 (Kitamoto et al., 1996). β-glucosidase of *A. niger* MA1 is more resistant to thermal denaturation with activity 60% after 24 h of exposure to 70°C which is more than that repoted by *A. niger* 322 which lost most of its activity at temperature higher than 50°C (Peshin and Mathur, 1999). These data encourage the application of *A. niger* MA1 cellulases in saccarfication trials of RH. For enhancing this process, pretreatment of RH make the process more efficient. In the present study, amount of reducing sugar produced from cellulases treatment of steam exploited RH are

Table 7. Comparing the results of the present investigation of reducing sugar and ethanol content with the previous work on RH.

Type of pretreatment	Enzyme used	Amount of reducing sugars	Amount of ethanol (g/L)	Strain of fermentation	Fermentation time (h)	Reference
Steam explosion	Cellulases enzyme (mix of CMCase, FBase and β-glucosidase) of *A. niger* MA1	16.36 g/l 163 mg/g hulls	9.42	*Saccharomyces cerevisae*	24	This study
Diluted H_2SO_4 1% pretreatment	Commercial enzyme (cellulase, xylanase, esterase, beta-glucosidase and Tween20)	42.0±0.7 g/l (acid, enzyme, overliming)	9.1±0.7	(1) *E. coli* strain FBR5 simultaneous saccharification and fermentation with recombinant *E. coli* FBR5	39	Saha and Cotta, 2005
Alkaline H_2O_2 pretreatment	Commercial enzyme (cellulase, beta-glucosidase and xylanase)	428±12 mg/g	8.2±0.2 (1) 8.0±0.2 (2)	(1) *E. coli* strain FBR5 (2)recombinant *E. coli* FBR5	(1) 24 (2) 48	Saha and Cotta, 2007
Lime pretreatment	Commercial enzyme preparations(cellulose, beta-glucosidase and hemicellulase)	154±1 mg/g 19.8±0.6 g/l	9.8±0.5 11.0±0.1	1) *E. coli* strain FBR5 simultaneous saccharification and fermentation with recombinant *E. coli* FBR5 2)	(1)19 (2) 53	Saha and Cotta, 2008
Fungal pretreatment with *A. awamori* and Pleurotus	FPU	34 mg/g	8.5	*Saccharomyces cerevisae*	168	Patel et al., 2007

16.36 g/L (163 mg/g RH). This result (Table 7) is much more than 34 mg/g RH recorded by Patel et al., (2007) with fungal pretreatment by *A. awamori* and *Pleurotus sajorcaju* and application of FPase enzyme and closer to 154 mg/g RH which recovered after lime pretreatment and hydrolysis by commercial enzyme preparations (cellulose, β - glucosidase and hemicellulase) (Saha and Cotta., 2008). On the other hand it lower than 428±12 mg/g RH that obtained after pretreatment with alkaline H_2O_2 and application of commercial enzyme (cellulase, β-glucosidase and xylanase) (Saha and Cotta, 2007). With respect to variation in amount of reducing sugar produced pretreatment with steam explosion is preferred because it save method, have no chemical wastes that can be produced from acid and alkalia treatment.

In continual improvements in the yield of ethanol the amount of ethanol produced from steam explosion pretreated RH are 9.42 g/L higher and faster than that exhibited by Patel et al (2007) who produce (8.5 g/L) from fungal pretreatment of RH with *A. awamori* and *P. sajorcaju* and fermentation with *Saccharomyces cerevisae* after 168 h. other researchers who used commercial cellulases in scarification of RH like Saha et al., (2005) who obtained similar yield (9.1 g/L) by simultaneous saccharification and fermentation of H_2SO_4 treated RH using recombinant *E. coli* FBR5 after 39 h. as well as Saha and Cotta (2008) when used *E. coli* strain FBR5 in fermentation of lime pretreatment RH with simultaneous saccharification they obtained 9.8 and 11.0 g/L ethanol after 19 and 53 h, respectively.

Conclusion

Simple optimization of RH as medium for *A. niger* MA1 can be used in rapid production of considerable amount and mixture of cellulases which used in scarification of RH and subsequently fermented to bioethanol.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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