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Improved Sugar Yield for Bioethanol Production by Modelling Enzymatic Hydrolysis of *Peganum Harmala* Biomass through Response Surface Methodology

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ABSTRACT

Alternative lignocellulosic substrates to produce high value-added products such as biofuel have been attractive. A Box-Behnken design was used to evaluate the effects of three parameters namely L/S ratio (50–100 mL/g), cellulase concentration (10–60 U/g) and incubation time (4–44h), on the enzymatic hydrolysis yield of physically pretreated Peganum harmala leaves. The fitted mathematical model allowed us to plot response surfaces as well as isoresponse curves and to determine optimal saccharification conditions. Statistical results indicated that the hydrolysis time and the enzyme concentration were the main factors influencing the release of reducing sugars. The selected optimal saccharification conditions were: L/S ratio of 75.0 mL/g, enzyme concentration of 35.0 U/g, and reaction time of 44.0h. These conditions allowed 39.6% of enzymatic hydrolysis yield versus 37.8±2.9%, respectively for the predicted values. The saccharification efficiency using enzyme treated biomass under optimized conditions was about 20-fold higher than before optimization. Fermentation of optimized cellulosic hydrolysate containing 12.6% glucose was performed using Saccharomyces cerevisiae yielded 4.75% ethanol production within 48h. These results showed a promising future of applying Peganum harmala leaves as potential lignocellulosic biomass for second generation bioethanol production.

Keywords: *Biomass conversion, cellulosic bioethanol, Peganum harmala, enzymatic saccharification, statistical optimization.*

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I. INTRODUCTION

In the last few decades, the demand for alternative fuel sources is accelerated as world population continues to grow and the limited amount of fossil fuels begin to diminish [1]. Renewable technologies to supplement or replace liquid fossil fuels are still in their early developmental stages. Bioethanol is currently produced primarily from sugar and starch sourced from crops (first generation biomass) such as sugar cane, wheat and corn, which have a high concentration of sugar [2]. However, they have social issues associated with the exploitation of potential food or feed resources [1]. In contrast, second-generation lignocellulosic biomass as residues from wood or dedicated energy crops constitute an attractive alternative because there is no competition with food and animal feed production, and these materials are also cheaper than first-generation biomass [2]. These advantages make them one of the most promising technological approaches available for supplementing the current fuel source.

Current technology for conversion of lignocellulose to ethanol requires biomass pre-treatment, cellulose hydrolysis (saccharification), fermentation, separation, and effluent treatment [3]. The goal of any pretreatment process is to alter or to remove structural and compositional impediments hydrolysis in order to improve the rate of enzyme hydrolysis and increase yields of fermentable sugars from cellulose or hemicelluloses [4]. Enzymatic hydrolysis of cellulosic biomass has been considered as an environmentally friendly process that replaces harsh acid treatment. The saccharification is the most important step for maximum sugar yield, with enzyme, substrate loading, incubation time, pH, and temperature constituting important parameters for optimization of saccharification process [1]. Therefore, optimization of hydrolysis process is thus necessary to obtain high yield of monomeric sugars which can be fermented into ethanol. Response surface methodology (RSM) is an efficient experimental modelization technique dedicated to the determination of optimum conditions for a multifactor experimental design rather than optimizing by the conventional method which involves changing one

independent factor while keeping the others constant. These conventional methods are time-consuming and incapable of detecting the true optimum, due especially to the interactions among the factors [5]. The RSM has been successfully applied in optimization of the enzymatic saccharification conditions of various lignocellulosic biomasses for bioethanol production [1,6,7].

Peganum harmala L. (Fam. Zygophyllaceae) is a wild-growing flowering medicinal plant mainly distributed in North Africa, the Middle East, central Asia, South America, Mexico, and southern USA [8]. Phytochemical studies of *P. harmala* led to the isolation of different types of value-added biomolecules such as alkaloids, steroids, flavonoids, anthraquinones, amino acids, and polysaccharides. *P. harmala* seed extracts which contain 2.5-4 % of alkaloids mainly harmaline, harmine, harmalol, vasicine, are used for their pharmaceutical and therapeutic effects [8,9]. However, *P. harmala* leaves can't be used to feed livestock. Indeed, all domesticated animals are susceptible to poisoning from *P. harmala*, camels especially young animals are the most affected as reported by Mahmoudian et al. [10]. The present work focused on applying *Aspergillus niger* cellulolytic enzymes, to hydrolyse pretreated dried *P. harmala* leaves. The main objectives of this work were to better understand relationships between the enzymatic hydrolysis variables (liquid to solid ratio L/S, cellulase concentration, and incubation time) and the response (released reducing sugars); and to obtain the optimum saccharification conditions for bioethanol production using a three-level Box-Behnken design and the RSM. All the results obtained in this study would provide a sound basis for assessing the valorization of *P. harmala* biomass into biofuel.

II. MATERIALS AND METHODS

2.1 Biomass preparation

Aerial parts of *P. harmala* were collected from El-Kef region, northwest Tunisia in the month of August, 2014. Fresh plant leaves were air-dried, powered with a blender and stored in cellophane bags at room temperature until further use.

2.2 Enzyme preparation

Cellulase from *Aspergillus niger* (0.8 enzyme units/mg solid, Sigma C1184-25KU, Sigma-Aldrich, USA) was used for the hydrolysis of pretreated *P. harmala* biomass. One unit of enzyme activity was defined as the amount of enzymes which liberates 1µmol glucose from carboxymethylcellulose per minute at pH 5.0 and 37°C.

2.3 Physical pretreatment of P. harmala biomass

One gram of dry powered leaves were put into 250 ml Erlenmeyer flask and moistened with 0.05 M sodium acetate buffer (pH 5.0), then steam treated by autoclaving at 121˚C and 1.5 bars for 20 min [11]. After the autoclaving period, the flasks content were extracted, filtrated and reducing sugars were determined.

2.4 Enzymatic saccharification of pretreated substrate

Enzymatic hydrolysis of pretreated *P. harmala* biomass was carried out following the experimental design given by table 1 and 2. The experiments were performed in 250 ml Erlenmeyer flasks with 1.0 g of *P. harmala* pretreated biomass moistened with the required volume of buffer (sodium acetate buffer 0.05 M, pH 5) containing various enzymes doses. This was supplemented with 0.01% sodium azide to prevent microorganism contamination. The reaction mixtures in flasks were incubated in orbital shaker for 4 to 44h at 37°C at 100 rpm. After regular time intervals, samples were taken from each flask and kept in boiling water to inactivate the enzyme. Each sample was filtered on a whatman filter paper and subsequently analyzed for reducing sugars.

Table 1 Experimental domain of the Box–Behnken design

Variable	Factor	Unit	Center	Step of variation
X_1	L/S ratio	mL/g	75.0	25.0
X_2	Enzyme conc.	U/g	35.0	25.0
X_3	Time	h	24.0	20.0

Table 2 Conditions of the Box-Behnken design in coded and natural variables and the corresponding experimental responses

N°Exp	X1	X2	X3	L/S ratio (ml/g)	Enzyme (U/g)	Time (h)	Reducing sugars in enzymatic hydrolyzate (mg/l)	Experimental enzymatic hydrolysis yield (%)
1	-1.00	-1.00	0.00	50.00	10.00	24.00	104	2.08
2	1.00	-1.00	0.00	100.00	10.00	24.00	158	1.58
3	-1.00	1.00	0.00	50.00	60.00	24.00	370	7.40
4	1.00	1.00	0.00	100.00	60.00	24.00	520	5.20
5	-1.00	0.00	-1.00	50.00	35.00	4.00	400	2.00
6	1.00	0.00	-1.00	100.00	35.00	4.00	520	5.20
7	-1.00	0.00	1.00	50.00	35.00	44.00	3620	18.10
8	1.00	0.00	1.00	100.00	35.00	44.00	1800	18.00
9	0.00	-1.00	-1.00	75.00	10.00	4.00	500	6.66
10	0.00	1.00	-1.00	75.00	60.00	4.00	1340	17.86
11	0.00	-1.00	1.00	75.00	10.00	44.00	1520	20.26
12	0.00	1.00	1.00	75.00	60.00	44.00	3050	40.66
13	0.00	0.00	0.00	75.00	35.00	24.00	2130	28.39
14	0.00	0.00	0.00	75.00	35.00	24.00	2710	36.12
15	0.00	0.00	0.00	75.00	35.00	24.00	2100	27.99
16	0.00	0.00	0.00	75.00	35.00	24.00	2760	36.79
17	0.00	0.00	0.00	75.00	35.00	24.00	1980	26.39

2.4.1. Analysis of reducing sugars

Reducing sugars produced after pretreatment and during hydrolysis of *P. harmala* biomass were determined by using the dinitrosalicylic acid method (DNS) [12]. The samples were analyzed using a spectrophotometer (Shimadzu, Columbia, MD, USA) at 540 nm. The absorbance readings were then converted into equivalent sugar concentration (mg/mL) using a standard glucose solution curve. Sugar yield was calculated on *P. harmala* biomass, using the following equation [13]:
Sugar Yield (%) = 100 (sugar produced during hydrolysis/gram of *P. harmala* biomass).

2.4.2. Experimental design and statistical analysis

In this work, a Box-Behnken design was set up to look for the best experimental conditions of three independent factors affecting the efficiency of the saccharification of *P. harmala* biomass namely: L/S ratio, enzyme concentration and hydrolysis time (Tables 1 and 2). The relationship between the response and the three quantitative variables was approximated by the following second order polynomial function:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Where: Y represents the measured response (Sugar yield); β_0, β_j, β_{jk} and β_{jj} are model coefficients.
The three-variable Box-Behnken design with 17 experiments was used to estimate the model coefficients. The experimental points are located in the middle of a cube ridges (12 experiments: runs n° 1 to 12) and at the center of the cube (5 experiments: runs n° 13 to 17) (Table 2). The five replicates at the center point are carried out in order to estimate the pure error variance. The significance of the fitted model was tested using the analysis of variance (ANOVA). The fitted model was used to study the relative sensitivity of the response to the variables in the whole domain and to look for the optimal experimental conditions. The relationship between the response and the experimental variables was illustrated graphically by plotting the isoresponse curves and the response surfaces [5]. In this study, regression analysis, estimation of the coefficient, generation and data treatments of the Box-Behnken design were performed using the experimental design software NemrodW [14].

2.4.3. Fermentation of cellulosic hydrolysate

Fermentation of cellulosic hydrolysate was conducted as described by Kuttiraja et al. [15] with slight modifications. A nutrient supplement was added to the hydrolysate so that it contained finally 0.3% Yeast extract, 0.025 M $(NH_4)_2SO_4$, 0.01 M $MnCl_2$, 0.01 M $MgSO_4$, 0.05 M K_2HPO_4 and 0.05 M NaH_2PO_4. 100mL of the optimized hydrolysate in 250 mL Erlenmeyer flasks was inoculated with 5% v/v of a 12 h old seed culture of *Saccharomyces cerevisiae*. Incubation was carried out at 30°C without agitation for 48 h. Fermentation broth was centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was then analyzed for glucose and ethanol

content using Glucose HK Assay Kit purchased form Sigma Aldrich [16] and ethanol FS kit marketed by Diagnostic System International, respectively [17].

III. RESULTS AND DISCUSSION

Various woody and herbaceous materials like Agave [18], Cassava [19], *Populus nigra*, *Eucalyptus globules* and *Brassica carinata* [20], have been exploited as lignocellulosic biomasses for biofuel purposes. The recalcitrant nature of these raw materials and the high cost of enzymes for saccharification are the major bottlenecks in commercial production of bioethanol from lignocellulosic biomasses [1]. Therefore, it is imperative to optimize the treatment conditions along with enzymatic hydrolysis variables in order to achieve maximum saccharification efficiency. In this study, an attempt was made to optimize enzymatic hydrolysis conditions of physically pretreated *P. harmala* biomass using RSM.

3.1 Development of a model for enzymatic saccharification

The experimental saccharification conditions of pretreated *P. harmala* biomass, shown in Table 2, were arranged according to the three variable Box-Behnken design. The observed values of sugar yields were used to compute the model coefficients using the least square method [21,22]. The overall second-order polynomial equation describing the relationship between the variables and the sugar yield from enzymatic hydrolysis of pretreated *P. harmala* biomass in terms of coded values is of the form:

$$\hat{y} = 31.13 + 0.05\ X_1 + 5.07\ X_2 + 8.16\ X_3 - 18.80\ X_1^2 - 8.27\ X_2^2 - 1.51\ X_3^2 - 0.42\ X_1X_2 - 0.82\ X_1X_3 + 2.30\ X_2X_3$$

where the coded variables were $\hat{y}$: enzymatic hydrolysis yield (%); X_1: L/S ratio, X_2: enzyme concentration and X_3: hydrolysis time

3.2 Statistical analysis and predictability of the model

The analysis of variance for the fitted model (Table 3) showed that the regression sum of squares was statistically significant at the level 99.9% and the lack of fit is not significant. Thus, it can be concluded that the model represents well the measured data. The *P* values for model terms X_2, X_3, X_1^2, X_2^2 were less than 0.05 indicating that they were the significant variables influencing the sugar yield (%) response than the others (data not shown). The R^2 value (0.94) was in good agreement with the adjusted R^2 value (0.86) and well adapted to the response. From the R^2 value, it was concluded that only 6% of the variation for response could not be explained by the model. High *F* and R^2 values and low P value for hydrolysis yield indicated the model predictability.

Table 3 Analysis of variance

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio	Significance
Regression	2652.00	9	294.66	11.9789	**
Residuals	172.19	7	24.60		
Validity	75.42	3	25.14	1.0392	N.S.
Error	96.77	4	24.19		
Total	2824.19	16			

**Significant at the level 99.0% N.S.: non significant

3.3 Interpretation of the response surface model

The interaction effect of the process parameters on the total yield of reducing sugar for enzymatic saccharification of *P. harmala* biomass can be illustrated graphically by plotting three-dimensional response surface plots and the two dimensional isoresponse curves (Figs. 1-3). In these plots, the factor not represented by the two axes was fixed at its center level. Such plots are helpful in studying the effects of the variation of the factors and consequently, in determining the optimal experimental conditions [22,23]. Figure 1 presents the sugar yield as a function of cellulase concentration and L/S ratio. It is observed from the figure, the yield of reducing sugar increases as one move from the lower to the middle levels of the factors and then there is a reduction in the yield. Either too low or too high factor levels are not appropriate for enzymatic hydrolysis. These findings are in line with the observations of Maurya et al. [6] who reported a decrease in the efficiency of enzymatic hydrolysis at low levels of biomass loading and enzyme concentration and that middle levels of these two factors showed maximum reducing sugar yield. Figure 2 shows the effect of the interaction between L/S ratio and hydrolysis time on total sugar concentration. It can be observed that for all the values of L/S ratio, the concentration of total reducing sugars obtained generally increased with treatment time. High hydrolysis yields 30-40% can be achieved when using medium L/S ratios (56-85 mL/gds) and relatively high incubation times (>

30h). The effect of hydrolysis time and cellulase concentration on the sugar yield is presented in Fig. 3. The contour and surfaces plots support the important role of incubation time. Indeed, the sugars yield increases two folds when the hydrolysis time increases from its low level (4h) to its high level (44h). Also, it was evident from Fig. 3 that the sugar yield increased with the increase in enzyme concentration from 10 to 35 U/gds. This could be attributed to the fact that as the concentration of enzyme was increased; the more freely it was available for reaction with lignocellulosic substrate [23,24]. However further increase in the cellulose concentration beyond 35 U/g has negative effect on the sugar yield. As reported by Singh et al. [25] and Zheng et al. [26], high cellulase doses could reduce the absorption efficiency of the enzyme on cellulose due to high viscosity that could also contribute to a lower sugar yield. However, minimizing enzyme consumption is an important way to reduce the process cost [27].

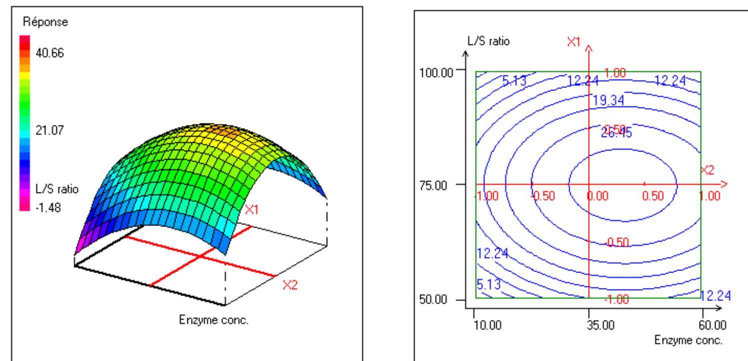

Fig. 1 Three-dimensional response surface and contour plots for the effect of cellulase concentration and L/S ratio at constant incubation time (24h) on the enzymatic hydrolysis yield.

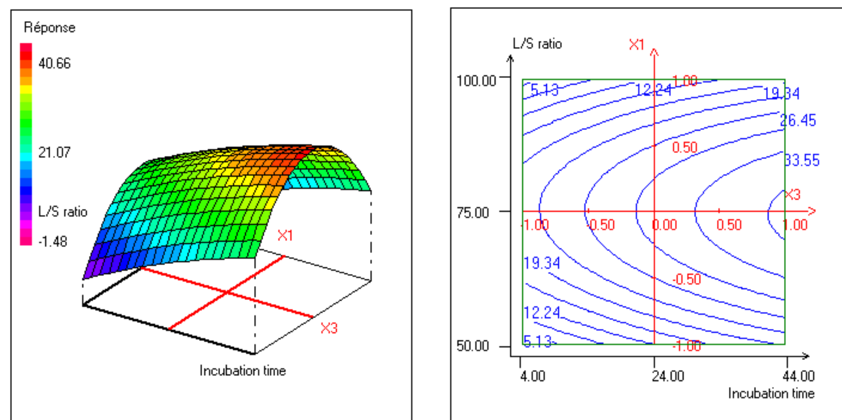

Fig. 2 Three-dimensional response surface and contour plots for the effect of L/S ratio and incubation time at constant cellulase concentration (35U/g) on the enzymatic hydrolysis yield.

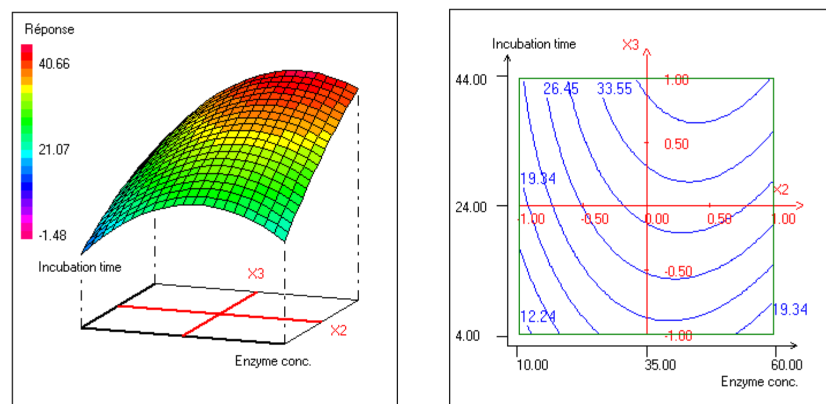

Fig. 3 Three-dimensional response surface and contour plots for the effect of cellulase concentration and incubation time at constant L/S ratio (75ml/g) on the enzymatic hydrolysis yield.

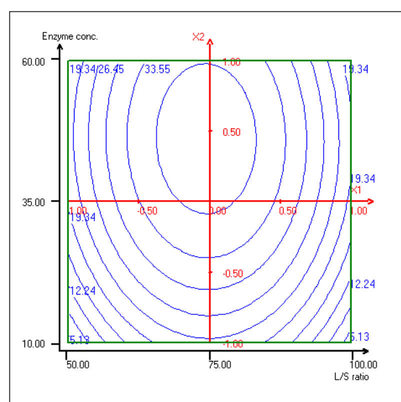

Fig. 4 Contour plots for the effect of L/S ratio and cellulase concentration at constant incubation time (44h) on the enzymatic hydrolysis yield.

3.4 Optimization of saccharification conditions for ethanolic fermentation

Optimization of enzymatic hydrolysis treatment of pretreated *P. harmala* biomass was carried out numerically by using NemrodW software. As the sugar yield can be maximized when using an incubation time in the range of 10 h–25 h (Figs 2 and 3), we fixed the incubation time at its high level (44.0h), and we ploted enzyme concentration versus redox L/S ratio (Fig. 4) to look for the highest sugar yield. The optimal conditions selected were: L/S ratio 75.0 mL/g, enzyme concentration 35.0 U/g, and reaction time 44.0h. Under these conditions, the expected value of the saccharification yield was $\hat{y}_{op}$ = 37.8% ± 2.9. To validate the predicted saccharification yield, an experiment was conducted with the mentioned optimum conditions of each variable as developed by the model. The optimal experimental sugar yield response for pretreated *P. harmala* biomass was 39.6% and it was in good agreement with predicated value. The saccharification efficiency using enzyme treated biomass under optimized conditions (396 mg/gds, 39.6%) was about 20-fold higher than the yield (1.76 mg/gds, 1.7%) obtained before optimization (pretreated biomass). These results revealed the superiority of *P. harmala* biomass in yielding high amount of sugars under enzymatic optimized conditions in comparison to other weedy lignocellulosic biomasses. For examples, Lee et al. [28] when enzymatically hydrolyzed *Pinus densiflora* observed only 3.53 mg/g sugar release. Saccharification of pretreated rice straw and wheat straw by *Fomitopsis* sp. RCK2010 cellulase resulted in release of 157.160 and 214.044 mg/g of reducing sugar, respectively [29]. The maximal amounts of reducing sugars released from corn stover and rice straw were 0.678 and 0.502 g/gds, respectively as reported by Yu and Li [30]. Enzymatic hydrolysate containing 12.6% (w/v) glucose was fermented after supplementation with necessary nutrients for yeast growth. Fermentation was completed after 48h, where ethanol reached a maximal concentration of 4.75% with approximately complete depletion of glucose (data not shown). The alcohol production corresponded to 74% of the maximum theoretical yield.

IV. CONCLUSION

In the present study, the potential of *P. harmala* biomass as a source of fermentable sugars useful for bioethanol production was evaluated by estimating the sugar yields during enzymatic hydrolysis. Analyze of the effects of three factors namely, L/S ratio, enzyme concentration and incubation time on the enzymatic hydrolysis yield was conducted using three factors Box-Behnken design and response surface methodology. Sugar yield was mainly enhanced by hydrolysis time and enzyme concentration. Under the optimum saccharification conditions (L/S ratio 75 mL/g; enzyme concentration, 35 U/g and reaction time 44 h), the measured reducing sugar, glucose and ethanol yields were 37.8, 12.6 and 4.7%, respectively. Thus, it is concluded that *P. harmala* leaves can serve as potent biomass for bioethanol production due to its high sugar content and ample availability.

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