Modelling the Effect of Cellulase Loading and Substrate Concentration on Enzymatic Saccharification of Indian Mango (*Mangifera indica*) Peelings for Bioethanol Production

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ABSTRACT

The main objective of the study was to develop a mathematical model to show the effect of cellulase loading and substrate concentration on the reducing sugar during enzymatic saccharification of Indian mango peelings for bioethanol production. This investigation also determined the effect of three pretreatment methods namely: dilute acid, dilute alkaline, and dilute alkaline peroxide pretreatment on the properties if powdered unripe Indian mango peelings. Likewise, the effect of the variation of two saccharification parameters - enzyme loading and substrate concentration on the reducing sugar - was sought.

The powdered dried unripe mango peel was subjected to three pretreatment methods: dilute acid using 0.8M H_2SO_4 solution, dilute alkaline using 1M NaOH solution, and dilute alkaline peroxide (2%) pretreatment. The properties of the peels were compared before and after treatment. During enzymatic saccharification, varying substrate concentration- 3 g/mL, 4 g/mL, and 5 g/mL of mango peelings were used and varying enzyme loading- 10 mL, 15 mL, and 20 mL of cellulase enzyme were used to determine the effect of such variation in the reducing sugar concentration. Using the obtained values of reducing sugar at varying cellulase loading and substrate concentration as input data in the Minitab 16 software, the RSM Regression Model equation was developed.

The study revealed that dilute sodium alkaline pretreatment significantly decreased the lignin content of Indian mango peels. The optimal saccharification condition of indian mango peel were identified at 20 mL of cellulase and 3 g/mL of substrate. Using Response Surface Methodology (RSM) via Regression, a mathematical model was developed in order to determine the optimum saccharification condition in terms of cellulase loading and substrate concentration, and their effect on the reducing sugar concentration.

Dilute acid pretreatment has the greatest effect in increasing reducing sugar and starch, while dilute alkaline pretreatment has the greatest effect in increasing the lignocellulosic properties of Indian mango peelings and likewise caused greatest delignification of the sample. The percent reducing sugar after enzymatic saccharification implied that at lower substrate concentration and higher cellulase loading, a greater amount of reducing sugar concentration can be achieved. The mathematical model generated explains the effects of substrate concentration and enzyme loading on the reducing sugar concentration.

Keywords: *Indian mango peelings, enzymatic saccharification, cellulase loading, substrate concentration, mathematical model.*

INTRODUCTION

At present, there is a great interest in using lignocellulosic materials for the production of bioethanol. Bioethanol produced from non-food lignocellulosic waste products such as wood strips and straw, fruit peelings such as cassava wastes, pineapple peelings and other waste materials from fruit processing industries have been investigated. Mango peelings are some of the non-food sources of bioethanol, and the peel forms about 20 percent of the whole fruit. Due to their cellulosic property, mango peels have been a potential substrate for bioethanol.

Being a lignocellulosic biomass, mango peel has a complex structure that is mainly composed of three polymeric fractions: cellulose, hemicellulose, and lignin. Cellulose is a linear homopolymer of D-glucose linked by glycosidic linkage, hemicellulose is a heteropolymer of D-xylose, D-glucose, D-mannose and L-arabinose[1]. The cellulose is associated with hemicellulose and other structural components and is surrounded by lignin sheath, thus hindering the enzymes to penetrate into the reactive fibers of the substrate.

Several chemical pretreatment methods such as dilute acid, dilute alkaline and dilute alkaline pretretreatment can be used prior to enzymatic hydrolysis. Dilute acid pretreatment is done at an elevated temperature of 140^{o} C to 190^{o}C using a low acid concentration like 0.1 – 1 percent sulfuric acid. Alkaline pretreatment uses a dilute base such as sodium hydroxide, while dilute alkaline peroxide uses 2 % hydrogen peroxide solution to cause delignification of the substrate.

Recently, interest has been shown in the use of enzymes for the conversion of cellulose to fermentable sugars in a process called enzymatic saccharification. This is accomplished by cellulytic enzymes wherein different kinds of cellulases may be used to cleave the cellulose and hemicelluloses.

According to Mussatto, et.al.,[2] there are two main factors that affect the yield and initial rate of enzymatic hydrolysis: the substrate concentration and the enzyme loading to substrate loading ratio. The use of high substrate concentrations increases the problem of product inhibition, which results to lower performance of the enzymes during the enzymatic saccharification of lignocelluloses. When the substrate concentration is gradually increased, the reaction increases until it reaches the maximum point. As the maximum is reached, the available enzyme is converted to the enzyme substrate (ES) complex. The maximum point is reached at relatively low substrate concentrations. The impact of the substrate loading on the reducing sugar concentration was also depicted in the results of this study.

The second factor affecting the reducing sugar concentration is the ratio of enzyme to substrate used. The application of more cellulase up to a certain level increases the rate of hydrolysis. This was also manifested in the results of this study.

Relative to the ratio of enzyme to substrate used, another factor that affects the enzymatic saccharification is enzyme loading. Enzyme affects the reaction by catalyzing the reaction during lignocellulosic degradation. Lignocellulosic material breaks down into simple sugar by using the appropriate enzyme. Enzymes such as cellulase, pectinase, and glucoamylase access the substrate surface which results to increased reducing sugar concentration. This further means that a decrease in the reactivity of cellulosic material in the course of hydrolysis can contribute to the low degree of carbohydrate conversion at high substrate concentration, mainly when low enzyme loadings are employed.

Optimization of saccharification parameters is important to ensure higher bioethanol yield. But optimization usually entails time, effort and money to shoulder the amount of chemicals and enzymes needed for the experimental procedures. Application of mathematical models will abridge the problem on spending too much time and money in performing experiments for the purpose of optimizing different parameters for a specific response needed.

The main objective of this study is to develop a mathematical model to show the effect of cellulase loading and substrate concentration on the reducing sugar during enzymatic saccharification of Indian mango peelings for bioethanol production.

MATERIALS AND METHODS

Materials Preparation

The Indian mango peelings used in this study were obtained from Pinagsibaan Multi-purpose Cooperative in Rosario, Batangas. The material was washed, sun and then oven-dried at 60^{0}C for five hours and it was pulverized to fine powder.

Chemical Pretreatments

The powdered dried unripe mango peel was subjected to three pretreatment methods: dilute acid using 0.8M H_2SO_4 solution, dilute alkaline using NaOH solution having a pH of 11.5, and dilute alkaline peroxide (2%) pre-treatment. The pre-treated samples were washed with distilled water until the pH was 7. The lignocellulosic composition of the untreated and pre-treated mango peels such as cellulose, hemicellulose, lignin, extractives, moisture and ash and the reducing sugar were analyzed using the standard

methods and procedures of National Renewable Energy Laboratory[3]. The best pre-treatment method based on the properties were determined and it was the one used prior to enzymatic saccharification.

Enzymatic Saccharification

The neutralized substrate pre-treated with the best pre-treatment method was hydrolyzed with various enzymes such as glucoamylase, cellulase and pectinase, which are all prepared and supplied by BIOTECH Enzyme Laboratory. Cellulase from *Trichoderma sp* having an enzyme activity of 2000μ/mL, gluco-amylase from *Bacillus sp.* having an activity of 300μ/mL were added consecutively to the substrate. Constant stirring was carried out to properly mix the enzymes and to prevent the settling of Indian mango peelings particles at the bottom of the flask.

Several hydrolysis conditions were tested to determine the best substrate concentration and cellulase loading. Varying substrate concentration- 3 g/mL, 4 g/mL, and 5 g/mL of mango peelings were used together with varying enzyme loading- 10 mL, 15 mL, and 20 mL of cellulase enzyme were used. Saccharification was performed in a water bath shaker at 50 ^{o}C for 72 hrs. The supernatant was separated from the mixture by centrifugation at 1400 rpm for 30 min for the determination of the reducing sugar concentration. Reducing sugar concentration was determined using the Dinitrosalicylic (DNS) Acid Method.

Mathematical Model

Using the obtained values of reducing sugar at varying cellulase loading and substrate concentration, a mathematical model was developed. Response Surface Methodology (RSM)[4] was used to determine the mathematical model that best represents the effect of substrate concentration and enzyme loading on the reducing sugar concentration of Indian mango peelings. The responses and variables were correlated by the Response Surface Analysis using the Statistica Minitab 16 software.

RESULTS AND DISCUSSIONS

Comparison of the Properties of Untreated and Pretreated Indian Mango Peelings

The comparison of the chemical properties of Indian mango peelings before and after different pretreatment methods can be seen in Table 1. As seen in the results, alkaline pre-treatment gave the highest increase in alpha-cellulose, hemi-cellulose and holocellulose, indicating that there is also an increase in the supply of glucose which can be further fermented and be the source of bioethanol. Dilute alkaline pretreatment also gave the greatest decrease both for the acid soluble and insoluble lignin.

Table 1. Comparison of the Properties of Indian Mango Peelings Before and After Three Different Pretreatment Methods

Properties	Untreated Mango Peels (% w/w)
Alpha-Cellulose**	17.44
Hemicellulose**	13.19
Holocellulose**	30.63
Acid-Soluble Lignin**	6.77
Acid-Insoluble Lignin***	25.07
Starch	6.43
Reducing Sugar	21.60
Extractives*	41.38
Moisture	11.5
Ash	7.18

Properties	Pretreatment Methods (% w/w)		
	Dilute Acid	Dilute Alkali	Dilute Alkaline Peroxide
Alpha-Cellulose**	29.72	37.62	35.14
Hemicellulose**	29.11	39.95	24.76
Holocellulose**	58.44	77.57	50.90
Acid-Soluble Lignin**	3.32	3.04	4.04
Acid-Insoluble Lignin***	24.68	14.77	18.87
Starch	6.23	3.91	2.48
Reducing Sugar	41.16	14.39	15.67
Extractives*	9.69	3.88	11.75
Moisture	11.20	13.13	17.80
Ash	2.27	5.89	2.95

The removal of lignin improves the reactivity of the remaining polysaccharides. It increases enzyme effectiveness by eliminating non-productive adsorption sites and by increasing access to cellulose and hemicellulose. With these results, in terms of the increase in the cellulosic composition of the sample and the extent of delignification, dilute alkaline pretreatment was found to be the best alternative.

In terms of extractive component, dilute alkaline pre-treatment also gave the highest percentage reduction. Extractives are wood components which are usually present in minor fraction of lignocellulosic materials. They are classified into terpenoids and steroids, fats and waxes, phenolic constituents and organic substances[5,6].

Removal of extractives is important because it interferes the further analysis of biomass. Reduction of extractives would also minimize the possibility of forming inhibitory compounds in fermentation like phenolic/aromatic compounds, which are believed to be degradation products of lignin during hydrolysis. However, the aromatic compounds may also form as a result of sugar degradation and are present in lignocelluloses as extractives[7]. Therefore, extractive content reduction also means more precision on the determination of carbohydrate component of the sample.

Dilute acid pretreatment gave the best retained percentage of the starch content, which is 6.23 percent. This means that compared to the other pretreatments, dilute acid pretreatment could give the highest result of reducing sugar that could be converted from starch.

In terms of the reducing sugar component of the sample, the dilute acid pretreatment, compared with the other two pretreatment methods, gave the highest increase, which was from 21.60 percent to 41.16 percent. This could be a good indication that there is a high yield of fermentable sugar after the acid pretreatment. By deeply analyzing the results, it seemed that partial hydrolysis already happened during the dilute acid pretreatment. This may not be favorable for succeeding processes since the main purpose of pretreatment is delignification- that is, to break the bonds that cement the cellulose and hemicelluloses, thus making the substrate more susceptible to enzymatic action.

Using the alkaline peroxide pretreatment, its effect on the chemical properties of Indian mango peelings is intermediate of the acid and alkaline pretreatment. To generalize, dilute acid pretreatment gave the most favorable result in increasing the reducing sugar and starch composition. Dilute alkaline pretreatment, however, gave the best reduction both for acid-soluble and insoluble lignin, and also gave the biggest increase in the percentage of the lignocellulosic composition. Alkaline peroxide pretreatment comes intermediate of the first two pretreatments with respect to its effect on the chemical properties of the dried powdered mango peelings.

Analyzing the results, the dilute alkaline can be considered the best pretreatment because it is the one that served the main purpose of pretreatment- that is delignification. Although it did not give the highest increase in reducing sugar composition, it could also be a good indication that no partial hydrolysis occurred during dilute alkaline pretreatment. This pretreatment also gave the highest increase in cellulosic component of the sample, hence producing more glucose source for fermentation and for bioethanol production.

Effect of Varying the Amount of Cellulase and Substrate Concentration in the Reducing Sugar Concentration during Enzymatic Saccharification

Table 2 presents the percent reducing sugar when substrate concentration and cellulose loading were varied during enzymatic saccharification.

Table 2. Reducing Sugar Concentration at Varying Parameters

Substrate Concentration (g/ml)	Cellulase Loading (ml)	Reducing Sugar Concentration (%)
3	10	39.72
3	15	39.46
3	20	41.37
4	10	37.82
4	15	38.78
4	20	38.19
5	10	19.99
5	15	30.18
5	20	38.36

As seen from the table, 3g/mL of substrate with 10 mL, 15mL and 20mL of enzyme produced 39.72 percent, 39.46 percent and 41.37 percent reducing sugar, respectively. These results indicate that as the same concentration of mango peelings were acted upon by increasing the amount of cellulase, the greater the amount of reducing sugar produced. This means that as the amount of enzyme goes higher, the greater will be the absorption onto the surface of the cellulose part of mango peelings[8].

On the other hand, a substrate concentration of 4g/mL using an increased cellulase loading of 10, 15 and 20 g/mL resulted to 37.82 percent, 38.78 percent and 38.19 percent reducing sugar, respectively. The increase in reducing sugar concentration was noted as the cellulase loading increased from 10 to 15 mL, but the amount of reducing sugar decreased a bit from 38.78 to 38.19 percent using 10, 15 and 20 mL cellulase loading, respectively.

a bit from 38.78 to 38.19 percent using 10, 15 and 20 mL cellulase loading, respectively. This indicated that the effect of increasing substrate loading in the reducing sugar concentration was not that much as compared to enzyme loading concentration. It also indicated that the optimum substrate concentration and enzyme loading was achieved.

The same trend was observed upon using 5 g/mL substrate concentration which yielded 19.99 percent, 30.18 percent and 38.36 percent reducing sugar using 10.15 and 20 mL of cellulase enzyme, respectively. The substrate with the least amount of concentration and with the largest amount of the enzyme loading yielded the highest conversion of lignocellulosic component of Indian mango peelings to reducing sugar after enzymatic saccharification. This was achieved at substrate concentration of 3g/mL and enzyme loading of 20mL obtaining the highest recovery of reducing sugar concentration amounting to 41.37 percent. This result is the same as the findings of Mussatto[2], when the researchers found out that in the enzymatic hydrolysis of brewer's spent yeast, the highest glucose yield was obtained at the lowest substrate concentration and highest enzyme loading.

For a more visible comparison of the effects of varying parameters in the reducing sugar concentration, Figure 1 is presented below. It shows the effect of cellulase loading in the reducing sugar at varying substrate concentration.

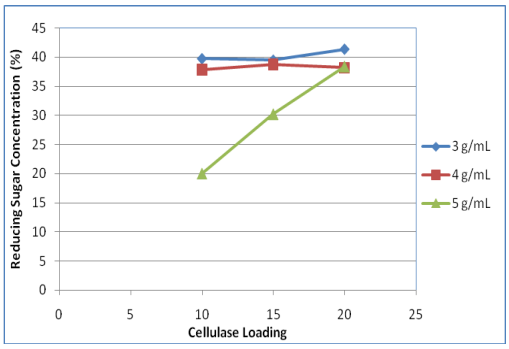

Figure 1. Effect of Cellulase Loading in the Reducing Sugar Concentration at Varying Substrate Concentration

As shown in the figure, reducing sugar concentration generally increases with higher cellulase loading at varying substrate concentration. Using 5g/mL substrate concentration, the highest percentage of 38.36 percent for reducing sugar was achieved in 20 mL of cellulase loading, which is also true in the case of 3 g/mL substrate concentration yielding 41.37 percent reducing sugar. This is the maximum amount of reducing sugar produced at 20 mL cellulase loading. However, using 4 g/mL substrate concentration, reducing sugar increased from 37.82 to 38.78 percent using 10 to 15 mL of cellulase loading. The figure decrease in the percentage of reducing sugar from 38. 78 to 38.19 percent reducing sugar at 20 mL cellulase loading. This means that saturation happened at 4 mL substrate concentration and 15 mL cellulase loading as indicated by the dropping of the line graph at that point. This is the point wherein the substrate is already saturated with the enzyme, and that introducing more enzyme in the reaction will no longer increase the reducing sugar concentration. Increasing the substrate concentration indefinitely does not increase the rate of an enzyme-catalyzed reaction beyond a certain point. This point is reached when there are enough substrate molecules to completely fill (saturate*)* the enzyme's active sites.

This observation is in parallel to the previous study[2], which stated that the larger the quantity of enzymes used, the better the hydrolysis. Furthermore, it was also found out that the effect of enzyme loading is significantly greater than other two studied variables (agitation speed and substrate concentration).

The effect of substrate loading in the reducing sugar concentration at variable cellulase loading is shown in Figure 2.

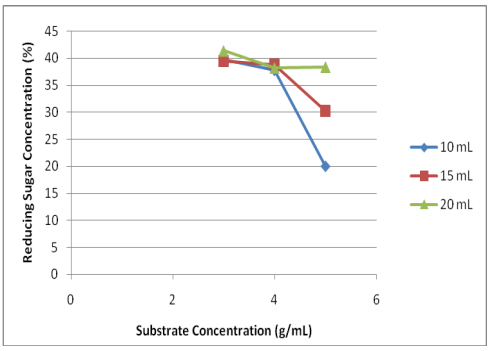

Figure 2. Effect of Substrate Concentration in Reducing Sugar at Varying Cellulase Loading

The trend depicted in the figure is opposite to the one shown in Figure 1 - that is, at lower substrate concentration, higher percentage of reducing sugar was attained at different amounts of cellulase. Specifically, using 10 mL of cellulase, the maximum reducing sugar concentration is at 3 g/mL. On the other hand, the least amount was obtained in 5 g/mL. Likewise, taking into account cellulase loading of

15 mL, similar observations had been noted. Increasing substrate concentration made the reducing sugar concentration decline. Such effect is mainly due to the lower cellulase loading at higher substrate concentration.

Substrate concentration is one of the main factors that affect the yield and initial rate of enzymatic saccharification of cellulose. At low substrate levels, an increase of substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis. However, high substrate concentration can cause substrate inhibition, which substantially lowers the rate of the hydrolysis, and the extent of substrate inhibition depends on the ratio of total substrate to total enzyme[9].

As manifested in the results of the present study, substrate loading had the greater impact on the variation of reducing sugar concentration, since it affected the behavior of the enzyme. At the same time, it had a direct impact on the enzymatic saccharification wherein an increase in substrate loading led to the increase in the variation of the reducing sugar concentration. This can be seen in the contour and surface plots of the actual data as shown in Figures 3 and 4.

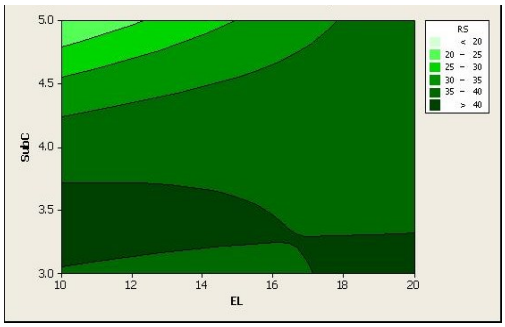

Figure 3. Contour Plot of Reducing Sugar Concentration (RS) Relative to Varying Substrate Loading Concentration (SubC) and Enzyme Loading (EL)

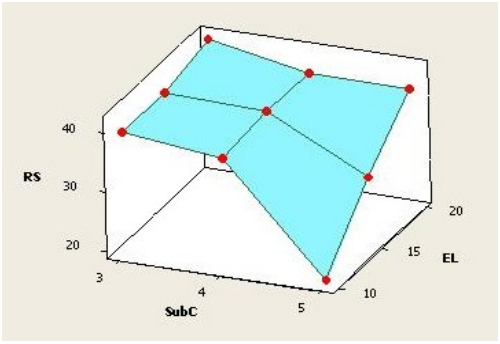

Figure 4. Surface (3D) Plot of Reducing Sugar Concentration (RS) Relative to Varying Substrate Loading Concentration (SubC) and Enzyme Loading (EL)

The contour and surface plots also show that the optimum condition to which highest reducing sugar is produced lies somewhere between a substrate concentration between 3 to 3.5 g/mL and cellulase loading of 20 mL. This is the area (represented by dark green color) to which around 40 percent reducing sugar is produced.

Mathematical Model Showing the Effect of Cellulase Loading and Substrate Concentration on Reducing Sugar Concentration

Using the obtained values of reducing sugar at varying cellulase loading and substrate concentration as input data in the Minitab 16 software, the RSM Regression Model equation developed was:

*RS (%) = (38.4178) - (5.3367)*A + (3.3983)*B + (4.18) * AB - (3.4167) * AA - (0.2317) * BB*

Where: RS = the reducing sugar concentration (%)
A = the substrate concentration (g/ml)
B = the cellulase loading (ml) a negative coefficient of A (-5.33670) representing the substrate loading indicates that keeping the cellulase loading constant decreases reducing sugar. In terms of the effect of cellulase loading having a positive (+) coefficient (+5.3367), the value indicates that increasing the cellulase loading also means an increase in the reducing sugar concentration, keeping the substrate loading constant. Therefore, cellulase loading has a greater effect in increasing the reducing sugar concentration than the substrate concentration. In terms of the variation in the reducing sugar, the substrate concentration has a greater effect while in terms of increasing the reducing sugar concentration, the cellulase loading has a greater effect.

When the interaction of both parameters - cellulase loading and substrate concentration - is considered, the + 4.18 AB value of parameters means that the interaction of the cellulase loading and substrate concentration results to an increase in the reducing sugar.

The coefficients of the regression model equation developed can be seen in Table 3, which explains the effect of enzyme loading and substrate concentration on the reducing sugar concentration. Shown in the table are the values obtained for the constant, substrate concentration (A), cellulase loading B and the interaction between and among parameters as obtained from the derived model equation.

Table 3. Parameter Estimates of the Regression Model

Term	Coefficient	Standard Error	p-value
Constant	38.4178	2.462	0.001
A: Substrate Concentration (g/ml)	-5.3367	1.348	0.029
B: Cellulase Loading (ml)	3.3983	1.348	0.086
AB	4.1800	1.651	0.085
AA	-3.4167	2.336	0.240
BB	-0.2317	2.336	0.927

Data show that the constant and substrate concentration (A) has p-values less than 0.05, while the cellulase loading (B) and the interaction between and among parameters had a p-value greater than 0.05. This indicates that the constant and substrate loading coefficients do not have a significant impact on the reducing sugar concentration based on model developed using RSM via regression.

Findings also show that increasing the substrate concentration does not necessarily mean an increase in the reducing sugar. This is caused by the saturation of substrate particles by the enzymes acting on it and when the reaction comes to the point wherein the cellulytic enzyme can no longer biodigest additional substrate, thus reducing sugar concentration ceases to increase. For a given enzyme concentration, the rate of reaction increases with increasing substrate concentration up to a point above which any further increase in substrate concentration produces no significant change in reaction rate or in this case, in increasing the reducing sugar concentration. This is because the active sites of the enzyme molecule at any given moment are virtually saturated with substrate.

For cellulase loading, the p-value obtained which was higher than 0.05 indicates that an increased amount of enzyme during saccharification also increases the reducing sugar concentration. This is an expected result since cellulytic enzymes are responsible for breaking cellulose chains into fermentable sugars or reducing sugars. Provided that the substrate concentration is high and that temperature and pH are kept constant, the rate of the reaction, or in this case, the reducing sugar concentration, is proportional to the enzyme or cellulase concentration.

On the other hand, the p-values of the interaction between and among parameters (AB, AA, BB) were all higher than 0.05. This means that the square of the enzyme loading, the square of the substrate loading concentration, and the interaction of enzyme loading and substrate loading concentration have a significant impact on the reducing sugar concentration based on the developed model.

Using the said model, a contour and surface plot were generated as indicated in Figures 5 and 6. As seen in the said figures, an optimum, depicted by the highest reducing sugar concentration (> 40%), can be obtained at substrate loading concentration 3 to 3.5 g/mL and enzyme loading of around 20 mL at specific points within the said limits.

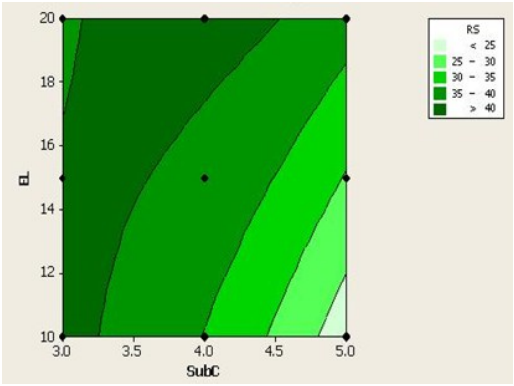

Figure 5. Contour Plot of Reducing Sugar Concentration (RS) Relative to Varying Substrate Loading Concentration (SubC) and Enzyme Loading (EL) using Regression Model via Response Surface Methodology

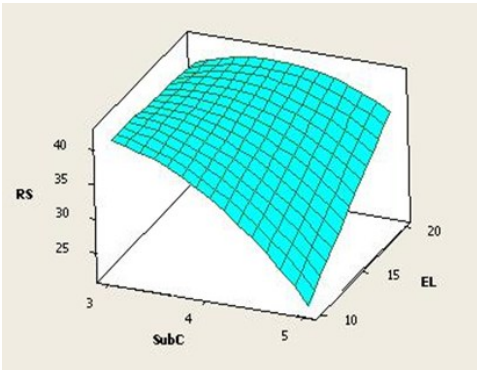

Figure 6. Surface (3D) Plot of Reducing Sugar Concentration (RS) Relative to Varying Substrate Loading Concentration (SubC) and Enzyme Loading (EL) using Regression Model via Response Surface Methodology

The contour plot also shows the greater variation in the reducing sugar concentration at different substrate concentrations as compared to the variation caused by different enzyme loadings.

The mathematical model obtained an R-square (R^2) value of 91.06 percent. This value indicates the accuracy result of the Minitab software used or the accuracy of the actual data as compared to the trend projected by the model. The value of 91.06 percent is already high enough and the accuracy of the derived model equation can be established.

CONCLUSIONS

The physical and chemical properties of mango peelings manifest their potentiality as a source of bioethanol. Dilute acid pretreatment has the greatest effect in increasing reducing sugar and starch, dilute alkaline peroxide pretreatment significantly decreases the lignin content, and dilute alkaline pretreatment has the greatest effect in increasing the lignocellulosic properties of Indian mango peelings, and likewise caused greatest delignification of the sample. The three pretreatment methods significantly affect the properties of the dried/powdered mango peelings.

Enzyme loading and substrate concentration significantly affect the reducing sugar concentration during enzymatic saccharification. The percent reducing sugar after enzymatic saccharification implies that at lower substrate concentration and higher cellulase loading, a greater amount of reducing sugar concentration can be achieved.

The mathematical model generated explains the effects of substrate concentration and enzyme loading on the reducing sugar concentration.

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