OPTIMIZATION OF MICROWAVE ASSISTED H$_2$SO$_4$ HYDROLYSIS OF COCOA POD SHELLS: COMPARISON BETWEEN RESPONSE SURFACE METHODOLOGY AND ARTIFICIAL NEURAL NETWORK AND PRODUCTION OF BIOETHANOL THEREOF

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ABSTRACT

To release reducing sugars from cocoa pod shell, sulphuric acid pre-treatment was adopted where the variables affecting H$_2$SO$_4$ pre-treatment i.e., cocoa pod shell weight, H$_2$SO$_4$ concentration, microwave irradiation time and power were screened using one factor at a time approach. The weight of cocoa pod shell, concentration of H$_2$SO$_4$ showed a significant effect on the pre-treatment process and the levels of these factors were further optimized by central composite design using response surface methodology. The optimized conditions were found to be 15.65g ofcocoa pod shell, 6% v/v H$_2$SO$_4$ at 8 min of irradiation, released maximum reducing sugar of 9.10 g/L. A second order model was generated and validated, which was found to be a good fit with $R^2$ value of 0.89. Artificial neural network modelling proved validation $R^2$ of 0.94 comparatively better than Response surface methodology $R^2$ of 0.89. The reducing sugars released after acid hydrolysis at optimized conditions were subjected to fermentation by Pichia stipitis to produce bioethanol. The bioethanol produced was 3.2g/L at 2% (v/v) inoculum concentration after 72h of fermentation.

Keywords: Cocoa pod shell, Acid hydrolysis, Optimization, Microwave

INTRODUCTION

Green house gas emission, global warming due to environmental pollution, depleting petroleum reserves and fluctuating oil prices has stimulated research in the field of renewable fuels as an alternative. Due to their low cost and abundance, lignocellulosic materials have attracted attention as renewable feedstocks for the production of biofuel as an alternative to petroleum fuel (Choi et al., 2015). Bioethanol production from lignocellulosic biomass is preferred because it does not compete with food sources such as starchy and sugary residues hence food versus fuel competition could be avoided (Farrel et al., 2006). Acid hydrolysis is an effective technology to breakdown polymerized cellulose with a partial removal of lignin and hemicellulose. It helps primarily, partial solubilisation of hemicellulose after pre-treatment and in specific increases the digestibility of cellulose (Hu et al., 2008). Diluted or concentrated acid treatment can be used to hydrolyze the lignocellulosic biomass to sugars (Wyman and Charles 1994). With the advent of commercial microwave oven, since 1970s, microwave irradiation has been used for pre-treatment of lignocellulosic feedstock as a non-conventional way of heating (Liet et al., 2016). Microwave-assisted pre-treatment process can be an alternative to accelerate the acid hydrolysis of carbohydrate polymers. Microwave heating can offer energy saving up to 85-folds, when compared to conventional heating by shortening reaction time and reduce chemical consumption (Yemisi and Mazza 2012, Yoshida et al., 2010). A number of undesired compounds are formed during the acid hydrolysis process, such as sugar derived furans and lignin derived phenolics. These compounds will hinder the fermentation process. Production of such compounds can be minimized adopting appropriate sets of pre-treatment conditions. Hence, detail study of parameters is essential prior to designing of the acid hydrolysis process of the lignocellulosic material. Statistical tools and design of experiments provide more information about the optimization of conditions in reduced trials. They provide efficient and systematic plan for lignocellulosic biomass hydrolysis considering the interactive effects among the significant control factors (Scordiaiet al., 2010). Most of the control factors can be simultaneously studied and optimized by statistical experimental designs. Thus, in the present study, cocoa pod shells (CPS), a lignocellulosic residue of horticultural origin, hitherto being underutilized as a source for bioethanol production, has been investigated because they are found to contain carbohydrates (Samah et al., 2011). Since microwave-assisted hydrolysis involves many variables that affect the process, a statistically designed experimental protocol such as, response surface methodology (RSM) could be used, which offers advantages like reduction in time and number of experiments. RSM demonstrates a relationship between variables and responses over a relatively broad factor domain, which is practical in determining the optimum conditions. Design expert and MATLAB tools were used in the study. In the current investigation, H$_2$SO$_4$ hydrolysis of CPS was carried out to release reducing sugars. The initial screening of factors that affect the process was carried by using one factor at a time (OFAT) approach, which was later optimised by RSM to obtain maximum reducing sugars. To compare the results obtained from RSM, artificial neural network (ANN) approach was also adopted for modelling the response of acid hydrolysis. The maximum sugars released at optimized conditions were subjected to fermentation by Pichia stipitis to produce bioethanol.

MATERIAL AND METHODS

Chemicals and raw material

Glucose, H$_2$SO$_4$, NaOH, 3, 5- dinitrosalicylic acid, yeast extract, malt extract, peptone, ammonium sulphate, di-potassium hydrogen phosphate, manganese sulphate, magnesium sulphate and 5-(hydroxymethyl)furfural were purchased from Sigma Aldrich. CPS was collected from Peruvai village of Vittla taluk situated in Dakshina Kannada district, Karnataka, India.

Processing of CPS

CPS was sun dried and kept in hot air oven at 90°C to remove the moisture content. To reduce the size of CPS, milling technique was adopted. Sieving was
further carried out using Taylor number 10 mesh and the powder was stored in closed containers under refrigerator to prevent any microbial growth.

EXPERIMENTAL DESIGNS AND OPTIMISATION STRATEGY

One factor at a time approach

In order to optimize conditions for the release of reducing sugars to facilitate the fermentation process, pre-treatment of CPS was carried out by microwave assisted (MA) acid hydrolysis. To identify significant parameters for pre-treatment of CPS, one factor at a time (OFAT) analysis was carried out. The conventional OFAT approach was used to select the significant physical parameters and the initial test range of the four variables i.e. H$_2$SO$_4$ concentration (X$_1$, % v/v), weight of CPS (X$_2$, % w/v), irradiation time (X$_3$, min) and microwave power (X$_4$, W) for both the pre-treatment processes. Significant physical parameters and the levels selected from OFAT approach for pre-treatment process are given in Table 1. All the experiments were conducted in 250 mL conical flask with working volume of 100 mL in triplicates. Each flask of MA acid hydrolyzed samples was neutralized using sodium hydroxide and pH of all the samples were adjusted to around 7.0. Released fermentable sugar was quantified by DNSA method (Miller 1959). The parameter levels at which maximum reducing sugars were released, were chosen as the centre point values to enhance the pre-treatment process by RSM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Notation</th>
<th>Test range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of H$_2$SO$_4$ (% v/v)</td>
<td>X$_1$</td>
<td>2-10</td>
</tr>
<tr>
<td>Weight of CPS (% w/v)</td>
<td>X$_2$</td>
<td>2-16</td>
</tr>
<tr>
<td>Irradiation time (min)</td>
<td>X$_3$</td>
<td>1-10</td>
</tr>
<tr>
<td>Microwave power (W)</td>
<td>X$_4$</td>
<td>300-600</td>
</tr>
</tbody>
</table>

Central composite design

Two experimental factors: Concentration of H$_2$SO$_4$ (X$_1$, % v/v) and weight of CPS (X$_2$, % w/v), were chosen for RSM optimization based on OFAT results. These factors showed significant effect on the pre-treatment process during OFAT studies and their levels were optimized for maximum release of reducing sugars from CPS using central composite design (CCD). The concentration of sugars released by MA acid pre-treatment was determined as the response (Y) and was designated as Y$_r$ for MA acid hydrolysis. Based on significant two factors, the CCD consisted of five levels (Table 2) and 12 experimental runs (Table 3). The experiments were conducted in random order and tabulated in standard order. The levels of other non-significant factors were kept constant at the tested centre values during the OFAT studies. These optimization experiments were designed by using Design expert (version 9). The data of pre-treatment studies obtained was subjected to analysis of variance (ANOVA) using the same software. Polynomial model of second order was utilized to obtain the mathematical response between the response and the independent variables

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_i X_i^2 + \sum \beta_{ij} X_i X_j$$

(1)

Where, Y is the dependent response, the $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are estimates of polynomial coefficients and $X_i$, $X_j$ represent independent variables. The optimization was carried out with 100 mL acid solution taken in 250 mL conical flasks as shown in Table 3. The microwave power of 300 W, irradiation time of 5min derived from OFAT studies were kept constant for all the experimental runs. Released reducing sugars (RRS) from MA hydrolysis were estimated by DNSA method.

Table 2

<table>
<thead>
<tr>
<th>Factors</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of acid (%)</td>
<td>X$_1$</td>
</tr>
<tr>
<td>Weight of CPS (%w/v)</td>
<td>X$_2$</td>
</tr>
</tbody>
</table>

Run No. X$_1$ X$_2$ Y$_1$

1. 2 2 6 5.837
2. 2 14 7.712
3. 10 6 6.819
4. 10 14 8.577
5. 0.34 10 6.264
6. 1.66 10 4.906
7. 6 4.34 6.502
8. 6 15.66 9.575
9. 6 10 8.279
10. 6 10 8.301
11. 6 10 8.203
12. 6 10 8.399

Validation of the second order polynomial model

The second order polynomial model obtained from RSM was validated by conducting a series of experiments generated by choosing random values of parameters within the optimized levels. Also, experiments were conducted at the optimized conditions generated by the software. The experimental output was then compared with the predicted values by the second order model obtained from CCD, to estimate the goodness of fit of the model.

Artificial neural network

Artificial Neural Network is a computational framework consisting of neurons and connections. Neural network has an input and output layer. There are one or more layers between them named hidden layers. Highly interlinked bundles of elements called neurons are present in the ANN architecture (Sarkar et al., 2009). Defined transfer and a summing function are controlled by neurons. Neurons are simple processing units that are grouped into layers and connected by weighted relations. The most commonly used transfer functions are: tan sig, purel and log sig (Hagan et al., 1996).

The function of the input layer is to present the scaled input data to the hidden layer through weights. The hidden layer then sums up the weighted inputs along with the biases as:

$$Sum = \sum_{i=1}^{n} X_i W_i + \Theta$$

(2)

where, $W_i$ (i=1,n) represents the weights of the connection between the neurons of the input and the hidden layer, $X_i$ signifies the input parameter and $\Theta$ is defined as the bias. To a non-linear domain, an activation function is used to transfer the weighted output. The data set formed after hidden layer operation was considered as the input for the output layer. The final predicted response by the ANN model was generated in the output layer. This study makes use of Multi Layered Perceptron (MLP) Neural Network (Das et al., 2015).

In the current study, the model is developed using neural network tool box in MATLAB 2014b. Levenberg-Marquardt (trainlm) training algorithm has been used. Model was developed (Fig.1) by considering two input variables namely concentration of H$_2$SO$_4$ (%v/v) and weight of CPS (%w/v). The tan sigmoid transfer function has been used in the hidden layer and the hidden neurons have been varied between 5 -15, in order to achieve the highest $R^2$. Model was trained using data set as shown in Table 3. In this study, the mean squared error (MSE) has been set at 0.001 as the desired goal and the network is trained until maximum $R^2$ is achieved. Maximum number of epoch of 1000 and the learning rate of 0.01 has been set. 12 experimental data have been considered as training data and 5 validation data have been considered as test data

Estimation of RRS by DNSA method

The MA acid hydrolysates were neutralized to pH 7.0 using NaOH. The expected products of hydrolysis are glucose, galactose, cellobiose and minor saccharides as xylulose and arabinose. All of these saccharides are reducing sugars (Brunneret et al., 2014). Therefore, the concentration of RRS was estimated by UV Visible spectrophotometer at 540 nm using 3, 5-dinitrosalicylic acid (DNS) reagent (Miller 1959).

Fermentation and estimation of bioethanol

Freeze dried culture of Pichia stipitis (NCIM 3498) was obtained from National Collection of Industrial Microorganisms (NCIM) Pune, India, were maintained in a medium of the following composition (g/L): glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5; pH 6.5. Stock culture of Pichia stipitis was prepared by growing it in liquid medium at 30°C. The inoculum was developed from stock culture by growing yeast cells in suspension culture for 24 h at 30±0.2°C and 100 rpm. After 24 hours, 2% v/v culture was transferred to side arm Erlenmeyer flask.
containing sterilised media. Optical density was measured at 600nm for the interval of 30min to determine the growth kinetics. The fermentation media was developed from the CPS hydrolysate derived from the MA pre-treatment at optimized conditions were supplemented with 1 g/L of ammonium sulphate, disodium hydrogen phosphate; 0.5g/L of manganese sulphate and magnesium sulphate. CPS hydrolysate was neutralized to pH 7 by using 5N NaOH solution, autoclaved and 2% (v/v) of Pichia stipitis was inoculated during the log phase. Fermentation was carried out at 30°C for 72 hours. Ethanol thus obtained was estimated using gas chromatography (Shimadzu GC) fitted with ZB-Wax column (Pasha et al., 2007). Injection port; column and FID detector temperature was set 180ºC, 150ºC and 160ºC respectively. Flow rate of carrier gas nitrogen was maintained at 2.5ml/min.

RESULTS AND DISCUSSION

OFAT analysis for MA acid pre-treatment for screening of significant parameters

Power of microwave was varied from 300W to 600W by keeping other factors constant (X2=5% (v/v); X3=5%w/v and X4=2min). The maximum reducing sugar concentration was found to be at 600W but the rate of evaporation was high. At 300W, the rate of evaporation was negligible. Thus reducing sugar obtained (X0) at 300W was considered as a significant value (Fig.2). Time was varied from 5 minutes to 10 minutes by keeping other factors fixed (X2=5% (v/v); X3=8min; X4=300W). Maximum reducing sugar of 4.35 mg/mL was released at 10g weight of CPS (Fig.4). Concentration of the acid was varied from 2% to 14% (v/v) by keeping other factors constant (X0=10%w/v; X1=8min; X2=300W). The released reducing sugar concentration of 5.13mg/mL was found to be maximum at 6% (Fig.5), hence, 6% acid and 10%w/v biomass were considered as the center points through OFAT analysis. These values were further used for central composite design (CCD).

Effect of hydroxymethyl furfural

Fermentation experiments were carried out using commercially available sugar. Three Erlenmeyer flask containing fermentation media with 9.1g/L of sugar was used. Hydroxymethyl furfural of 100µL and 500µL of was incorporated to separate fermentation media. Erlenmeyer flask without furfural was marked as control.2% (v/v) of Pichia stipitis was inoculated during the log phase and fermentation was carried out at 30±0.2°C for 72 hours. Ethanol generated during fermentation was estimated using GC.

Figure 1 ANN Model for released reducing sugars

Figure 2 Effect of microwave power on reducing sugar

Figure 3 Effect of irradiation time on reducing sugar

Figure 4 Effect of weight of CPS on reducing sugar

Figure 5 Effect of concentration of sulphuric acid on reducing sugar

Optimization of parameters by CCD for release of reducing sugars by microwave assisted acid hydrolysis

The influence of concentration of acid (X0) and weight of CPS (X2) to release reducing sugars was determined by CCD results, which are shown in Table 3. This table also presents the observed values for RRS concentration by acid hydrolysis (Y1) using different combinations of the independent parameters. The regression equation for release of reducing sugars by MA acid hydrolysis of CPS, as a function of the two independent variables (X0 and X2) and their linear and quadratic interactions is represented by the following:

\[ \text{Y}_1 = 2.828945 + 0.944391 \times \text{X}_0 - 0.07737 \times \text{X}_0^2 + 0.274215 \times \text{X}_2 - 0.0007 \times \text{X}_0 \times \text{X}_2^2 - 0.00183 \times \text{X}_0^2 \times \text{X}_2^2 \]

Table 4 indicates the values obtained by ANOVA for the release of reducing sugars on acid hydrolysis of CPS. Table 4 shows that the linear effects of the independent variables concentration of acid and the quadratic effect concentration of H2SO4 were significant. The second order models obtained were validated using a random set of experiments other than the experimental runs (Table 5). R² for the correlation between the observed and predicted RRS for MA acid hydrolysis was 0.89612. The observed values of RRS were compared with the RRS values as predicted by the second order model. These results indicate that there is excellent correlation between experimental and predicted values and in turn proves the validity of the model.
Table 4 ANOVA table for release of reducing sugar on MA acid hydrolysis of CPS

<table>
<thead>
<tr>
<th>X1 (L)</th>
<th>SS</th>
<th>Df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000675</td>
<td>0.000675</td>
<td>0.001837</td>
<td>0.9672</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.806941</td>
<td>9.806941</td>
<td>26.683131</td>
<td>0.002083</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.957812</td>
<td>7.957812</td>
<td>21.65195</td>
<td>0.003492</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.000792</td>
<td>0.000792</td>
<td>0.002155</td>
<td>0.964479</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.003422</td>
<td>0.003422</td>
<td>0.009311</td>
<td>0.926269</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2052</td>
<td>6</td>
<td>0.367533</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SS</td>
<td>20.34677</td>
<td>11</td>
<td>R²=0.89162</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5 Validation runs with observed and predicted values of RRS from CPS on microwave assisted pre-treatment with acid

<table>
<thead>
<tr>
<th>Run no.</th>
<th>X1</th>
<th>X2</th>
<th>Experimental yield (mg/mL)</th>
<th>Predicted yield (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>8</td>
<td>5.7936</td>
<td>7.69</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>10</td>
<td>6.4619</td>
<td>8.19</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>14</td>
<td>7.5918</td>
<td>9.45</td>
</tr>
<tr>
<td>4</td>
<td>9.5</td>
<td>12</td>
<td>6.5562</td>
<td>7.8</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>6</td>
<td>5.1755</td>
<td>7.26</td>
</tr>
</tbody>
</table>

The optimized levels of variables X1 and X2 for the maximum release of reducing sugars by both the hydrolysis were determined by desirability profiles (Fig 6). Based on the desirability plots (Fig 7), the microwave-assisted hydrolysis of CPS under optimized conditions (300W, 8min irradiation time, 6% v/v H₂SO₄, 15.657% w/w of weight of CPS) offered a maximum RRS of 9.10mg/mL. As reported by Awolu et al. (2015) 40g of cocoa bean shell treated with 0.5M of 160mL H₂SO₄ at 80°C for 150 min had released 45.06mg/g of reducing sugar. The cocoa pods were grinded to fine powder weighing 200g was hydrolyzed using 500mL of 1M HCl for 4 hours at 75°C to release reducing sugar of 135g/L (Mhajuika et al., 2015). Cocoa pod shells weighing 7.82g was treated with 0.58N Na₂CO₃ has released reducing of 0.94g/L (Shet et al., 2016). Previous report has depicted release of reducing sugar up to 1.51g/100g biomass using dilute H₂SO₄ treatment (Zhang et al., 2016). Saha et al. (2016) reported reducing sugar yield of 4.9g/L from wheat straw. RSM was found to be an efficient methodology for the determination of conditions leading to effective pre-treatment of CPS. This work demonstrated that the MA hydrolysis process is an effective method for the acid-catalyzed conversion of CPS to RRS with sugar yield of 0.58g/g of CPS. Time taken for hydrolysis is 8min; it is very less with comparison to the available literature.

Figure 6 Response surface plot showing effect of acid concentration and weight of CPS on the reducing sugar concentration released by acid hydrolysis of CPS

Figure 7 Profiles for predicted pretreatment process and the desirability levels for different parameters for optimum hydrolysis X₁(6%/ v/v H₂SO₄) and X₂(15.65%) of CPS.

ANN Modelling

Table 6 shows the results obtained through neural network toolbox. It can be seen that trainlm provided the best possible R² at 5 epochs for 6 hidden neurons and the error has been reached to 0.00163. The R² for training data is 0.99 whereas for test data it is 0.9607. ANN model developed for releasing reducing sugar shows better R² compared to RSM (Table 7).

Table 6 ANN Model for release of reducing sugars

<table>
<thead>
<tr>
<th>Training algorithm</th>
<th>No. of hidden neurons</th>
<th>Epochs reached</th>
<th>Error reached</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>trainlm</td>
<td>06</td>
<td>5</td>
<td>0.00163</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 7 Comparison of RSM with ANN

<table>
<thead>
<tr>
<th>RSM</th>
<th>ANN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trail Validation</td>
<td>Trail Validation</td>
</tr>
<tr>
<td>R²</td>
<td>0.89</td>
</tr>
<tr>
<td>0.99</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Production of bioethanol using batch fermentation

After the neutralisation of acid hydrolysat, 250mL flasks containing 100mL of sample was inoculated with 2% (v/v) inoculum of Pichia stipitis. Flasks were kept in 30°C rotary shaker maintained at 100rpm. Ethanol produced during fermentation was estimated to be 3.2g/L. The lower yield of ethanol may be due to the presence of inhibitory products which may be present in the hydrolysate. There are numerous reports which reveal that the presence of furfural or hydroxyl methyl furfural causing intense inhibitory effect on ethanol production. These substances act by inhibiting the major enzymes like hexokinase, phosphofructokinase and trios-phosphate dehydrogenase responsible for ethanol production in the yeasts (Palmqvist et al., 1996). The ethanol generated during the fermentation of cocoa pod shell hydrolysate was estimated to be 3.2g/L. The study determined the potential of ethanol yield to be 4.1g/L using fermentation media containing 9.1g/L of sugar and 100µL of hydroxymethyl furfural, whereas without the presence of hydroxymethyl furfural, the yield was observed to be 4.3g/L, respectively. However, the growth of Pichia stipitis was inhibited with 500µL of hydroxymethyl furfural. By comparing the ethanol yield as produced in the method of about 4.3g/L, 4.1g/L and 3.2 g/L in the present work, the studies concluded that due to the generation of furfurals the yield was lower during the fermentation of acid hydrolysate of cocoa pod shells. In the absence of furfural generation during sulphuric acid hydrolysis, the ethanol yield should have been 4.3g/L. From the current investigation, it is evident that furfural generated during the acid hydrolysis has inhibited the fermentation.
CONCLUSION

The primary focus was on enhanced release of fermentable sugars from CPS by H$_2$SO$_4$ pre-treatment process. The CPS was first pre-treated under acidic condition to release reducing sugars. The process parameters selected for the acid hydrolysis were optimised. Thus, it is concluded that acid pre-treatment is an effective method. ANN modelling proved R$^2$ comparatively better than RSM. The studies were designed to explore the feasibility of CPS as biomass for ethanol production. H$_2$SO$_4$ pre-treatment showed the maximum reducing sugar release of 9.10g/L. Further fermentation of CPS hydrolysate using P. stipitis NCIM3498 exhibited 3.2g/L of ethanol production.500µL of hydroxymethyl furfural was found to significantly inhibit the growth of Picha stipitis. The results clearly demonstrate that CPS is an economically and environmentally benign renewable source for the RRS generation as it is a cheap and abundantly available resource in Dakshina Kannada district, Karnataka, India.

REFERENCES


