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Original Article

Alkaline pretreatment of spent coffee grounds for oligosaccharides production by mannanase from *Bacillus* sp. GA2(1)

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ABSTRACT

Spent coffee grounds (SCGs) are solid wastes from the manufacturing of fresh coffee beverages which are globally popular. SCGs are composed of lignocellulosic materials which can be digested by lignocellulolytic enzymes and oligosaccharide is a product from the hydrolysis of cellulose and hemicellulose. However, the presence of lignin in lignocellulose leads to resistance in the activity of lignocellulolytic enzymes. Therefore, it is necessary to add a pretreatment step to increase the efficiency of the enzymes. Four factors were examined that impact on the SCGs pretreatment process: concentration of NaOH (0.5–2.5 Normality); ratio of SCGs to NaOH (1:1–1:5); temperature (50–121 °C); and reaction time. Afterward, untreated SCGs and treated SCGs were digested by crude mannanase from *Bacillus* sp. GA2(1). The amounts of reducing sugar were analyzed using the dinitrosalicylic acid method. The results showed the optimum SCG-treated conditions were 0.5 N NaOH, 1:2 and 50 °C for 6 h which produced 520 ± 0.01 μg/mL of reducing sugar. Mannobiose and mannotriose were detected when the oligosaccharide products were compared to the standard sugar solution using thin layer chromatography. Features of the surface of untreated SCGs and treated SCGs were observed using scanning electron microscopy and the surface of treated SCGs at 50 °C still had a good appearance, with only some parts destroyed, and pores had formed. This feature would promote access into the SCGs and make the digestion process much more efficient while was also supported by the results from the reducing sugar analysis.

Introduction

Nowadays, utilizing agricultural and agro-industry wastes such as sugarcane bagasse, straw, soybean grounds, and spent coffee grounds by several industries has become accepted and is widely practiced (Ballesteros et al., 2014). These wastes mostly contain lignocellulose as the main component from which hemicellulose and cellulose are digested by lignocellulolytic enzymes to produce oligosaccharides (Mussatto et al., 2011). These oligosaccharides can be used as a substrate in various industries, for example, for the production of energy, food, and animal feed (Kim et al., 2016). However, lignocellulose has a complex structure (Iroba et al., 2013). Therefore, to increase the efficiency of lignocellulolytic enzymes, a pretreatment process is important and necessary for using agricultural and agro-industry wastes as a substrate to produce oligosaccharide at the industrial level (Kim et al., 2016).

There are many methods of pretreatment, for example, physical pretreatment, steam explosion, ammonia fiber explosion (AFEX), ozonolysis, acid hydrolysis, and alkaline hydrolysis (Mosier et al., 2005). Different pretreatment methods and conditions can be chosen according to the type of materials, cost, and suitability. Among pretreatment methods, alkaline hydrolysis was applied in this work because NaOH solutions are carried out under milder conditions, are less polluting and use a less corrosive chemical than acid (Kim et al., 2016), while furthermore, alkaline pretreatment has an important role in the breakdown of lignin (Iroba et al., 2013). In addition, previous research by Chantorn et al. (2012) found that pretreatment of coffee grounds using alkaline

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hydrolysis was better than applying acid hydrolysis. They found that when treated SCGs (using alkaline hydrolysis and acid hydrolysis) were used as a substrate for digestion using mannanase, under experimental conditions, treated SCGs from alkaline hydrolysis could produce more reducing sugar than treated SCGs using the acid hydrolysis method. In the current research, spent coffee grounds as solid waste was used as it was easy to acquire, had a cheap cost and could be found nearby in massive amounts due to the rise in fresh coffee consumption. In addition, SCGs have other beneficial uses, for example, as fertilizer, insect pest repellent, for deodorizing unwanted smells and as material in mushroom cultivation (Ballesteros et al., 2014). SCGs were found to be rich in sugars (45.3%, weight per weight, w/w), among which hemicellulose and cellulose corresponded to 36.7% (w/w) and 8.6% (w/w), respectively, and the hemicellulose extracted from SCGs contained 21.1% mannans, 13.8% galactans and 1.7% arabinans (Mussatto et al., 2011). Differences in the amounts of each component depending on the species of coffee bean. As indicated, coffee grounds mostly contain mannans. Thus, it is possible for them to be digested by mannanase and to then use this as a substrate to produce manno-oligosaccharide (MOS). Mannanase can be found in microorganisms largely isolated from natural environments such as bacteria, actinomycetes, yeasts, and fungi (Dhawan and Kaur, 2007). Bacteria that produced mannanase were isolated from soil using copra meal as a carbon source and most were identified as *Bacillus* (Abe et al., 1994). Chantorn et al. (2015) found *Bacillus* sp. GA2(1) produced mannanase when SCGs were used as the carbon source. Therefore, mannanase from *Bacillus* sp. GA2(1) was used in the current study. The objectives of this research were to investigate the optimum conditions for the pretreatment process of SCGs using alkaline hydrolysis and then to use the substrate produced as a substrate to produce oligosaccharide, and then to analyze the type of sugar found in SCGs using mannanase from *Bacillus* sp. GA2(1) and to observe the surface features of the SCGs.

Materials and methods

Material preparations

Spent coffee grounds

The SCGs used in the research were sponsored by Bake@Dome Café, Office of the Rector, Thammasat University, Rangsit campus, Thailand. First, the SCGs were dried at 60 °C for 24 h. After that, the SCGs were sieved to be smaller than 45 μm and the selected SCGs were stored for later use in all the research.

Bacterial strain

Bacillus sp. GA2(1) stored at −20 °C in nutrient broth was grown in nutrient broth at 37 °C and 200 rpm for 16–18 h. Then, the product was used as an inoculum to produce mannanase.

Mannanase production

First, *Bacillus* sp. GA2(1) inoculum (5 mL) with an absorbance level of 600 nm = 0.5 were added into 500 mL of mineral salt broth (applied from Berg et al., 1972) containing 0.20% weight per volume, w/v of $NaNO_3$, 0.05% w/v of K_2HPO_4, 0.02% w/v of $MgSO_4 \cdot 7H_2O$, 0.02% w/v of $MnSO_4 \cdot 2H_2O$, 0.02% w/v of $CaCl_2 \cdot H_2O$, 0.02% w/v of $FeSO_4 \cdot 7H_2O$, 1% w/v of peptone and 0.5% w/v of SCGs. After that, the solutions were incubated at 37 °C and 200 rpm, for 16–18 h. Lastly, the incubated solutions were centrifuged at 7000 rpm and 4 °C for 15 min. The supernatant or crude enzyme was kept for mannanase activity analysis.

Pretreatment methods

Determination of pretreatment factors

This research examined four factors that impacted on the SCGs pretreatment process: the concentration of NaOH (0.5–2.5 Normality); the ratio of SCGs to NaOH (1:1–1:5); the temperature (50–121 °C); and the reaction time. The experiments were performed serially with triplicate experiments. After each pretreatment process, treated SCGs (in each experiment) were collected in a cheesecloth and the pH of the sample was adjusted to 7 using distilled water (pH paper was used to measure the pH level). Afterward, each treated SCG sample was dried at 60 °C overnight and stored in a plastic bottle for use in the next analysis of digestion of the SCGs by mannanase from *Bacillus* sp. GA2(1). A summary of the pretreatment process of SCGs is shown in Fig. 1.

Sodium hydroxide concentration

SCGs samples were added to NaOH solution with concentrations of 0.5 N, 1.0 N, 1.5 N, 2.0 N and 2.5 N and incubated at 60 °C for 96 h. The ratio of liquid to solid was 1:1. For each concentration, samples were collected at 2 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, and 96 h to study the impact of reaction time.

The ratio of SCGs to NaOH solution

SCGs samples were incubated with NaOH at the optimum concentration at ratios of SCGs to sodium hydroxide solution equal to 1:1, 1:2, 1:3, 1:4, and 1:5, at 60 °C for 6 h. The final volume of all samples was 60 mL. After that, samples were collected every 1 h to analyze the digestion process of the SCGs using mannanase from *Bacillus* sp. GA2(1).

Temperature

SCGs samples were incubated with NaOH at the optimum concentration and the optimum ratio of SCGs to sodium hydroxide solution, at temperatures of 50 °C, 60 °C, 70 °C, and 80 °C for 6 h, at 110 °C for 15 min, and at 121 °C with a pressure rate of 15 psi for 30 min. For each experiment, samples were collected every 1 h.

Enzymatic hydrolysis of treated-spent coffee grounds

A sample of 5 mL of crude mannanase and 5 mL of 50 mM pH 6.0 citrate-phosphate buffer were added into a test tube that contained 0.05 g treated SCGs and then incubated at 50 °C for 300 min. The amount of reducing sugar released was analyzed by using the 3, 5-dynotrosalicylic acid (DNS) method (Miller, 1959). The amounts of reducing sugar of samples were compared with the control that used untreated SCGs.

Analysis

Mannanase activity analysis

The mannanase activity was analyzed by calculating the amount of reducing sugar with 3, 5-dynotrosalicylic acid (DNS) method (Miller, 1959), including 0.5% of 50 mM pH 6.0 citrate-phosphate buffer with 0.5% (w/v) of locust bean gum, mixed with 0.5 mL of crude enzyme solution and the solutions were incubated at 50 °C for 15 min. Next, the reaction was stopped by adding 1 mL of DNS and the mixtures were immediately immersed in boiling water at 100 °C for 10 min to inactivate the enzymes. Afterward, 5 mL of distilled water was added and the mixtures were determined for absorbance at 540 nm using spectrophotometry as well using the calculated activity of mannanase.

One unit of mannanase activity was defined as the amount of enzyme producing 1 μmol of mannose per minute under the experimental conditions.

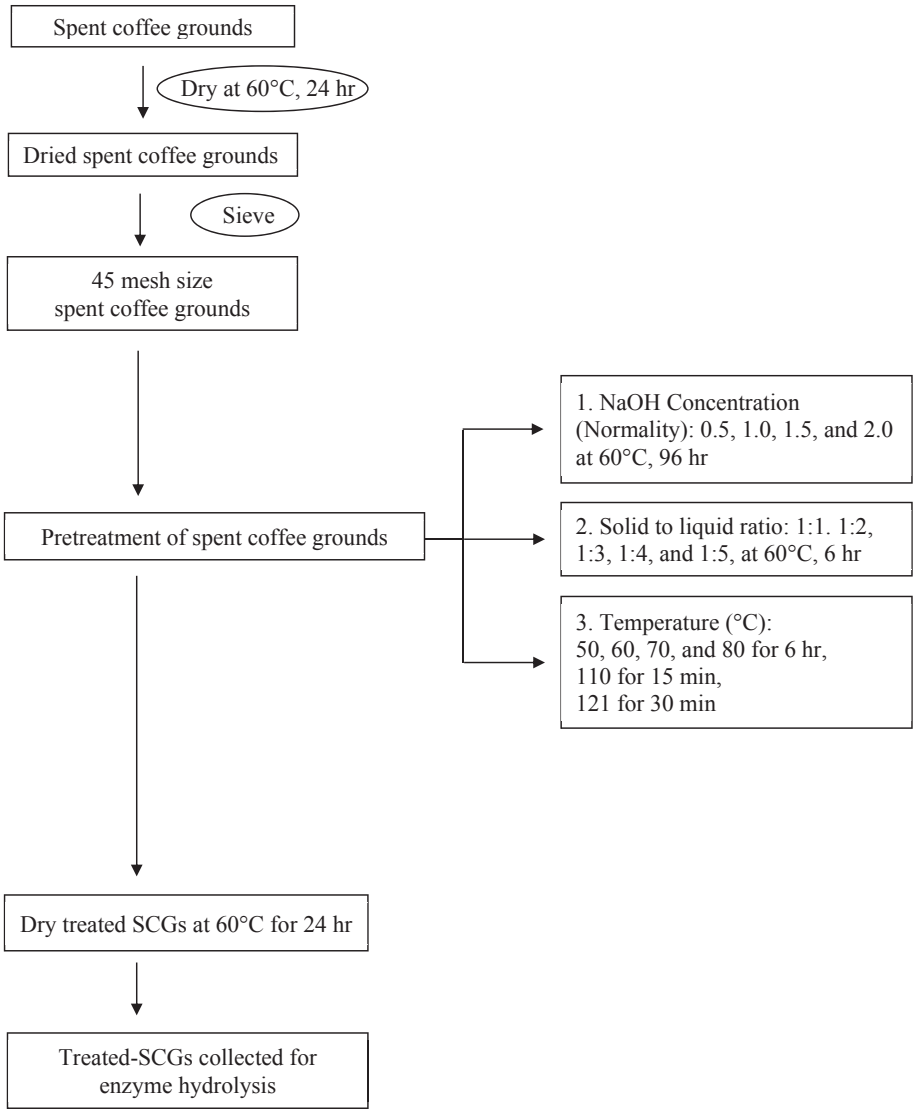

Fig. 1. Summary pretreatment process of spent coffee grounds (SCGs).

Analysis of treated-spent coffee ground hydrolysates using thin layer chromatography technique

Types of oligosaccharide sugar that are the product of sample substance were analyzed using thin layer chromatography technique (TLC) modified from Chantorn et al. (2013). The solvents used as the mobile phase were composed of butanol:isopropanol:ethanol:deionized water in the ratio of 2:3:3:2, respectively. a sample of 2 μL of each degradation product was applied on Kieselgel 60 (Merck) and developed for 90 min in developing solvent. The brown spots of sugars were developed by dipping in 0.2% (w/v) orcinol in 10% (v/v) sulfuric acid in ethanol and incubated at 100 °C until visible. The samples were compared with 0.1 M of standard sugar solutions of mannohexose, mannopentose, mannotetraose, mannotriose, mannobiose, mannose, galactose, glucose, and xylose.

Analysis of treated-spent coffee ground surface using scanning electron microscopy

The surfaces of treated SCGs were analyzed under a scanning electron microscope at the Central Scientific Equipment Laboratory, Institute of Research and Development, Kasetsart University, Bangkhen campus, Bangkok, Thailand.

Results and discussion

Mannanase activity analysis

There are diverse microorganisms that can be used as a substance to produce mannanase including bacteria, yeast, fungi, and protozoa. Abe et al. (1994) reported most of the bacteria that can be used to produce mannanase are in the *Bacillus* genus. The mannanase activity produced from *Bacillus* sp. GA2(1) was 0.72 ± 0.15 U/mL. In this research, SCGs were used as the carbon source and inductor for enzyme production. Since SCGs contain mannan and arabinogalactan as main components, the main enzyme from the production is mannanase which has the ability to digest mannan (other enzyme activities such as cellulase and xylanase are not present). Therefore, mannanase that had an enzyme activity of 0.72 ± 0.15 U/mL was chosen to be used in the further studies of this research.

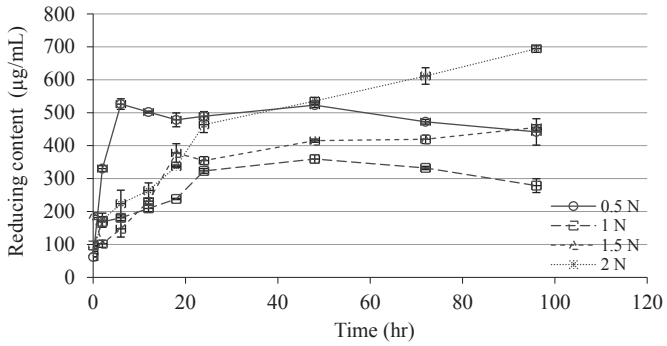

Fig. 2. Amount of reducing sugar from treated spent coffee grounds with various NaOH concentrations and hydrolysis by mannanase from *Bacillus* sp. GA2(1) at 50 °C for 300 min; error bars indicate mean ± SD (n = 3).

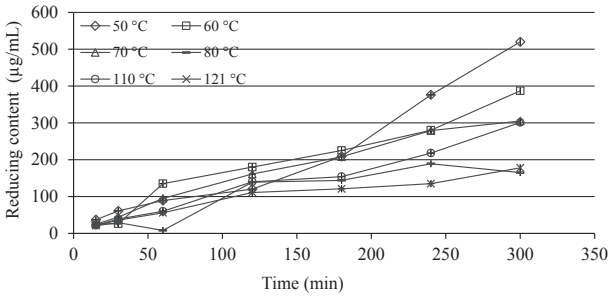

Fig. 4. Amount of reducing sugar from treated spent coffee grounds with various temperatures and hydrolysis by mannanase from *Bacillus* sp. GA2(1) at 50 °C for 300 min; error bars indicate mean ± SD (n = 3).

Effect of NaOH concentration

The primary research that compared the efficiency of SCG pretreatment between acid hydrolysis and alkaline hydrolysis, and used treated SCGs as a substrate for mannanase from *Bacillus* sp. GA2(1), found that using alkaline-treated SCGs as a substrate-enzyme produced more reducing sugar than with untreated SCGs and acid-treated SCGs. The alkaline method, especially with NaOH, was more efficient than the acid method when used for enhancing enzymatic hydrolysis. This action creates pores and increases the surface of the substrate so enzymes will able to easily digest the substrate (Donohoe et al., 2011). Consequently, further studies used the optimized conditions suitable for alkaline pretreatment of SCGs, with 0.5 N, 1.0 N, 1.5 N and 2.0 N NaOH concentrations, for 96 h.

Fig. 2 shows the concentration of 2 N at 96 h provided the best conditions which released the maximum amount of reducing sugar. However, the amount of reducing sugar readily increased early in the incubation periods and the concentration of 0.5 N released the maximum amount of reducing sugar at 6 h, so these conditions (0.5 N at 6 h and 2 N at 96 h) were chosen for estimating other significant factors (the ratio of SCGs to NaOH solution). The results indicated that treated SCGs using 2 N and 96 h released a lower amount of reducing sugar than at the concentration of 0.5 N NaOH solution. Shuhaida and Soh (2016) reported that the hemicellulose components were easily solubilized under mild pretreatment conditions (at lower NaOH concentrations and a shorter time) but were easily degraded under harsh pretreatment conditions (higher NaOH concentrations and longer times) as a high NaOH concentration caused the hemicellulose to degrade to other unknown byproducts which result in a low hemicellulose composition. Siti et al. (2013) reported the hemicellulose content was significantly higher at a lower NaOH concentration and diminished at higher NaOH concentration. This was probably due to the solubilization of hemicellulose compounds that increased when treated with concentrated NaOH. The reducing sugar content also showed similar trends to the hemicellulose contents. This might have been due to the fact that the alkali pretreatment process generally utilized the lignocelluloses content at a lower temperature. Then, the concentration of 2 N NaOH at 96 h proved harsh pretreatment conditions resulting in a low hemicellulose component as well as a low amount of reducing sugar. In addition, treated SCGs from the concentration of 2 N NaOH at 96 h required a lot of time for pretreatment and adjustment of the pH. Moreover, the high NaOH concentration caused a pungent smell during the process and the mixture of SCGs and 2N NaOH were more viscous

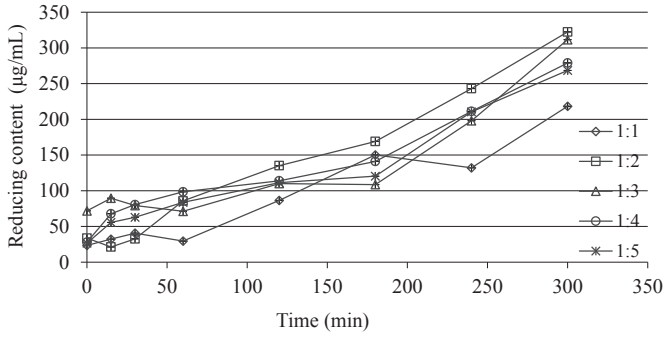

Fig. 3. Amount of reducing sugar from treated spent coffee grounds with various ratio of SCGs to NaOH solution and hydrolysis by mannanase from *Bacillus* sp. GA2(1) at 50 °C for 300 min; error bars indicate mean ± SD (n = 3).

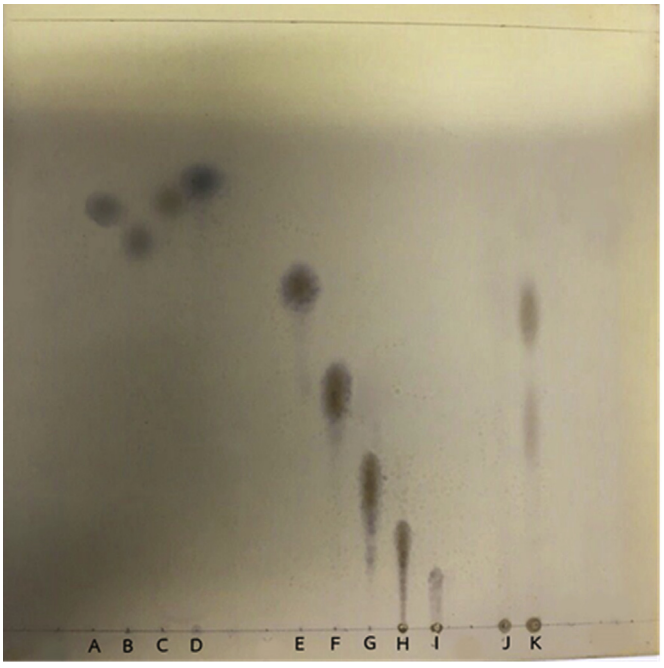

Fig. 5. Oligosaccharide patterns from spent coffee ground (SCG) hydrolysates by crude mannanase from *Bacillus* sp. GA2(1). Lane A: glucose, Lane B: galactose, Lane C: mannose, Lane D: xylose, Lane E: mannobiose, Lane F: mannotriose, Lane G: mannotetraose, Lane H: mannopentaose, Lane I: mannohexaose, Lane J: untreated SCGs degradation by mannanase *Bacillus* sp. GA 2(1) and Lane K: treated SCGs degradation by mannanase *Bacillus* sp. GA 2(1).

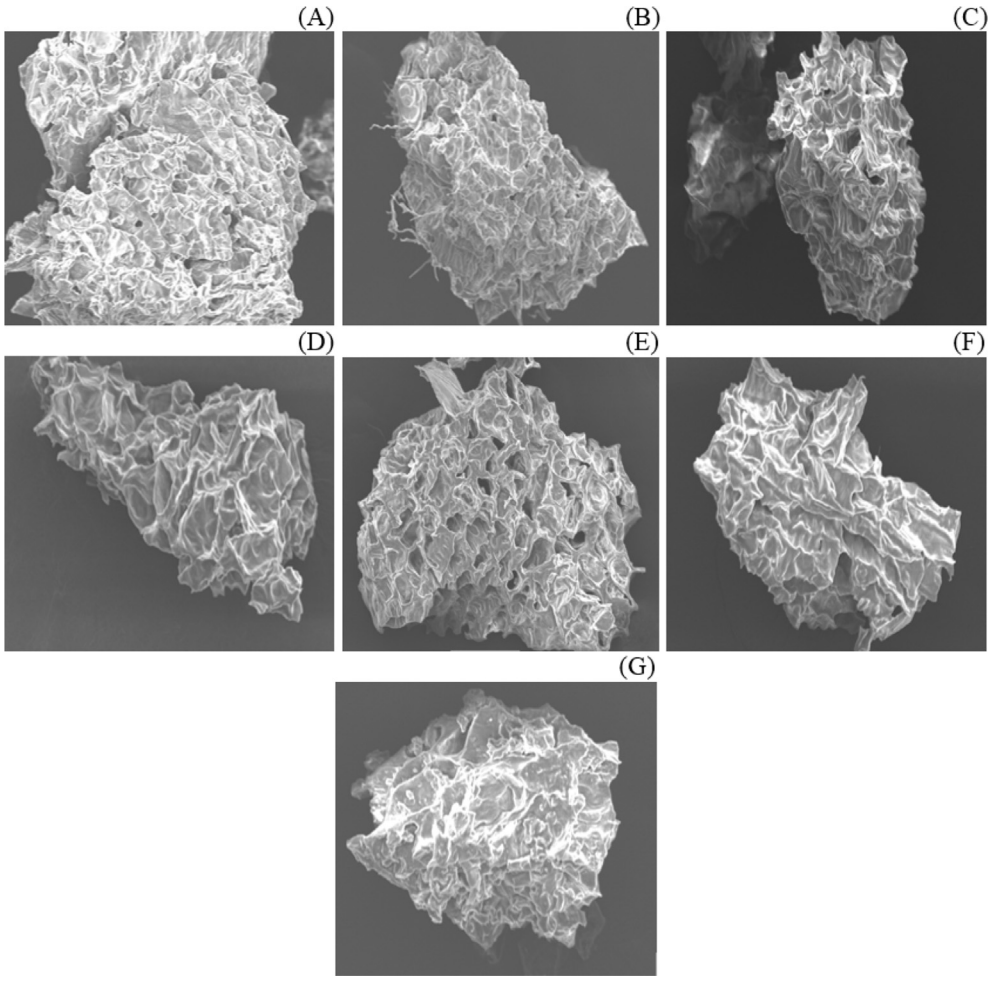

Fig. 6. Surface appearance of spent coffee grounds (SCGs) using scanning electron microscopy: (A) 50 °C treated SCGs; (B) 60 °C treated SCGs; (C) 70 °C treated SCGs; (D) 80 °C treated SCGs; (E) 110 °C treated SCGs; (F) 121 °C treated SCGs; (G) untreated SCGs.

than the concentration with 0.5 N. Therefore, the concentration of 0.5 N and the reaction time of 6 h that released the highest amount of reducing sugar (526.25 ± 16 μg/mL) within a short time were chosen as the optimum conditions for the SCGs pretreatment.

Effect of the ratio of spent coffee grounds to NaOH solution

Fig. 3 illustrates that the SCGs to NaOH solution at a ratio of 1:2 provided the best conditions for the SCG pretreatment process to produce the most reducing sugar (514 ± 0.005 μg/mL). Arantes et al. (2011) reported that the enzyme activity will increase when the substrate has a sufficiently surface area for enzymatic hydrolysis. The increased enzyme activity enhanced the efficiency of SCG hydrolysis so the amount of reducing sugar produced increased as well. Moreover, pore size and shape of the substrate after pretreating are also important. Nevertheless, at the ratio of 1:1 even with a low amount of SCGs, there was the lowest amount of reducing sugar produced perhaps because the volume of NaOH that reacted with the low amount of SCGs initiate unsuitable surface conditions for the hemicellulose so when the enzyme reaction occurred, the amount of reducing sugar was very low compared with other ratios. As a result, the ratio of 1:2 was appropriately selected for the pretreatment of SCGs.

Effect of temperature

Physical factors in terms of the temperature are important for the SCG pretreatment process. To improve the efficiency of enzymatic hydrolysis, using a higher temperature will destroy the structures of cellulose and lignin. In addition, the ability of an enzyme will be more efficient when the pretreatment process is conducted at high temperature (Ohgren et al., 2005). However, the current study found that SCGs treated at higher than 60 °C produce less reducing sugar (Fig. 4). This might have been a result of the excessive temperature level destroyed the hemicellulose structure of the SCGs. Chen et al. (2012) reported that higher temperatures increased carbohydrate loss through random chain cleavage, which can greatly reduce the sugar yield from the overall process. Therefore, the amount of reducing sugar was lower when the SCGs treated at high temperature were digested by crude mannanase from *Bacillus* sp. GA2(1). From observation, the optimum temperature for the SCG pretreatment process was at 50 °C and the amount of reducing sugar increased to 520 ± 0.01 μg/mL when digested was by crude enzyme solution from *Bacillus* sp. GA2(1).

Treated spent coffee ground hydrolysate types using thin layer chromatography

TLC was used to analyze the type of hydrolysate products from the treated SCGs using the crude enzyme from *Bacillus* sp. GA2(1). By comparing products to standard sugar solutions, it was found that the oligosaccharide products from digestion of treated SCGs were mannobiose and mannotriose (Fig. 5). The pretreatment processes caused changes to the SCG structure which made it easier for the enzymes from *Bacillus* sp. GA2(1) to access the SCGs and activate. The TLC results indicate that the amount of reducing sugar was higher as well as producing more mannobiose and mannotriose as an oligosaccharide product (Fig. 5, lane K). However, it can be seen that the bands of product from treated SCGs was slightly lower than for the standard sugars. In order to confirm the TLC result, the step of further analyzing types and amounts of oligosaccharide will be conducted by using a chromatography technique such as high-performance liquid chromatography and high-performance anion exchange chromatography which are the technologies that can be used to analyze both qualitative and quantitative aspects. In addition, the oligosaccharide products demonstrated that the main enzyme extracted from *Bacillus* sp. GA2(1) was mannanase (Fig. 5), as the only mannobiose and mannotriose were detected which are products from the reaction of mannanase only.

Analysis of treated spent coffee ground surface using scanning electron microscopy

The efficiency of pretreatment processes can be measured not only by the amount of reducing sugar from treated SCG digestion with the crude enzyme solution but also by observing the surface area of pretreated substrates. Fig. 6 illustrates the surface features of SCGs prepared from different pretreatment conditions. Under SEM, it was observed that more SCGs were destroyed when the temperature was higher. Parts of the surface of SCGs were clearly ruined and even the inner structure also can be seen. This could impact the accessibility of enzyme so the amount of reducing sugar would be decreased. The temperature results showed that 50 °C was the optimum temperature for pretreatment. Fig. 6A shows the surface of treated SCGs are less affected at this temperature compared to the other treated-SCG conditions and there are many pores. Therefore, 50 °C is the best temperature for SCGs pretreatment.

In conclusion, the alkaline pretreatment process is necessary to improve enzyme efficiency in the production of the oligosaccharide. The optimum conditions for the pretreatment process of SCGs are: a concentration of NaOH solution equal to 0.5 N, a ratio of SCGs to NaOH solution of 1:2, the optimum temperature is 50 °C and the incubation periods is 6 h. When the amount of reducing sugar from treated SCGs and untreated SCGs under the studied conditions were compared, with untreated SCGs it was found that alkaline pretreatment of SCGs had a substantial effect on increasing the amount of reducing sugar. As demonstrated by SEM, this method can change the structure of SCGs but under the optimum conditions, there was no breakage of the structure of SCGs so that it became unsuitable for the accessibility of enzyme. According to the TLC results, the pretreatment process of SCGs under the optimum conditions will produce mannobiose and mannotriose. Therefore, it is possible to prepare oligosaccharide sugar products from treated SCGs which will also potentially add value for agricultural and agro-industry wastes.

Conflict of interest

The authors declare that there are no conflicts of interest.

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