Optimization of Pretreatment and Enzymatic Hydrolysis of Spent Coffee Ground for the Production of Fermentable Sugar

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Abstract: The aim of this work was to optimize the condition of pretreatment and enzymatic hydrolysis for high yield of sugar production of spent coffee ground (SCG). Acid and alkaline pretreatment method were compared and the method with more sugar produced was selected. Response surface methodology was use for the analysis of conditions such as concentration of alkali, temperature and weight of SCG. The optimized condition obtained was 0.5% (v/v) of alkali, temperature of 100°C and 5% (w/v) of SCG. Enzymatic hydrolysis was carried out after the optimized condition of alkaline pretreatment. The conditions were pH, temperature and enzyme dosage. The optimized condition obtained was at pH 4.8, 0.01 ml of enzyme and temperature of 55°C.

1. Introduction
Coffee is one of the world’s most consumed beverages, it is the second most profitable product in the world [1]. In 2015, 9.1 billion kg of coffee was being consumed by the people around the world [2]. The requirement for coffee is huge. Hence, it produces large quantity of residues called spent coffee ground (SCG) which will cause the environmental pollution. Brewing of coffee and preparation of instant coffee by treating coffee powder with hot water produce the spent coffee grounds. Six million tons of SCG produced annually from the coffee brewing and instant coffee production [3]. SCG contains various type of organic compounds which are carbohydrates, lipids, protein, lignin, ashes and polyphenols.

Pretreatment is an important step for the biochemical conversion of lignocelulosic materials into fermentable sugar. The structure of the lignocellulose which contains cellulose, hemicellulose and lignin is needed to change for the ease of enzyme to convert the carbohydrate polymers into sugar monomers [4]. Acid and alkaline pretreatment are the examples of chemical pretreatment. Acid pretreatment use concentrated or diluted acids as the reagents and it is usually carried out at high temperature [5]. Alkaline pretreatment usually involves calcium, sodium, ammonium and potassium hydroxide. This pretreatment method involves partial solvation of hemicellulose, changing in the structure of lignin and partial decrystallization of cellulose [6]. The other method for converting carbohydrate to fermentable sugar is enzymatic hydrolysis. The advantages of enzymatic hydrolysis are higher yield of fermentable sugar, milder working conditions higher selectivity and cheaper energy costs as compared to chemical pretreatment [7].

2. Materials
2.1 Raw material
Spent coffee ground (SCG) were obtained from McCafé of McDonald’s at Kangar, Perlis. The material was dried in an oven at 60°C for 72 hours.
2.2 Chemicals and Reagents
Sodium hydroxide, sodium potassium tartrate, glucose monohydrate, sodium hydrogen phosphate and sodium dihydrogen phosphate from HmbG Chemicals. Cellulase, citric acid and sodium citrate from Merck. Dinitrosalicylic acid (DNS) reagent from ALDRICH and sulphuric acid from QReC.

2.3 Apparatus and equipment
Erlenmeyer flasks (250 mL), test tubes, UV-Vis spectrophotometer, fume hood, oven, electronic balance, water bath, hot plate, micropipette, centrifuge, pH meter and incubator were used in the research work.

3. Procedures
3.1 Comparison of pretreatment method
Sulphuric acid and sodium hydroxide solution were common acid and alkali used in acid and alkali pretreatment. Working volume of 10ml was prepared by mixing 10% (w/v) of dried SCG with 1% (v/v) of sulphuric acid (H2SO4)/sodium hydroxide (NaOH) solution and put in the water bath of 60˚ C for 60 minutes [8]. Pretreated solid and liquid were separated by centrifuging at 8000 rpm for 5 minutes [9], whereas the pretreated SCG was washed with distilled water [10] to remove insoluble matter and the supernatant was used for sugar analysis by DNS method.

3.2 Reducing sugar analysis by DNS method
Sufficient amount of the mixtures obtained from the pretreatment and enzymatic hydrolysis were placed in test tubes. After that, 0.5 ml of DNS reagent was added and mixed well. Then, the test tubes were placed in boiling water bath for 5 minutes and was cooled to room temperature by washing through tap water. About 4.5 ml of distilled water was added into each test tube. Sufficient amount of solution was taken out from the test tubes and was put in cuvettes. The cuvettes were placed in UV-Vis spectrophotometer at absorbance of 540nm [11].

3.3 Optimization of alkaline pretreatment method
Alkaline pretreatment method was chose as it produced more fermentable sugar when compared to acid pretreatment method. Three parameters which were concentration of alkali, concentration of SCG weight and temperature were considered. For alkali pretreatment method, the range of studied parameters were 0.5-5% (v/v) of sodium hydroxide [12], 5-20% (w/v) of SCG weight [8] and temperature (50-100˚ C) [12]. For studying effect of temperature, water bath was used. Working volume of 10ml was prepared and put in the water bath of 60˚ C for 60 minutes [8].

The following steps were the same in the method of conducting alkaline pretreatment in comparing the pretreatment methods. These parameters was ran by using Central Composite Design (CCD). The three factors resulted experiment runs for the optimization of selected pretreatment method. Table 1 showed the experimental design matrix generated by DOE software. There was 20 runs of the experiments and the respond obtained was the sugar concentration.
Table 1. Optimization of alkaline pretreatment’s parameters by using CCD method.

| Run | Concentration of alkali (%) | Temperature (°C) | Weight of SCG (%) | Sugar concentration (mg/ml) |
|-----|-----------------------------|------------------|------------------|---------------------------|
| 1   | 4.09                        | 60.1             | 8.04             |                            |
| 2   | 4.09                        | 60.1             |                 | 16.96                     |
| 3   | 4.09                        | 89.9             | 8.04             |                            |
| 4   | 2.75                        | 100.0            |                 | 12.50                     |
| 5   | 2.75                        | 75.0             |                 | 12.50                     |
| 6   | 2.75                        | 75.0             |                 | 12.50                     |
| 7   | 2.75                        | 75.0             |                 | 20.00                     |
| 8   | 1.41                        | 60.1             |                 | 16.96                     |
| 9   | 1.41                        | 89.9             |                 | 16.96                     |
| 10  | 0.50                        | 75.0             |                 | 12.50                     |
| 11  | 2.75                        | 75.0             |                 | 12.50                     |
| 12  | 4.09                        | 89.9             |                 | 16.96                     |
| 13  | 2.75                        | 75.0             |                 | 12.50                     |
| 14  | 5.00                        | 75.0             |                 | 12.50                     |
| 15  | 2.75                        | 75.0             |                 | 12.50                     |
| 16  | 1.41                        | 89.9             |                 | 8.04                      |
| 17  | 2.75                        | 50.0             |                 | 12.50                     |
| 18  | 2.75                        | 75.0             |                 | 12.50                     |
| 19  | 2.75                        | 75.0             |                 | 5.00                      |
| 20  | 1.41                        | 60.1             |                 | 8.04                      |

3.4 Citrate buffer preparation

Concentration of 0.1M solution of citric acid and 0.1M solution of sodium citrate were prepared by dissolving 21.01g of citric acid and 29.41g of sodium citrate in 1L of distilled water respectively. The volume of solution needed to prepare citrate buffer with different pH was shown in Table 2. The total volume was made up to 100ml by adding of distilled water [13].

Table 2. Volume of solutions needed for preparing different pH of citrate buffer.

| Citric acid solution (ml) | Sodium citrate solution (ml) | pH    |
|---------------------------|------------------------------|-------|
| 33.0                      | 17.0                         | 4.0   |
| 31.5                      | 18.5                         | 4.2   |
| 28.0                      | 22.0                         | 4.4   |
| 25.5                      | 24.5                         | 4.6   |
| 23.0                      | 27.0                         | 4.8   |
| 20.5                      | 29.5                         | 5.0   |
| 18.0                      | 32.0                         | 5.2   |
| 16.0                      | 34.0                         | 5.4   |
| 13.7                      | 36.3                         | 5.6   |
| 11.8                      | 38.2                         | 5.8   |
| 11.8                      | 38.2                         | 5.8   |
| 9.5                       | 41.5                         | 6.0   |
| 7.2                       | 42.8                         | 6.2   |
3.5 Sodium phosphate buffer preparation

Concentration of 0.2M solution of sodium dihydrogen phosphate and 0.2M solution of sodium hydrogen phosphate were prepared by dissolving 27.8g of sodium dihydrogen phosphate and 71.7g of sodium hydrogen phosphate in 1L of distilled water respectively. The volume of solution needed to prepare sodium phosphate buffer with different pH was shown in Table 3. The total volume was made up to 200ml by adding of distilled water [14].

| Sodium dihydrogen phosphate solution (ml) | Sodium hydrogen phosphate solution (ml) | pH  |
|------------------------------------------|----------------------------------------|-----|
| 77.5                                     | 22.5                                   | 6.3 |
| 73.5                                     | 26.5                                   | 6.4 |
| 68.5                                     | 31.5                                   | 6.5 |
| 62.5                                     | 37.5                                   | 6.6 |
| 56.5                                     | 43.5                                   | 6.7 |
| 51.0                                     | 49.0                                   | 6.8 |
| 45.0                                     | 55.0                                   | 6.9 |
| 39.0                                     | 61.0                                   | 7.0 |
| 33.0                                     | 67.0                                   | 7.1 |
| 28.0                                     | 72.0                                   | 7.2 |
| 23.0                                     | 77.0                                   | 7.3 |
| 19.0                                     | 81.0                                   | 7.4 |
| 16.0                                     | 84.0                                   | 7.5 |
| 13.0                                     | 87.0                                   | 7.6 |
| 10.5                                     | 90.5                                   | 7.7 |
| 8.5                                      | 91.5                                   | 7.8 |
| 7.0                                      | 93.0                                   | 7.9 |
| 5.3                                      | 94.7                                   | 8.0 |

3.6 Optimization of enzymatic hydrolysis

The optimized condition from the selected pretreatment method was carried out and the mixture obtained was filtered and washed with distilled water to remove the alkali. The filtered SCG was dried in oven at 60°C for 24 hours. Working volume of 15ml was prepared by mixing dried SCG with 10ml of citrate buffer or sodium phosphate buffer and distilled water. Cellulase was used for enzymatic hydrolysis. The solutions was filled in test tubes and the test tubes were put in incubator for 24 hours [15]. The parameters were 0.01-0.03 ml of cellulase [16], pH (4-8) and temperature (35-55°C) [17]. The pH is adjusted during the mixing of the buffer with solutions and the cellulase was added after the pH was adjusted. These parameters were run by using Central Composite Design (CCD). Table 4 showed the experimental design matrix generated by DOE software. There was 20 runs of the experiments and the respond obtained was the sugar concentration.
Table 4. Optimization of enzymatic hydrolysis’s parameters by CCD method.

| Run | pH  | Enzyme dosage (ml) | Temperature (˚C) | Sugar concentration (mg/ml) |
|-----|-----|--------------------|------------------|----------------------------|
| 1   | 7.2 | 0.026              | 50.9             |                            |
| 2   | 6.0 | 0.020              | 55.0             |                            |
| 3   | 6.0 | 0.020              | 45.0             |                            |
| 4   | 4.8 | 0.026              | 50.9             |                            |
| 5   | 6.0 | 0.020              | 45.0             |                            |
| 6   | 6.0 | 0.010              | 45.0             |                            |
| 7   | 4.0 | 0.020              | 45.0             |                            |
| 8   | 4.8 | 0.014              | 50.9             |                            |
| 9   | 6.0 | 0.020              | 45.0             |                            |
| 10  | 6.0 | 0.030              | 45.0             |                            |
| 11  | 6.0 | 0.020              | 35.0             |                            |
| 12  | 4.8 | 0.014              | 39.1             |                            |
| 13  | 7.2 | 0.014              | 50.9             |                            |
| 14  | 7.2 | 0.014              | 39.1             |                            |
| 15  | 7.2 | 0.026              | 39.1             |                            |
| 16  | 8.0 | 0.020              | 45.0             |                            |
| 17  | 6.0 | 0.020              | 45.0             |                            |
| 18  | 6.0 | 0.020              | 45.0             |                            |
| 19  | 6.0 | 0.020              | 45.0             |                            |
| 20  | 4.8 | 0.026              | 39.1             |                            |

4. Results and Discussion
4.1 Pretreatment Method Selection
Acid and pretreatment method were compared and Figure 1 showed the comparison of two pretreatment method investigated in the work. It was observed that alkaline pretreatment had a better result of sugar production which is 1.438 mg/ml while sugar production of acid pretreatment obtained was 0.453 mg/ml. The result obtained agreed with the previous work carried out by [18]. Alkaline pretreatment was better than acid pretreatment as it was more effective in breaking the bonds between the lignocellulose [19].

![Comparison of pretreatment methods](image-url)

**Figure 1.** Bar chart of comparison of two pretreatment methods.
4.2 Optimization of parameters of alkaline pretreatment

CCD was used to find the optimum condition of the alkali pretreatment of SCG. Table 5 shows the sugar concentration obtained after alkaline pretreatment with different combination between parameters (concentration of alkali, temperature and weight of SCG) had been carried out. It can be seen that the highest sugar concentration obtained was 48.32 mg/ml at 4.09% (v/v) sodium hydroxide, temperature of 60.1˚ C and 16.96% (w/v) of weight of SCG. The calculation of sugar concentration was based on the glucose standard curve.

| Run | Parameters | Sugar concentration (mg/ml) |
|-----|------------|-----------------------------|
|     | Concentration of sodium hydroxide (%) | Temperature (˚C) | Weight of SCG (%) |
| 1   | 4.09       | 60.1                        | 8.04                | 10.69 |
| 2   | 4.09       | 60.1                        | 16.96               | 48.32 |
| 3   | 4.09       | 89.9                        | 8.04                | 20.76 |
| 4   | 2.75       | 100.0                       | 12.50               | 20.81 |
| 5   | 2.75       | 75.0                        | 12.50               | 17.43 |
| 6   | 2.75       | 75.0                        | 12.50               | 16.21 |
| 7   | 2.75       | 75.0                        | 20.00               | 29.88 |
| 8   | 2.75       | 60.1                        | 16.96               | 13.64 |
| 9   | 1.41       | 89.9                        | 16.96               | 19.30 |
| 10  | 0.50       | 75.0                        | 12.50               | 15.58 |
| 11  | 2.75       | 75.0                        | 12.50               | 19.09 |
| 12  | 4.09       | 89.9                        | 16.96               | 45.93 |
| 13  | 2.75       | 75.0                        | 12.50               | 13.40 |
| 14  | 5.00       | 75.0                        | 12.50               | 38.33 |
| 15  | 2.75       | 75.0                        | 12.50               | 16.34 |
| 16  | 1.41       | 89.9                        | 8.04                | 28.16 |
| 17  | 2.75       | 50.0                        | 12.50               | 10.96 |
| 18  | 2.75       | 75.0                        | 12.50               | 16.90 |
| 19  | 2.75       | 75.0                        | 5.00                | 12.87 |
| 20  | 1.41       | 60.1                        | 8.04                | 16.39 |

Figure 2a shows the 3D surface plot on the interaction of the parameters. This graph shows the interaction between concentration of alkali and temperature at the fixed 12.5% (w/v) weight of SCG. Based on the graph, the optimum region for concentration of alkali was 1.65% to 5% while for temperature was 50˚C to 90˚C. At concentration of 2.75%, the sugar concentration increased when temperature increased. It was due to removal of lignin increased when the temperature increased [20]. At temperature of 75˚C, the sugar concentration increased when concentration of alkali increased as the solubilization of lignin was increased [20]. Figure 2b shows the interaction between concentration of alkali and weight of SCG at the fixed temperature of 75˚C. At concentration of 2.75%, the sugar concentration increased when weight of SCG increased. At 12.5% weight of SCG, the sugar concentration increased when concentration of alkali increased as the solubilization of lignin was increased [20]. It could be observed that the sugar concentration increased when the concentration of alkali was increased from 0.5% (v/v) to 5% (v/v) and the weight of SCG was increased from 5% (w/v) to 20% (w/v). Figure 2c shows the interaction between temperature and weight of SCG at the fixed 2.75% (v/v) concentration of alkali. The optimum region for weight of SCG was 5% to 12.5% while for temperature was 50˚C to 75˚C. At temperature of 75˚C, the sugar concentration increased when the weight of SCG decreased. On the other hand, at 12.5% weight of SCG, sugar concentration increased when the temperature decreased.
4.3 Validation of optimization of alkaline pretreatment

Various experiments were carried out on the given optimum condition to validate the model. The optimized condition for alkaline pretreatment was 0.5% (v/v) of alkali, temperature of 100°C and 5% (w/v) of SCG was carried out and performed in triplicate. The results were shown in Table 6. The average sugar concentration yields obtained was 57.0 mg/ml. The value obtained was near to the value predicted by the models which was 58.58 mg/ml of sugar concentration. According to [20], the optimum condition of alkali pretreatment was 90°C. This is near to the temperature of the optimum condition obtained.

Table 6. Validation of the model for optimization of alkaline pretreatment.

| Run | Concentration of alkali (%) | Temperature (°C) | Weight of SCG (%) | Sugar concentration (mg/ml) |
|-----|-----------------------------|------------------|-------------------|----------------------------|
|     | Actual                      | Predicted        | % Error           |                            |
| 1   | 0.5                         | 100              | 5                 | 56.50                      |
|     |                             |                  |                   | 58.58                      |
|     |                             |                  |                   | 3.55                       |
| 2   | 0.5                         | 100              | 5                 | 54.78                      |
|     |                             |                  |                   | 58.58                      |
|     |                             |                  |                   | 6.49                       |
| 3   | 0.5                         | 100              | 5                 | 59.78                      |
|     |                             |                  |                   | 58.58                      |
|     |                             |                  |                   | 2.04                       |
|     | Average                     |                  |                   | 57.02                      |
|     |                             |                  |                   | 58.58                      |
|     |                             |                  |                   | 4.03                       |

4.4 Optimization of parameters of enzymatic hydrolysis

CCD was used to obtain the optimum condition of the enzymatic hydrolysis of SCG. Table 7 shows the sugar concentration obtained after enzymatic hydrolysis with different combination between parameters (pH, temperature and enzyme dosage) had been carried out. It can be seen that the highest sugar concentration obtained was 2.76 mg/ml at pH 4.8, temperature of 50.9°C and 0.026ml of enzyme while the lowest sugar concentration obtained was at the condition at pH 6, temperature of 35°C and 0.020 ml of enzyme. The calculation of sugar concentration was based on glucose standard curve.

Figure 3a shows the 3D surface plot on the interaction of the parameters. The graph shows the interaction between pH and enzyme dosage at the fixed temperature of 45°C. The optimum region for pH was 4.8 to 6 while for enzyme dosage was 0.01ml to 0.023ml. It could be observed that the sugar concentration increased when the enzyme dosage was increased from 0.01 ml to 0.03 ml. The increasing pH from 6 to 8 caused the decrease of sugar concentration. This was because the pH disrupt the charge of enzymes and the enzymes became denatured and unable to carry out chemical reactions [21]. Figure 3b shows the interaction between pH and temperature at the fixed 0.02 ml of enzyme used. The optimum region for pH was 5.4 to 6.4 while for temperature was 35°C to 45°C. At pH 6, sugar concentration increased when the temperature increased [17]. It could be observed that the highest sugar concentration had achieved with increasing of temperature from 35°C to 55°C as the enzyme activity increased when the temperature increased. Decreasing of pH from pH 7.2 to pH 4.8 obtained the highest sugar production as the enzyme became more active when it near to the optimum pH [22]. Figure 3c shows the interaction between enzyme dosage and temperature at the pH 6.0. It could be observed that the highest sugar concentration occurred when the temperature increased from 35°C to 55°C and enzyme
dosage increased from 0.01 ml to 0.03 ml as the enzymatic hydrolysis required high enzyme dosage for the production of fermentable sugar [23].

Table 7. Data obtained for the response of enzymatic hydrolysis based on the CCD matrix.

| Run | pH  | Enzyme dosage (ml) | Temperature (°C) | Sugar concentration (mg/ml) |
|-----|-----|-------------------|-----------------|-------------------------|
| 1   | 7.2 | 0.026             | 50.9            | 2.62                    |
| 2   | 6.0 | 0.020             | 55.0            | 2.33                    |
| 3   | 6.0 | 0.020             | 45.0            | 1.41                    |
| 4   | 4.8 | 0.026             | 50.9            | 2.76                    |
| 5   | 6.0 | 0.020             | 45.0            | 1.23                    |
| 6   | 6.0 | 0.010             | 45.0            | 1.36                    |
| 7   | 4.0 | 0.020             | 45.0            | 2.06                    |
| 8   | 4.8 | 0.014             | 50.9            | 1.85                    |
| 9   | 6.0 | 0.020             | 45.0            | 1.31                    |
| 10  | 6.0 | 0.030             | 45.0            | 1.47                    |
| 11  | 6.0 | 0.020             | 35.0            | 1.15                    |
| 12  | 4.8 | 0.014             | 39.1            | 1.51                    |
| 13  | 7.2 | 0.014             | 50.9            | 1.57                    |
| 14  | 7.2 | 0.014             | 39.1            | 1.51                    |
| 15  | 7.2 | 0.026             | 39.1            | 1.16                    |
| 16  | 8.0 | 0.020             | 45.0            | 1.73                    |
| 17  | 6.0 | 0.020             | 45.0            | 1.19                    |
| 18  | 6.0 | 0.020             | 45.0            | 1.35                    |
| 19  | 6.0 | 0.020             | 45.0            | 1.30                    |
| 20  | 4.8 | 0.026             | 39.1            | 1.44                    |

Figure 3. 3D surface plot towards sugar concentration: a) enzyme dosage versus pH, b) temperature versus pH, c) temperature versus enzyme dosage.

4.5 Validation of optimization of enzymatic hydrolysis
Various experiments were carried out on the given optimum condition to validate the model. The optimized condition for enzymatic hydrolysis was at pH 4.8, 0.01 ml of enzyme and temperature of 55°C was carried out and performed in triplicate. The results were shown in Table 8. The sugar concentration yields obtained was 1.824 mg/ml. The value obtained was near to the value predicted by the models which was 1.878 mg/ml of sugar concentration. From previous study, enzyme cellulase was found to be most active at temperature of 55°C and at pH 5.5 [17]. The optimum condition obtained was close to the result from the research.
Table 8. Validation of the model for optimization of enzymatic hydrolysis.

| Run | pH  | Enzyme dosage (ml) | Temperature (°C) | Sugar concentration (mg/ml) Actual | Sugar concentration (mg/ml) Predicted | % Error |
|-----|-----|--------------------|------------------|-----------------------------------|--------------------------------------|---------|
| 1   | 4.8 | 0.01               | 55               | 1.789                             | 1.878                                | 4.74    |
| 2   | 4.8 | 0.01               | 55               | 1.853                             | 1.878                                | 1.33    |
| 3   | 4.8 | 0.01               | 55               | 1.831                             | 1.878                                | 2.50    |
|     |     |                    |                  | Average                           | 1.824                                | 2.86    |

5. Conclusion

As a conclusion, the alkaline pretreatment method is better than acid pretreatment as it produced more fermentable sugar. RSM with CCD was successfully implemented to optimize the conditions of the process parameters (concentration of alkali, temperature and weight of SCG) for alkaline pretreatment and process parameters (temperature, pH and enzyme dosage) for enzymatic hydrolysis. The optimized condition obtained was 0.5% (v/v) of alkali, temperature of 100°C and 5% (v/v) of SCG for a maximum response of 57.02 mg/ml of sugar concentration while the optimized condition for enzymatic hydrolysis was at pH 4.8, 0.01 ml of enzyme and temperature of 55°C for a maximum response of 1.824 mg/ml of sugar concentration.

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