



Faculty of Resource Science and Technology

**Optimisation and Comparative Analysis of Alkaline Pre-treatment of
Defatted Spent Coffee Ground to Increase Sugar Recovery**

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Optimisation and Comparative Analysis of Alkaline Pre-treatment of Defatted Spent Coffee Ground to Increase Sugar Recovery

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

Spent coffee grounds (SCG) have the potential as a raw material for producing valuable products due to their cellulose and hemicellulose content. However, their complex lignocellulosic structure makes sugar extraction challenging. Effective pre-treatment and enzymatic hydrolysis are therefore essential to maximise sugar recovery. This study optimised the alkaline pre-treatment of defatted SCG following enzymatic hydrolysis using Response Surface Methodology (RSM) of 5-level, 3-factor Central Composite Design (CCD) by examining the effects of alkaline concentration, temperature, and incubation time on reducing sugar yield after enzymatic hydrolysis to maximise reducing sugar recovery. The optimal conditions for KOH pre-treatment were 3% (w/v) KOH concentration at 90 °C for 2 h, yielding a 2.97-fold increase, while NaOH pre-treatment at 3% (w/v) NaOH, 85 °C for 1 h achieved a 3.28-fold increase over the control. For second phase of the study, acid pre-treatment was combined with alkaline pre-treatment. Acid was included for its effectiveness in hydrolysing hemicellulose and was used synergistically with alkaline pre-treatment, which primarily removes lignin. Following pre-treatment, enzymatic hydrolysis with cellulase was applied to convert cellulose into glucose. To compare pre-treatment strategies, one-step pre-treatment methods using either alkali (KOH, NaOH) or acid (H₂SO₄), and two-step pre-treatment combinations (acid–alkali and alkali–acid), were statistically evaluated using One-Way ANOVA. Results showed that two-step pre-treatment achieved the highest sugar recovery (4.25–4.70 folds) which significantly greater than one-step pre-treatment methods (1.50–3.28 folds), demonstrating the synergistic effect of acid-base combinations to enhance lignin removal, hemicellulose solubilisation and enzyme accessibility, thereby improving reducing sugar release. This study explores the valorisation

of SCG into reducing sugars by offering a sustainable solution to mitigate the environmental impact of SCG disposal.

Keywords: Alkaline pre-treatment, acid-base combination pre-treatment, central composite design, response surface methodology, spent coffee grounds, sugar recovery

*Pengoptimuman dan Analisis Perbandingan Pra-Rawatan Beralkali Terhadap
Hampas Kopi Tanpa Lemak untuk Meningkatkan Perolehan Gula*

ABSTRAK

Hampas kopi (SCG) berpotensi sebagai bahan mentah untuk menghasilkan produk bernilai kerana kandungan selulosa dan hemiselulosanya. Walau bagaimanapun, struktur lignoselulosa SCG yang kompleks menjadikan pengekstrakan gula mencabar. Oleh itu, pra-rawatan yang berkesan dan hidrolisis enzimatik adalah penting untuk memaksimumkan perolehan gula. Kajian ini mengoptimumkan pra-rawatan beralkali bagi SCG tanpa lemak diikuti hidrolisis enzimatik menggunakan Kaedah Permukaan Gerak Balas (RSM) dengan Reka Bentuk Komposit Pusat (CCD) 5 aras, 3 faktor untuk menilai kesan kepekatan alkali, suhu dan masa inkubasi terhadap perolehan gula penurun selepas hidrolisis enzimatik. Keadaan optimum bagi pra-rawatan KOH ialah pada kepekatan 3% (w/v) KOH pada 90 °C selama 2 jam, yang menghasilkan peningkatan sebanyak 2.97 kali ganda, manakala pra-rawatan NaOH pada 3% (w/v) NaOH pada 85 °C selama 1 jam mencapai peningkatan sebanyak 3.28 kali ganda berbanding kawalan. Dalam fasa kedua kajian, pra-rawatan berasid digabungkan dengan pra-rawatan beralkali. Asid dimasukkan kerana keberkesanannya dalam menghidrolisis hemiselulosa dan digunakan secara sinergistik dengan pra-rawatan beralkali yang berfungsi menghapuskan lignin. Selepas pra-rawatan, hidrolisis enzimatik menggunakan selulase dijalankan untuk menukarkan selulosa kepada glukosa. Bagi membandingkan strategi pra-rawatan, kaedah pra-rawatan satu langkah menggunakan sama ada alkali (KOH, NaOH) atau asid (H_2SO_4), serta kombinasi pra-rawatan dua langkah (asid–alkali dan alkali–asid), telah dinilai secara statistik menggunakan ANOVA Sehalu. Keputusan menunjukkan pra-rawatan dua langkah mencapai perolehan gula penurun tertinggi (4.25–4.70 kali ganda) yang secara signifikan lebih tinggi

daripada kaedah pra-rawatan satu langkah (1.50–3.28 kali ganda). Ini membuktikan kesan sinergistik kombinasi alkali–asid dalam meningkatkan penyingkiran lignin, melarutkan hemiselulosa dan akses enzim, sekali gus meningkatkan pembebasan gula penurun. Kajian ini meneroka potensi SCG tanpa lemak menjadi gula penurun sebagai penyelesaian lestari untuk mengurangkan kesan alam sekitar akibat pelupusan SCG.

Kata kunci: *Pra-rawatan berakali, pra-rawatan kombinasi asid-bes, reka bentuk komposit pusat, kaedah permukaan gerak balas, hampas kopi, perolehan gula*

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- (h) NaOH- H_2SO_4 pre-treated sample
- (i) H_2SO_4 -NaOH pre-treated sample

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LIST OF ABBREVIATIONS

α	Alpha
%	Percentage
$\mu\text{g/g}$	Micrograms per gram
$\mu\text{g/mL}$	Micrograms per millilitre
5-HMF	5-Hydroxymethylfurfural
ADF	Acid detergent fiber
ADL	Acid detergent lignin
AOCS	American Oil Chemist Society
AOAC	Association of Official Analytical Chemists
ANOVA	Analysis of variance
CCD	Central composite design
CaCO_3	Calcium carbonate
C/N	Carbon-to-nitrogen
CBU/g	Cellobiase unit per gram
$^{\circ}\text{C}$	Degree Celsius
CTAB	Cetyltrimethylammonium bromide
CV	Coefficient of variation
df	Degree of freedom

DNS	Dinitrosalicylic acid
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acid
FFA	Free fatty acid
FPU/g	Filter paper unit per gram
GDP	Gross domestic product
g	Gram
g/g	Grams per gram
g/kg	Grams per kilogram
g/L	Grams per liter
g/mL	Grams per milliliter
GE	Glucose equivalent
h	Hour
H ₂ SO ₄	Sulfuric acid
H ₃ PO ₄	Phosphoric acid
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HSD	Highest significant difference

IUPAC	International Union of Pure and Applied Chemistry
ICO	International Coffee Organization
IDF	Insoluble dietary fiber
kg	Kilogram
KMnO ₄	Potassium permanganate
KOH	Potassium hydroxide
LCC	Lignin-carbohydrate complexes
lbs/sq.inch	Pounds per square inch
M	Molarity
mg	Milligram
mg/g	Milligrams per gram
mg/kg	Milligrams per kilogram
mg/mL	Milligrams per milliliter
min.	Minute
MNU/g	Mannanase unit per gram
mL	Milliliter
mL/min	Milliliters per minute
MS	Mean squares
mM	Millimolar

Na^+	Sodium ions
$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$	Sodium tetraborate decahydrate
Na_2CO_3	Sodium carbonate
Na_2SO_3	Sodium sulfite
NaOH	Sodium hydroxide
N	Normality
NDF	Neutral detergent fiber
Nm	Nanometer
OECD	Observatory of Economic Complexity
OH^-	Hydroxide ions
pH	Potential of hydrogen
PLA	Polylactic acid
PHAs	Polyhydroxyalkanoates
PSA	Phenol-sulfuric acid
rpm	Revolutions per minute
R^2	Coefficient of determination
RSM	Response surface methodology
SCG	Spent coffee ground
SDF	Soluble dietary fiber

SEM	Scanning electron microscope
SmF	Submerged fermentation
SS	Sum of squares
SSF	Solid state fermentation
<i>sp.</i>	Species
TDF	Total dietary fiber
TRS	Total reducing sugar
TS	Total sugar
U/g	Enzyme units per gram
U/mL	Enzyme units per milliliter
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

CHAPTER 1

INTRODUCTION

1.1 Study Background

According to The International Coffee Organization (ICO): Coffee Break Series No. 1 (April 2020), the consumption of coffee increased over the last 50 years pre-pandemic COVID-19. However, the coffee consumption has decreased significantly during global pandemic as countries were adopting a full or partial lockdown that forced coffee shops, restaurants and offices to close in order to avoid the spread of coronavirus. The regression results based on their analysis showed a one percent drop in GDP growth globally on average is associated with 0.95 percent or 1.6 million 60-kg bags lower growth in coffee consumption, in relative terms. During this period, low coffee consumption lead to the decrease in the import and export of coffee.

Fast forward to post-pandemic, the world coffee exports amounted to 9.38 million and 10.24 million in November 2021 and 2022 respectively. This shows the export increment by 1.6% from 19.56 million bags for the first two months of coffee year 2022/23 between October and November in comparison with 19.25 million bags for the same period in 2021/22. During the 2022/23 coffee year, consumption declined by 2.0% due to high living costs, reduced disposable income, and stock drawdowns, despite coffee's relatively inelastic demand. However, forecasts for 2023/24 showed a positive increment, with a projected 2.2% increase in consumption, driven by anticipated global economic growth exceeding 3.0% and restocking efforts (ICO, 2024).

Coffee is one of the most widely traded agricultural commodities in the world, with consumption and trade playing a significant role in both international and domestic markets (Chéron-Bessou *et al.*, 2024; Utrilla-Catalan *et al.*, 2022; McNutt and He, 2019). Developing countries serve as the main suppliers, while developed countries represent the largest consumers, with demand highly concentrated in certain regions (Vegro and de Almeida, 2020). Brazil, Vietnam, Colombia, and Indonesia account for about 80% of global coffee production, while Malaysia also participates in the international coffee trade including import and export. In 2021, Malaysia ranked 34th among global coffee exporters, generating approximately \$105 million, while also ranking 26th as an importer with imports worth \$229 million, primarily from Vietnam, Indonesia, Colombia, Brazil, and Ethiopia (Karmee, 2018; OEC, 2021). These figures illustrate the strong economic impact of the coffee industry, particularly in Malaysia where coffee trade significantly contributes to the national economy.

However, the global demand for coffee also results in the generation of substantial amounts of waste. During processing and brewing, large quantities of residues such as coffee pulp, husks, silverskin, and especially spent coffee grounds (SCG) are produced (Echeverria and Nuti, 2017; Arya *et al.*, 2021). The most abundant by-product is SCG, which generated after hot water extraction of ground coffee beans to prepare beverages. Although non-edible, SCG contains bioactive compounds such as caffeine and polyphenols that, if improperly disposed of, can pose environmental hazards. Common disposal methods including landfilling, composting, and incineration that contribute to soil contamination, greenhouse gas emissions, and air pollution (Cruz *et al.*, 2012; Taifouris *et al.*, 2021).

From a resource perspective, SCG is recognized as a lignocellulosic biomass with high potential for valorization. Its composition mainly consists of polysaccharides, including cellulose, hemicellulose, and lignin (Mussatto *et al.*, 2012; Ballesteros *et al.*, 2015). Cellulose is a homopolymer of glucose units, while hemicellulose is a heteropolymer containing sugars such as glucose, mannose, arabinose, and galactose. These structural polysaccharides represent an important feedstock for sugar recovery, which can be converted into value-added products such as biofuels, biochemicals, and bioplastics (Ballesteros *et al.*, 2015; Atabani *et al.*, 2019; Battista *et al.*, 2020).

Despite its potential, the efficient utilization of SCG faces challenges due to the recalcitrant structure of lignin, which restricts access to fermentable sugars during hydrolysis. This necessitates the use of effective pre-treatment methods and enzymatic hydrolysis to break down the complex lignocellulosic matrix and release fermentable sugars. Alkaline pre-treatment with KOH and NaOH has been shown to enhance delignification and improve sugar yield. Recently, the development of two-step pre-treatment strategies, which combine alkaline and acid methods sequentially, has been explored to further maximise sugar recovery by leveraging the complementary mechanisms of each treatment (Ravindran *et al.*, 2017; Trinh *et al.*, 2022). To optimise such processes, statistical tools such as response surface methodology (RSM) are employed to determine the best combination of parameters, including alkaline concentration, temperature, and incubation time. This to maximise the targeted yield while minimise cost and material use (Ostertagová and Ostertag, 2013; Rajewski and Dobrzyńska-Inger, 2021).

1.2 Problem Statement

SCG are rich in carbohydrates, yet the recovery of fermentable sugars is highly challenging due to their rigid and recalcitrant lignocellulosic structure (Trinh *et al.*, 2022). Structural barriers such as lignin, hemicellulose, and acetyl groups hinder enzymatic access to cellulose, making pre-treatment an essential step to enhance hydrolysis efficiency and improve biomass conversion (Zhang *et al.*, 2021; Kim *et al.*, 2023). Among various methods, alkaline pre-treatment has been widely studied because it promotes biomass swelling, disrupts hemicellulose, reduces cellulose crystallinity, and effectively removes lignin through delignification. These modifications increase enzyme accessibility and improve sugar recovery (Park and Kim, 2012; An *et al.*, 2021). Additionally, alkaline pre-treatment is cost-effective compared to other chemical methods, making it a promising approach for large-scale applications. Once the biomass structure is disrupted, enzymatic hydrolysis can efficiently convert carbohydrate polymers into fermentable sugars (Jin *et al.*, 2020).

Despite these advantages, research on pre-treatment optimization of SCG remains limited. Most studies have focused on raw SCG, while comparatively little attention has been given to defatted SCG, which may exhibit distinct structural properties. Trinh *et al.* (2022) demonstrated that defatting SCG through sonication before alkaline pre-treatment significantly improved sugar recovery by facilitating lignin disruption. However, comprehensive optimisation studies such as determining the best alkali type, concentration, temperature, and incubation time are still scarce for defatted SCG. Moreover, there is a lack of comparative evaluations between one-step pre-treatments (acid or alkali alone) and sequential two-step strategies that combine acid and alkaline treatments. These unexplored areas represent a key knowledge gap, as understanding the impact of different pre-treatment approaches is critical for developing more efficient and sustainable bioconversion processes.

1.3 Research Aim and Objectives

Therefore, this research aimed to determine the best conditions for alkaline pre-treatment of defatted SCG in order to maximise sugar recovery. This can be achieved through the following set of objectives:

- i. To optimise the pre-treatment of defatted SCG using KOH by focusing on key parameters: KOH concentration, temperature and incubation time.
- ii. To optimise the pre-treatment of defatted SCG using NaOH by focusing on key parameters: NaOH concentration, temperature and incubation time.
- iii. To conduct a comparative analysis between one-step pre-treatment (either using acid or alkali) and two-step pre-treatment (combining both acid and alkali) of defatted SCG.

1.4 Scope and Limitations

This study focuses on optimising the alkaline pre-treatment of defatted spent coffee grounds (SCG) using potassium hydroxide (KOH) and sodium hydroxide (NaOH) to maximise sugar recovery. The optimisation process specifically considers key parameters, including alkali concentration, temperature, and incubation time, with the aim of enhancing delignification and disrupting hemicellulose to improve enzyme accessibility. Enzymatic hydrolysis is subsequently performed using cellulase, which selectively targets cellulose for conversion into fermentable sugars. In addition, the research incorporates a comparative evaluation of one-step pre-treatments (acid or alkali alone) and two-step pre-treatments (acid combined with alkali) to better understand how different approaches influence sugar yield.

The scope of this work is restricted to alkaline pre-treatment optimisation, and no detailed optimisation of acid pre-treatment or enzymatic hydrolysis conditions was undertaken. While Soxhlet extraction is recognised as a more effective method for defatting SCG, its longer processing time and limited equipment availability necessitated the use of sonication as an alternative method. Similarly, enzymatic hydrolysis was carried out using a water bath with a shaker, which limited the process to using only cellulase and required longer incubation times. Furthermore, the results reflect laboratory-scale experimentation and may not fully represent industrial-scale applications.

1.5 Significance of Study

The environmental and economic costs of disposing SCG is undesirable as this method can leads to environmental pollution and increase the disposing cost for coffee waste. For these reason, both cost effective and environmental friendly alternatives for coffee waste management especially SCG are required. Utilizing the residue for these products not only can reduces its impact on the environment but also creates additional value by transforming waste from one industry into raw material for another. This aligns with the goal of zero-waste emissions, leading to a truly circular economy. Besides, SCG can be used to produce a variety of valuable products due to its composition. Several authors have investigated the use of SCG sugar and carbohydrate content to produce various types of biofuels, including biodiesel, bioethanol (Kondamudi *et al.*, 2008; Kwon *et al.*, 2013), bio-oil (Banerjee *et al.*, 2013; Vardon *et al.*, 2013), and bio-sugar (Gómez *et al.*, 2016; Nguyen *et al.*, 2019).

Additionally, sugar extractives of SCG such as manno-oligosaccharides can be utilized as food supplements and as bio-components in the pharmaceutical and cosmetic industries (Wongsiridetchai *et al.*, 2021) as it contain bioactive compounds such as phenolic extracts including chlorogenic, caffeic, and coumaric acids as well as carotenoids which shown to be beneficial for human health by acting as antibiotics, antioxidants, and anti-inflammatory agents (Bouhlal *et al.*, 2020). In the industry, SCG can be used as additives for industry such as polyphenolic compound including tannins (Low *et al.*, 2015), a monomer for producing polylactic acid (PLA) which is a biodegradable polymer for potentially replacing petroleum-based plastics (Bomfim *et al.*, 2023; Mak *et al.*, 2023) as well as a polymer including polyhydroxyalkanoates (PHA) (Obruca *et al.*, 2014; Obruca *et al.*, 2015). SCG also can be incorporated into compost (Santos *et al.*, 2017) and used as fertilisers (Ciesielczuk *et al.*, 2017).

CHAPTER 2

LITERATURE REVIEW

2.1 Coffee Beans Structure

Coffee plants, scientifically known as *Coffea*, are flowering plants that belong to the Rubiaceae family. The seeds of these plants, called coffee beans, are cultivated and harvested for people to drink. Coffee thrives in regions between the Tropic of Cancer and Tropic of Capricorn (Muthy and Naidu, 2012). There are about 70 known coffee species globally, with Arabica comprises of 75% and Robusta, which is more resistant to pests and damage, making up 24% of global coffee production. Coffee trees are perennial plants that start blooming within 5 years after planting.

As shown in Figure 2.1, coffee fruit or cherry contains two beans enclosed within a shell that resembles a thin parchment layer covered by pulp and produce bright red fruits (epicarp) containing a sticky substance (mesocarp), a thin parchment layer (endocarp), and a silverskin (spermoderm) covering the coffee beans according to the description by Esquivel and Jimenez (2012). Coffee trees are cultivated at higher altitudes between 600 and 1200 meters with average temperatures of 15 to 25 °C with moderate humidity, and cloud formation. Additionally, coffee plants can be harvested 6 years after maturity when the coffee turns red, which occurs 8 to 12 months after flowering while the peak yield can be obtained after 10 to 15 years after planting (Arya and Rao, 2007).

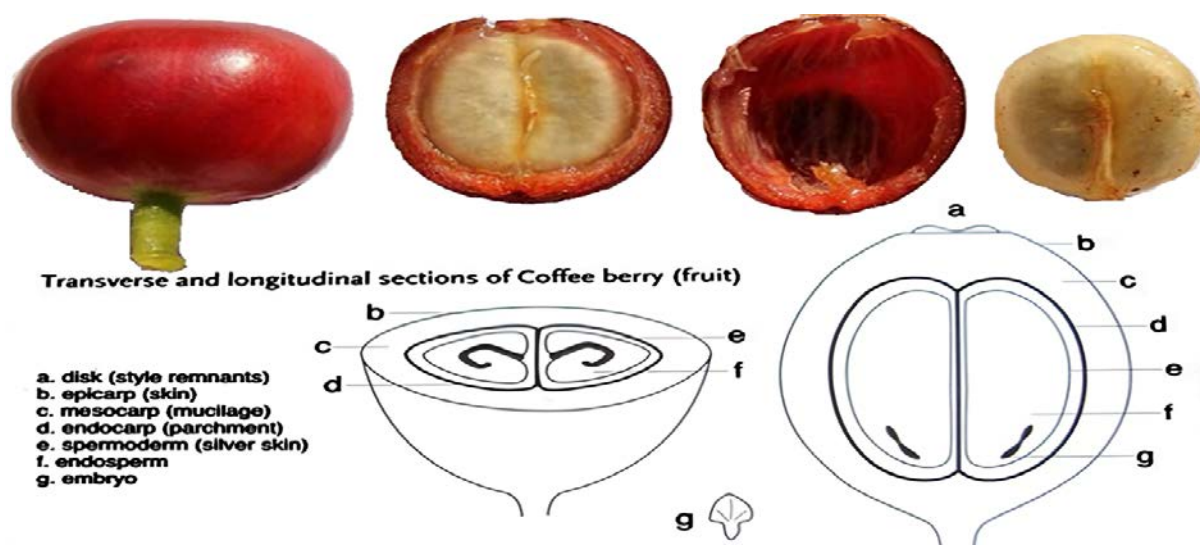


Figure 2.1: Structure of coffee beans. Adapted from Arya *et al.* (2021)

2.2 Coffee Beans Composition

The coffee bean contains a diverse array of components, including micronutrients, amino acids, fatty acids, minerals, and bioactive compounds such as antioxidant and organic acids. The specific composition of coffee beans is outlined in detail in Table 2.1. The components and the quantity stated in table below was obtained and compiled from several studies.

Table 2.1: Chemical composition of coffee bean. Adapted from Arya *et al.* (2021).

Components	Quantity	Reference
<i>Micronutrient</i>		
Glucose ($\mu\text{g/g}$)	300 – 2200	Murkovic and Derler (2006)
Mannose ($\mu\text{g/g}$)	< 300	
Galactose ($\mu\text{g/g}$)	100 – 600	
Fructose ($\mu\text{g/g}$)	1900 – 5900	
Sucrose (mg/g)	36 – 90	
Carbohydrates (g/kg)	450 – 650	Farah (2012)
Proteins (g/kg)	100 – 150	
Lipids (g/kg)	70 – 170	

Table 2.1 continued

<i>Amino acid (g/kg)</i>	78 – 140	Dong <i>et al.</i> (2017)
<i>Essential amino acids</i>	25.8 – 52.9	
Methionine (g/kg)	0.8 – 12.0	
Threonine (g/kg)	3.1 – 6.0	
Phenylalanine (g/kg)	4.0 – 8.5	
Lysine (g/kg)	4.7 – 7.3	
Isoleucine (g/kg)	2.9 – 5.3	
Leucine (g/kg)	4.9 – 8.1	
Valine (g/kg)	3.5 – 5.7	
<i>Non-essential amino acids</i>	52.1 – 95.2	
Serine (g/kg)	4.0 – 7.5	
Tyrosine (g/kg)	2.8 – 5.3	
Proline (g/kg)	3.9 – 11.9	
Glutamic acid (g/kg)	15 – 28.8	
Glycine (g/kg)	2.5 – 6.3	
Cysteine (g/kg)	1.3 – 2.5	
Alanine (g/kg)	3.7 – 8.1	
Asparagine (g/kg)	7.1 – 16.0	
Histidine (g/kg)	2.7 – 8.5	
Arginine (g/kg)	3.3 – 5.1	
<i>Fatty acids (FA) (%)</i>		Dong <i>et al.</i> (2017)
Saturated FA (%)	45 – 45.62	
Unsaturated FA (%)	1.18 – 1.21	
Mono unsaturated FA (%)	8.8 – 10.6	
Polyunsaturated FA (%)	43 – 45.5	

Table 2.1 continued

<i>Minerals</i>		
Titanium (µg/g)	0 – 5.3	Santato <i>et al.</i> (2012)
Cadmium (µg/g)	1.17 – 26	
Lead (µg/g)	3.05 – 152	
Molybdenum (µg/g)	0 – 436	
Chromium (µg/g)	17.3 – 87.2	
Nickel (µg/g)	25.7 – 3969	
Zinc (mg/kg)	6.95 – 21.0	
Copper (mg/kg)	6.95 – 21.0	
Manganese (mg/kg)	10.2 – 46.8	
Rubidium (mg/kg)	9.21 – 85.2	
Iron (mg/kg)	18.4 – 38.1	
Aluminium (mg/kg)	1447 – 44,431	
Calcium (mg/kg)	906 – 1572	
Sodium (mg/kg)	1.63 – 73	
Potassium (mg/kg)	14,827 – 21,570	
Magnesium (mg/kg)	1478 – 2476	
<i>Phenolic compounds and organic acids (mg/g)</i>		
Quercetin (mg/g)	0.01 – 0.13	Kroř <i>et al.</i> (2020)
Gallic acid (mg/g)	0.4 – 2.3	
Chlorogenic acid (mg/g)	4.0 – 8.0	
Salicylic acid (mg/g)	0.02 – 0.204	
Total polyphenols (mg/g)	8.6 – 8.8	
Total phenolics acids (mg/g)	0.59 – 8.74	
Total flavonoids (mg/g)	0.6 – 1.4	
Cafeic acid (mg/g)	0.007 – 0.16	
Caffeine (mg/g)	2.6 – 8.7	

2.3 Coffee Beans Processing

There are various methods to produce green coffee from freshly harvested coffee beans. One of the method is dry processing which involves sun-drying the cherries, followed by removing the dried pulp, parchment, and coffee silverskin. According to Arya *et al.* (2021), the wet method includes variations like pulped natural, semi-washed, and fully washed. In the pulped natural method, the mucilage is left on the parchment during drying, allowing sugars to infuse the bean, resulting in a sweeter coffee. Meanwhile, the semi-washed method utilized mechanical brushes and friction to remove the mucilage whereas the wet method involves significant bacterial, fungal, and yeast activity to digest pectin a sugar to produce a variety of by-product such as alcohols and acids to generate a wide flavour profiles (Klingel *et al.*, 2020). Roasting is another crucial step in coffee processing that affects the taste, smell, and colour of coffee which directly related to the quality of coffee drink, as stated by Cardoso *et al.* (2023).

In unroasted coffee beans, carbohydrates, nitrogen compounds including proteins, trigonelline, and caffeine as well as lipids, organic acids, and water are the precursors of aroma and flavour (Belitz *et al.*, 2009). During roasting, these precursors undergo thermal reactions to form aroma and flavour compounds (Worku *et al.*, 2018). The Maillard reaction and Strecker degradation are the main processes responsible for creating aroma compounds such as furanones, pyridines, pyrazines, furans, pyrroles, thiophenes and thiols while phenolic substances in coffee are converted into products like melanoidins, which change the biological activity and chemical composition of coffee (Lee *et al.*, 2015; Zhang *et al.*, 2019). Meanwhile, Lee *et al.* (2015) and Zhang *et al.* (2019) also reported that roasting can also create organic compounds such as browning compounds, aroma, and flavour compounds since amino acids and reducing sugars produced from the breakdown of proteins

and sucrose during roasting are precursors of key aroma compounds in roasted coffee. After roasting, coffee beans are ground and brewed with hot water to make coffee beverages.

After roasting, coffee beans must be cooled to prevent over-roasting and stop exothermic reactions (Baggenstoss *et al.*, 2007). The roasted beans are then ground using multi-stage grinders and packaged in vacuum seals. For instant coffee, the coffee must be extracted before roasting and grinding to obtain soluble solids and produce volatile compounds that improve flavour and aroma. To keep the extraction water liquid, it is heated to 175 °C under high pressure and then evaporated or freeze concentrated to increase the concentration of soluble compounds in the coffee extract (Mussatto *et al.*, 2012). The concentrated coffee extract can be dried using several method as described by Ballesteros *et al.* (2017). Sun-drying involves drying coffee fruits under the sun to remove the pulp, endocarp, and silverskin. In freeze-drying, the concentrated coffee extract is frozen, milled into granules, and passed through a sieve before drying. This method can change the aroma of coffee due to heating and oxidation that occurs in the vacuum chamber while spray-drying atomizes the concentrated coffee extract in a drying chamber, removing water at temperatures between 200 and 300 °C.

On the other hand, green coffee can be produced from fresh coffee beans using the wet method to de-pulp the fruits, which includes three distinct procedures: the pulped natural, semi-washed, and fully washed methods (Arya *et al.*, 2021). Soaking coffee beans in water for 36 to 40 hours during wet processing can reduce caffeine content through conventional and enzymatic methods. These processes involve microbial fermentation by yeast, bacteria, and fungi to break down pectin and sugars, resulting in the production of acids and alcohols as well as helps to minimize the caffeine content in coffee according to Widodo *et al.* (2023). In the pulped natural method, the mucilage remains on the parchment

during drying, allowing the sugars to infuse into the beans, resulting in a sweeter coffee. Conversely, the semi-washed method removes the mucilage through mechanical brushing and friction (Klingel *et al.*, 2020).

2.4 Chemical Composition of Coffee Waste and their Potential Application

The coffee industry produces significant amounts of harmful solid waste that negatively impact the environment. This necessitates the development of sustainable solutions to address these environmental problems (Moreira *et al.*, 2018; Peshev *et al.*, 2018; Catalán *et al.*, 2019; Yun *et al.*, 2020; Lee *et al.*, 2023). Every year, the coffee industry produces over 10 million tons of waste annually, which include husks, pulp, mucilage, silverskins, and spent coffee grounds (SCGs) based on the report by Arya *et al.* (2021) as well as Echeverria and Nuti (2017). The waste materials come from various stages of coffee production, such as harvesting, processing, roasting, and brewing (Arya *et al.*, 2021). SCGs are the most abundant coffee waste, comprising of 0.6 tons per ton of coffee. In contrast, coffee pulp and husks produce 0.50 and 0.18 tons per ton of fresh coffee during coffee processing, respectively Lee *et al.*, 2023. Coffee waste has been used to produce biofuel, bio-sugar, enzymes, fertilizer, dietary fiber, and bioactive compounds (Atabani *et al.*, 2019; Durán-Aranguren *et al.*, 2021). As a result, the by-products of coffee processing are no longer considered waste but rather valuable materials for producing many value-added products (Nguyen *et al.*, 2017; Nguyen *et al.*, 2019).

2.4.1 Coffee Pulps, Coffee Husks and Coffee Silverskin

Figure 2.2 illustrates the waste produced at each stage of coffee processing. Both wet and dry coffee processing methods produce a significant portion of coffee pulps and coffee husks, comprises about approximately 0.5 and 0.2 metric tons per metric ton of fresh coffee

respectively according to the report by Lee *et al.* (2023). Coffee pulp is the fleshy, mucilaginous substance that surrounds the coffee bean while coffee husk is the tough, fibrous outer layer of the coffee beans (Arya *et al.*, 2021). Apart from carbohydrates, proteins, and minerals, coffee pulps and coffee husk contain high amounts of caffeine, chlorogenic acid, and tannins, which can negatively affect animal health as well as seed germination and plant growth, limiting their use as animal feed or fertilizer. Nevertheless, coffee pulps and coffee husk biomass have been used for biogas production (Corro *et al.*, 2013).

Coffee pulps and coffee husk are also valuable for producing valuable products such as enzymes, biofuels and bioactive compounds (Janissen and Huynh, 2018). In the production of enzyme, a study by Muthy and Naidu (2012) explored the potential utilization of coffee husks as the carbon source of xylanase production using *Penicillium* sp. where the study achieved the highest xylanase activity rates of 9.475 U/g. Besides, another enzyme activity study conducted by Selvam *et al.* (2014) reported on the fermentation of coffee pulp with *Acinetobacter* sp. after alkali pre-treatment, which managed to produce about 888 U/mL of cellulase within 60 hours.

Apart from enzyme production, coffee pulp and coffee husk also can be used as substrate to produce bioethanol. Prior to saccharification and fermentation, coffee pulp or husk are often subjected to pre-treatment using alkali or dilute acid. Studies have shown promising results, such as Menezes *et al.* (2012) reported that about 70 g/L of ethanol was yield from a hydrolysate mixture of sugarcane molasses and coffee pulp while the hydrolysate mixture of coffee husk, coconut coir, and cassava stems produces about 55.2 g DW bioethanol per 300 g DW of mixed biomass after popping pre-treatment, which equivalent to 77.3% hydrolysis rate based on the study by Nguyen *et al.* (2017).

Additionally, Janissen and Huynh (2018) stated that coffee pulp and coffee husk are rich in chlorogenic acids, caffeic acids, caffeine, polyphenols, tannins, pectin, and carbohydrates. Torres-Mancera *et al.* (2013) utilized an enzymatic extraction approach through solid state fermentation using three fermentation agent, namely *Aspergillus tamaraii*, *Rhizomucor pusillus*, and *Trametes* sp., producing about 36.1% chlorogenic acid and 33.0% caffeic acid respectively. Besides, in-vitro experiments reported by Duangjai *et al.* (2016) and Magoni *et al.* (2018) had demonstrated the antioxidant, antibacterial, and anti-inflammatory properties of coffee pulps, primarily due to their chlorogenic and caffeic acids contents.

On the other hand, coffee silverskin is a thin layer that tightly covers the oval-shaped coffee bean and is surrounded by other parts of the coffee fruit, including the endocarp, a sticky pectic layer, pulp, and the outer skin. This coffee waste is formed during roasting, comprises a small portion of the coffee cherry (4.3%) but is a rich source of dietary fiber (80%), antioxidants, and phenolic compounds including polyphenols and tannins (Muthy and Naidu, 2012; Ballesteros *et al.*, 2014). Antioxidants extracts of coffee silverskin contains a high amount of chlorogenic acid (246 mg/100 g), caffeine (1.25 g/100 g), and 5-hydroxymethylfurfural (5.68 mg/ 100 g) based on the report by Costa *et al.* (2018). According to a study by Ballesteros *et al.* (2014), polysaccharides such as cellulose and hemicellulose are the most abundant components in coffee silverskin, comprising approximately 40.5% of its total dry weight. Furthermore, although coffee silverskin and SCG have been used to produce bio-butanol in previous studies, they can be less practical for this purpose compared to other abundant lignocellulosic materials (López-Linares *et al.*, 2021; Mirzoyan *et al.*, 2022).

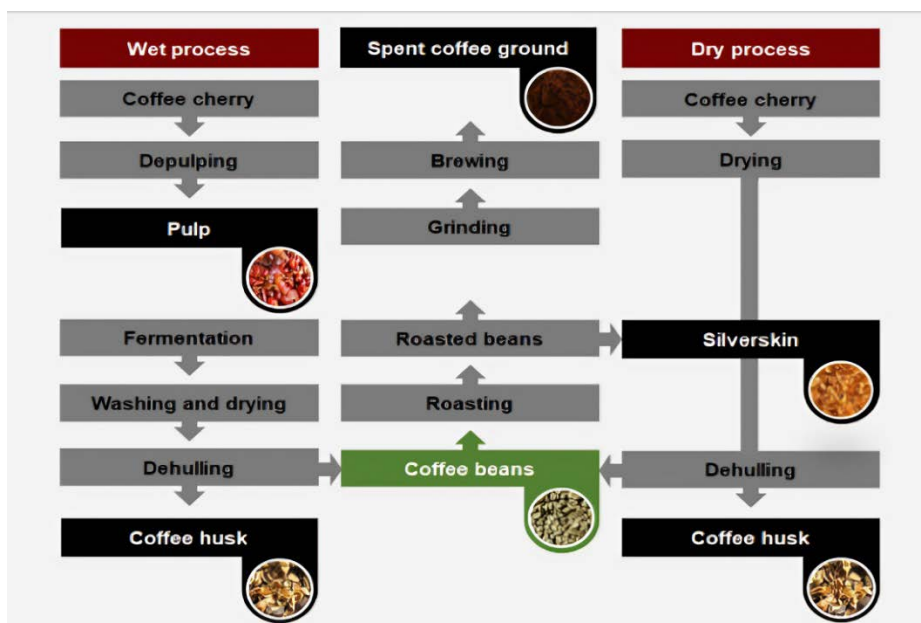


Figure 2.2: Waste generated during wet and dry processing of coffee.

Adapted from Lee et al. (2023)

2.4.2 Spent Coffee Grounds (SCG) and its Potential Industrial Applications

SCG is the by-product of coffee brewing and instant coffee production, making up about 50% dry weight of the coffee cherry biomass. Instant coffee is made by treating coffee powder with steam or hot water. The leftover material after the coffee is extracted is called spent coffee ground (SCG). Annually, an estimated 6 million metric tons of SCG are generated worldwide, equivalent to 650 kilograms of SCG per metric ton of green coffee beans while more than 2 kilograms of wet SCG is generated for each kilogram of instant coffee (Anastopoulos *et al.*, 2017; Lee *et al.*, 2023). In most cases, industries that have significant amounts of SCG dispose of them by incineration. However, incinerating SCG can negatively impact the air quality in nearby areas due to the particulate matter produced (Arya *et al.*, 2021). Given their potential hazardous environmental impact, proper treatment of SCG residues is essential before disposal, which has sparked growing interest in reusing these materials.

In addition, SCG contain high amount of organic compounds such as carbohydrates, lipids, proteins as well as phenolic compounds, including caffeine, chlorogenic acid, and caffeic acid according to Peshev *et al.* (2018). Contrary to other lignocellulosic materials, SCG contains a higher percentage of hemicelluloses (30-40%) and lignin (25-30%), but it has a lower cellulose content, ranging from approximately 8.6% to 13.3% (Musatto *et al.*, 2012; Ballesteros *et al.*, 2015; Obruca *et al.*, 2015). Table 2.2 shows the composition of SCG based on the study by Ballesteros *et al.* (2014). SCG composition vary significantly and influenced by a range of factors, including brewing method, growing conditions, and coffee type. But, most SCG content have a relatively similar composition with the combination of hemicellulose and cellulose make up approximately 50% DW of SCG while glucose is the component of cellulose (Musatto *et al.*, 2012). The primary components of hemicellulose are mannose, galactose, glucose and arabinose.

Table 2.2: Summary of chemical composition of SCG.

Adapted from Ballesteros *et al.* (2014).

Component	Composition (g/100g dry matter)
Hemicellulose	39.10
Mannose	19.07
Galactose	16.43
Arabinose	3.60
Cellulose (Glucose)	12.40
Lignin	23.90
Insoluble	17.59
Soluble	6.31
Total dietary fiber	60.46
Insoluble	50.78
Soluble	9.68
Ashes	1.30
Protein	17.44
Fat	2.29

Recent studies have investigated the potential of lignin from SCG as a fuel source to address the growing global energy needs including the utilization of organosolv pre-treatment method to extract the lignin from SCG as demonstrated by Lee *et al.* (2019). However, processing lignin biologically is not economically feasible because to its resistant nature (Petrosyan *et al.*, 2020; Saratale *et al.*, 2020). Due to its availability in large quantities, SCG is a promising resource for producing valuable products such as, biofuels, bio-sugar, bio-oils, enzymes and organic acids as well as antioxidants and bioactive compounds mentioned in numerous studies in the literatures (Martins *et al.*, 2011; Atabani *et al.*, 2019; Bouhlal *et al.*, 2020).

SCGs are an ideal raw material for producing biofuel, especially bioethanol because of their high carbohydrate content. Similar to other biomass used in bioethanol production, SCGs require pre-treatment process to solubilize their complex lignocellulosic structure and enhance sugar extraction efficiency. Common pre-treatment methods include using ammonia, supercritical carbon dioxide extraction, ionic liquids, organosolv, diluted acid and alkaline solutions, but only a few of these methods are potentially feasible (Campos-Vega *et al.*, 2015; Ravindran *et al.*, 2017; Karmee, 2018). Dilute acid pre-treatment stands out among these methods, producing high monosaccharide concentrations (58.4 g/L) and achieving a high ethanol conversion rate (0.46 g ethanol per gram of total fermentable saccharides) when fermented using *Saccharomyces cerevisiae* (Kwon *et al.*, 2013). In addition, many researches have reported high ethanol production from SCGs pre-treated with dilute acid, yielding 50% conversion yield rates (Mussatto *et al.*, 2012; Juarez *et al.*, 2017).

Another affordable and effective method is alkaline pre-treatment, which uses a highly concentrated alkaline such as sodium hydroxide solution to produce polysaccharides which can be further converted into bioethanol (Jin *et al.*, 2020; Lee *et al.*, 2021). This

method is highly practical because it involves a simple purification process and produces high-quality products. Apart from that, popping pre-treatment is another method that has been shown to enhance sugar extraction from SCGs where the ethanol concentration and yield were 15.3 g/L and 87.2 % respectively (Choi *et al.*, 2012). Oils extracted from SCGs can also be converted into biodiesel using chemical and biocatalytic methods, with alkaline including NaOH and KOH or acids such as H₂SO₄ and HCl which can serve as chemical catalysts. Previous researches achieved a maximum biodiesel yield of 73% using NaOH whereas KOH produced a yield of 96 % (Kondamudi *et al.*, 2008; Vardon *et al.*, 2013).

Kondamudi *et al.* (2008) reported on the production of biodiesel directly from SCGs by solvent extraction, which yielded 10-15% of oil content depending on the coffee species where the biodiesel subsequently synthesized through transesterification. In-situ transesterification combined with extraction method was utilized to produce biodiesel by skipping the oil separation step (Tuntiwiwattanapun *et al.*, 2017). However, high energy consumption needed for methanol recovery makes this process economically unfeasible for producing biodiesel from SCGs as transesterification of SCG oil had high free fatty acid (FFA) content that reacts with alkali catalysts to produce soap (Tuntiwiwattanapun *et al.*, 2017; Kim and Yeom, 2020).

To overcome this challenge, Caetano *et al.* (2014) proposed two-step pre-treatment process of acid esterification followed by alkaline transesterification for SCG oil with high FFA which manage to obtain higher biodiesel yields compared to direct transesterification. SCGs also can be converted into bio-oil since it contain considerable amount of lignin. Extraction methods using solvents such as hexane or diethyl ether in a Soxhlet extractor or supercritical extraction are the common method for oil extraction according to the report by Jenkins *et al.* (2014) and Vardon *et al.* (2013). Meanwhile, based on the experiment by

Banerjee *et al.* (2013) demonstrated that 6% oil was extracted with hexane under reflux, whereas 14% was obtained using the Soxhlet method. For the past few years, researches had explored the potential utilization of SCGs as alternative to produce bio-sugar by developing greener processes which involve pre-treatment and saccharification to yield high-quality value-added product. Bio-sugars are products of saccharification of lignocellulosic biomass and waste that are not utilized during fermentation have recently attracted significant investment from major corporations (Gómez *et al.*, 2016; Hu *et al.*, 2016). Several studies have mentioned on the chemical and physical methods that can be employed for the bio-sugar production, but enzymatic hydrolysis was still preferred as they are more safe, stable as well as highly effective and environmentally friendly.

In a study by Nguyen *et al.* (2017), the steps of producing bio-sugar involve several simultaneous processes which include ethanol pre-treatment, enzymatic hydrolysis using cellulase and pectinase, fermentation, colour removal via activated carbon and pervaporation producing 11.3 g DW of bioethanol and 15.7 g DW of D-mannose from 150 g DW of ethanol pre-treated SCG sample. The entire process can be considered eco-friendly because it only uses non-harmful chemicals and continuously recycles ethanol. Another study by the same researches upgraded the process which include pre-treatment methods such as delignification and defatting steps that fully removed lipids and non-saccharides from SCG, not only yield significantly more amount of D-mannose (25.6 g) and less amount of bioethanol (3.1 g) but also produces 15.9 g mannose-oligosaccharide (Nguyen *et al.*, 2017).

The excessive mannose and mannan content of SCGs made this coffee waste a great source for producing bio-sugars such as D-mannose and manno-oligosaccharides. Unfortunately, these resources are still significantly underused. The manno-oligosaccharides extracted from SCG could have potential applications as nutraceuticals, which can be used

as food additives to enhance health and immune function (McNutt and He, 2018). Evaluation study by Wongsiridetchai *et al.* (2021) found that manno-oligosaccharides from treated SCGs have prebiotic properties, including promoting the growth of beneficial bacteria, tolerating gastrointestinal conditions, and inhibiting the growth of pathogens. These findings suggest that manno-oligosaccharides from treated SCGs could also exhibit symbiotic properties.

Various industrial enzymes have been produced from SCGs, mainly through solid state fermentation (SSF) and submerged fermentation (SmF). Of these two methods, SSF is more commonly used for enzyme production due to its operational and economic benefits compared to SmF (Lee *et al.*, 2023). A study by Buntić *et al.* (2016) used *Paenibacillus chitinolyticus* to produce 71 % cellulase while another study Muthy and Naidu (2012) reported on the utilization of *Penicillium* sp. as fermentation agent to obtain xylanase under SSF process. SCG can also be utilized in the production of organic acid such as citric acid, gibberellic acids and chlorogenic acids (Burniol-Figols *et al.*, 2016; Lee *et al.*, 2023).

Meanwhile, recent study by Wysocki *et al.*, (2024) studied the biotechnological potential of lactic acid produced from SCG by using the *Lactocaseibacillus rhamnosus*. Through optimisation of acid hydrolysis, neutralization, enzymatic hydrolysis of SCG, and fermentation, the lactic acid concentration increased from 4.60 g/L to 48.6 g/L after 72 hours of culture. Lactic acid is a valuable substance with wide applications in the food, pharmaceutical, cosmetic, and chemical industries (Hudeckova *et al.*, 2018). Most of the lactic acid used in industry is produced through biological methods like microbial fermentation such as fermentation by *Lactobacillus* sp. that is known for its high tolerance to acid and its ability to selectively produce stereoisomers (Hudeckova *et al.*, 2018; Kim *et al.*, 2021; Lee *et al.*, 2021).

SCGs contain a high concentration of phenolic compounds such as caffeic acids as well as chlorogenic acid and its derivative including quinic acids, caffeoylquinic, feruloylquinic, p-coumaroylquinic, and esters of caffeic and ferulic acids. These compounds can be extracted via SSF method which produce high-quality extractives while offering a cleaner alternative to traditional chemical and thermal methods that use of toxic organic solvents (Zuorro and Lavecchia, 2012; Panusa *et al.*, 2013). Meanwhile, Zuorro and Lavecchia (2012) managed to extract about 90% of phenolic extracts from SCGs with aqueous ethanol solutions through solvent extraction method. Compared to the SSF method, ultrasound-assisted solid–liquid extraction can increase phenolic yields of 3.6% by nearly six times, making it a much more efficient extraction technique (Al-Dhabi *et al.*, 2017).

In addition, various extraction methods have been examined to produce polyphenolic compounds. For example, Low *et al.* (2015) studied the extraction of tannins from SCG using elevated temperatures and sodium hydroxide, while Conde and Mussatto (2016) explored hydrothermal pre-treatment as a method for removing polyphenols from SCG. Besides, microbes such as yeasts, filamentous fungi, bacteria, and algae can be used to ferment SCGs and produce beneficial compounds like carotenoids. For instance, the carotenogenic yeast *Sporobolomyces roseus* used SCG hydrolysate as a food source to produce polyhydroxyalkanoates (PHAs) and carotenoids following oil extraction (Obruca *et al.*, 2014; Obruca *et al.*, 2015). Due to their strong antioxidant and antimicrobial properties, these compounds are widely used in the food, pharmaceutical, and cosmetic industries. Although there are concerns about their environmental impact, these bioactive compounds have been shown to benefit human health by acting as antibiotics, antioxidants, and anti-inflammatory agents (Martins *et al.*, 2011; Bouhlal *et al.*, 2020).

Applying SCG directly to soil has been found to be harmful due to its high C/N ratio, phenol content, and acidity (Hardgrove and Livesley, 2016). However, Cruz *et al.* (2012) reported that small amounts of SCG in the soil helped boost mineral content, bioactive compounds, and antioxidant activities. Apart from that, Fenoll *et al.* (2014) discovered that the use of SCG and other organic wastes can potentially reduce pesticide leaching in soil. To address the negative effects of excessive SCG exposure on plants, Ciesielczuk *et al.* (2017) developed slow-release SCG fertilizer pellets that release elements gradually. Numerous studies have investigated the impact of incorporating SCG into compost at various ratios, and the results have generally been positive (Hachicha *et al.*, 2012; Santos *et al.*, 2017).

2.5 Challenges in Extracting Sugar from SCG

Lignocellulosic biomass encompasses all dry plant matter on Earth, making it the most abundant renewable raw material. SCGs are the example of lignocellulosic material since they primarily composed of polysaccharide components such as lignin, cellulose, and hemicellulose. This make SCG a promising and sustainable feedstock for producing value-added products as well as in renewable energy production. One of the main component in SCG is lignin, which consist of up about 20-30% of SCG dry weight (Mussatto *et al.*, 2012; Ballesteros *et al.*, 2015). Lignin is an amorphous, highly branched, and cross-linked three-dimensional polymer without any regular repeating structures. This component is chemically linked to hemicelluloses and part of cellulose while acting as a binding agent between these fibers, while providing structural support to the cell walls of SCG. It also protects the structural polysaccharides from being broken down by enzymes, thus hindering the enzyme accessibility to the sugar components (Yoo *et al.*, 2020; Sheng *et al.*, 2021).

Unlike most plant cell wall that have cellulose as main structure, the most abundant polysaccharide in SCG is hemicellulose, which account for between 36.7% to 39.1% of SCG dry mass (Iriundo-DeHond *et al.*, 2021). Hemicellulose structure in SCG is composed of sugars such as mannose, galactose, and arabinose. Based on the study by Mussatto *et al.* (2012), mannose is difficult to extract than galactose or arabinose from SCG hemicellulose as arabinogalactans can be easily dissolve during pre-treatment process than linear mannans, forming crystalline. The study also highlighted that side chains can be easily broken down than the main chains within a polymeric structure. Arabinogalactans in SCG are composed of a main chain of galactose units linked by 1→3 bonds, with side chains are connected to arabinose and galactose. Meanwhile, galactomannans have a main chain of mannan units linked by 1→4 bonds, with side chains of galactose units linked at C-6, and varying degrees of branching Mussatto *et al.* (2012). This composition make hemicellulose of SCG to have a random and amorphous, or only partially crystalline structure. Hemicelluloses are embedded in the cell wall of SCG and form bonds with adjacent cellulose microfibrils through hydrogen bonding and van der Waals interactions. This network of cellulose and hemicellulose serves as a structural support element that strengthens the cell wall (Zhang *et al.*, 2016; Broda *et al.*, 2022).

Another main structural polysaccharide of SCG is cellulose which contribute to its rigidity and strength. Cellulose is typically a highly crystalline, high-molecular-weight polymer that tends to form tightly packed crystalline fibers (Broda *et al.*, 2022). It is composed of long, linear chains of β -D-glucopyranose units, which are connected by β -1,4-glycosidic bonds. The disaccharide cellobiose is made up of two β -glucose molecules joined by a β (1→4) bond as shown in Figure 2.3. Cellulose promotes a crystalline structure and helps organize individual cellulose chains into bundles, forming microfibrils that are further

stabilized by van der Waals interactions (hydrophobic interactions), resulting in a fibrous state. Highly ordered crystalline regions in cellulose are interspersed with disordered amorphous regions. This structure of cellulose makes it insoluble in water and resistant to solubilisation (Heinze, 2015; Baghaei and Skrifvars, 2020).

Each of the primary structural polymers in the cell wall of SCG possesses distinct chemical structures and behaviours. They are also closely intertwined and connected through non-covalent forces, such as hydrogen bonds and van der Waals interactions, along with covalent cross-linkages, creating a strong and complex matrix. These characteristics, along with crystalline nature of cellulose and limited surface area for enzyme access, make lignin difficult to separate and degrade. As a result, sugar extraction of SCG is challenging and demands specialized knowledge and technologies that integrate heat, chemicals, enzymes and/or microorganisms (Mussatto *et al.*, 2012; Ravindran *et al.*, 2017; Zhang *et al.*, 2021).

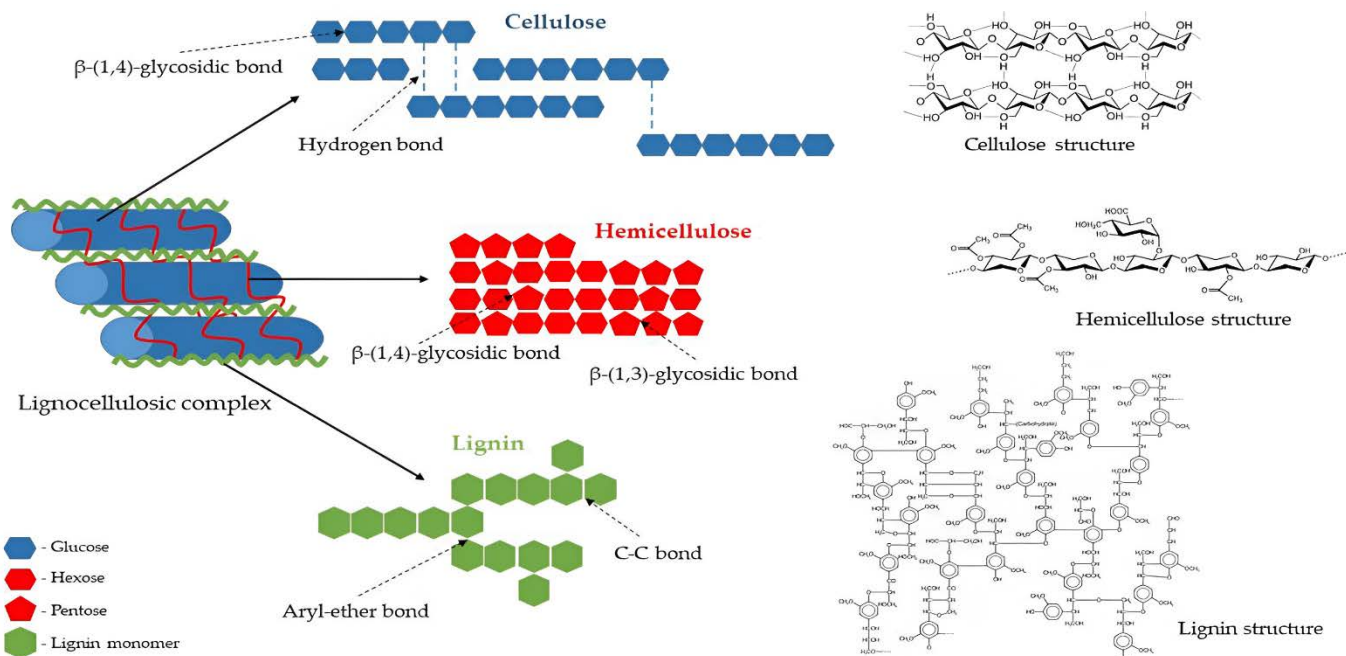


Figure 2.3: A diagrammatic representation of the lignocellulosic structure.

Adapted from Baruah *et al.* (2018)

Among the structural polymers in SCG lignocellulose, only cellulose and hemicelluloses contain sugars. These long-chain polysaccharides can be broken down into a mixture of fermentable sugar of pentoses and hexoses, which are then converted into valuable products such as bioethanol, lactic acid and chlorogenic acid (Burniol-Figols *et al.*, 2016; Kim *et al.*, 2019). However, due to the resilient nature of SCG biomass, achieving high efficiency in the bioconversion process requires an initial pre-treatment step. This step is essential to release cellulose and hemicelluloses from the complex matrix, making them more accessible for enzymatic hydrolysis (Zhang *et al.*, 2016; Trinh, 2022).

2.6 Pre-treatment Methods to Enhance Sugar Recovery from SCG

Unlike cellulose, which is difficult to hydrolyse, hemicellulose is relatively easier to degrade (Broda *et al.*, 2022; Bomfim *et al.*, 2023). Pre-treatment is an essential step for extracting sugars from the lignocellulosic complex of spent coffee grounds (SCG) before their conversion into valuable products. During pre-treatment, the lignocellulosic structure is disrupted by breaking the cross-linkages between structural polymers, facilitating the separation of carbohydrates from lignin. It also breaks the hydrogen bonds between cellulose chains, reducing cellulose crystallinity and the degree of polymerization (Das *et al.*, 2020; Awogbemi and Kallon, 2022). The main objective is to enhance the accessibility of carbohydrates to enzymes by reducing particle size, increasing porosity, and improving surface area, which collectively improve hydrolysis (Trinh *et al.*, 2017; Zhang *et al.*, 2021).

The major barrier to efficient sugar release is lignin. This polymer prevents hydrolytic enzymes from accessing cellulose microfibrils and hemicellulose chains, while also adsorbing enzymes and rendering them inactive (Haghighi *et al.*, 2013; Zabed *et al.*, 2017; Safarian and Unnthorsson, 2018). Although delignification is necessary, the process can produce inhibitory compounds such as furans (5-Hydroxymethylfurfural, furfural), phenolics, and organic acids (acetic, formic, levulinic) that interfere with microbial activity and enzymatic efficiency (Bomfim *et al.*, 2022; Pereira *et al.*, 2022). Therefore, pre-treatment methods must balance delignification efficiency with minimal inhibitor formation while remaining cost-effective and environmentally sustainable (Lee *et al.*, 2021; Broda *et al.*, 2022). Pre-treatment approaches are generally classified into physical, chemical, physicochemical, and biological methods, and often a combination of methods is required to achieve optimal results (Das *et al.*, 2020; Awogbemi and Kallon, 2022). Table 2.3 depicted various studies on the pre-treatment and enzymatic hydrolysis of SCG. For SCG, both acid and alkaline pre-treatments are commonly applied, often followed by enzymatic hydrolysis for bioconversion into valuable products.

Table 2.3: Comparison of multiple studies on the pre-treatment and enzymatic hydrolysis of SCG

Methods	Condition	Results	References
Acid hydrolysis	10 mL/g non-delipidated SCG, 4% (v/v) H ₂ SO ₄ , 95 °C, 120 min.	Sugar yield: 31 %	Go <i>et al.</i> (2016)
	8 g/mL non-delipidated SCG, 5% v/v H ₂ SO ₄ , 95 °C, 180 min.	Sugar concentration: 33.4 g/L 5-HMF: 0.056 g/L Furfural: 0.012 g/L	Juarez <i>et al.</i> (2017)
	10 g/g liquid-to-solid, 163 °C, 45 min.	Hydrolysis efficiency: Galactan: 100 %, Mannan: 77.4 % Arabinan: 89.5 %	Mussatto <i>et al.</i> (2011)
	20% solid loading, 5.3% H ₂ SO ₄ , 100 °C, 118 min.	Sugar concentration: 563 mg/g	Wang <i>et al.</i> (2016)
Alkaline hydrolysis	Defatted SCG, 4M NaOH, 25 °C, Overnight.	Sugar recovery: 4.57%	Ballesteros <i>et al.</i> (2015)
Enzymatic hydrolysis	30% substrate loading, 40-60 °C, 18 h, 100 rpm, enzymes loading: 0.5mg/g SCG using mannannase	Sugar yield: 17%	Jooste <i>et al.</i> (2013)

Table 2.3 continued

Alkaline pre-treatment followed by enzymatic hydrolysis	0.5% (v/v) NaOH concentration, 5% (v/v) of SCG, 100 °C. 0.01 cellulase 55 °C pH 4.8	Sugar concentration: 1.824 mg/ml	Jin <i>et al.</i> (2020)
	3% (w/w) KOH solution, 75 °C, 2.8 h. 30 FPU/g-biomass cellulase 15 CBU/g-biomass cellobiase 50 MNU/g-biomass mannanase	Glucan content: 18.9 % Mannan content: 47.5 % Enzymatic efficiency: 42.0 %	Lee <i>et al.</i> (2021)
	0.5 N NaOH concentration, 1:2 SCG-to-NaOH, 50 °C, 6 h Enzyme mannanase <i>Bacillus</i> sp. GA2(1)	Reducing sugar: 520 ± 0.01 µg/mL	Wongsiridetchai <i>et al.</i> (2018)
Steam explosion pre-treatment followed by enzymatic hydrolysis	Steam at 210 °C, 15min 2% (w/w) SCG, 0.01% (w/w) mannanase 0.91% cellulase 60 °C, 18 h	Sugar yield: 27.65 g/100g	Chiyanzu <i>et al.</i> , (2014b)

Table 2.3 continued

Two-step pre-treatment followed by enzymatic hydrolysis	Defatted SCG, 1:5 SCG-to-acetone. Sonication for 30 min. 1% NaOH at 1:5 SCG-to-NaOH solution, autoclave at 120 °C, 15 min 4% (w/v) of SCG, 50 mM citrate buffer, pH 5.0, 5 % (v/v) viscozyme to SCG ratio	Sugar concentration: 6120 mg/L Sugar yield: 161 mg/g	Trinh <i>et al.</i> (2022)
Sequential pre-treatment using concentrated phosphoric acid-acetone and ammonia fibre explosion pre-treatment followed by enzymatic hydrolysis	Concentrated H₃PO₄ in the ratio of 1:10 50 °C for 1 h at 120 rpm Mixed with acetone centrifuge at 8000 rpm 10 min Mixed and dried overnight Add 25 mL of ammonia Autoclave at 120 °C under pressure of 15 (lbs/sq.inch), 30 min 50 mL 1% w/v SCG, 1.5mL cellulase 0.37mL hemicellulose 50 °C, 24h	Sugar yield: 350.12 mg/g	Ravindran <i>et al.</i> (2017)

2.6.1 Alkaline Pre-treatment

Alkaline pre-treatment has been extensively studied for SCG because of its effectiveness in disrupting the lignocellulosic structure, particularly by solubilising lignin, which acts as a protective barrier around cellulose and hemicellulose. For example, NaOH pre-treatment of SCG was reported to reduce biomass weight from 50 g to 30.3 g due to the removal of lignin, lipids, and polyphenolics through saponification, demonstrating the strong delignification capacity of alkaline media (Kim *et al.*, 2023; Ribeiro *et al.*, 2023). The removal of lignin is essential as it improves accessibility of structural carbohydrates for subsequent hydrolysis. The cleavage of cross-linkages between lignin and carbohydrates is another important outcome of alkaline pre-treatment. In SCG, NaOH treatment was found to selectively remove lignin while retaining most of the polysaccharide fraction, indicating that lignin–carbohydrate linkages were effectively disrupted (Limayem and Ricke, 2017; Kim *et al.*, 2023; Ribeiro *et al.*, 2023). This loosening of the rigid lignocellulosic matrix enhances the digestibility of cellulose and hemicellulose, both of which are otherwise locked within the dense SCG cell wall structure.

In addition to delignification, alkaline pre-treatment also promotes biomass swelling and increases porosity. Studies using NaOH on SCG showed that the treatment creates pores and expands surface area, facilitating better enzyme penetration during hydrolysis (Kim *et al.*, 2023; Ribeiro *et al.*, 2023). This structural modification is critical for improving the efficiency of enzymes such as β -mannanases, which target the abundant mannan component in SCG. Alkaline pre-treatment also reduces cellulose crystallinity by disrupting intramolecular hydrogen bonding, converting it into a more amorphous structure. While few SCG-specific studies have directly measured crystallinity reduction, recent work optimising NaOH pre-treatment conditions (0.06% NaOH at ~99.5 °C for 60 min) achieved substantial

lignin removal (35.7%) with minimal glucan and hemicellulose loss (2.5%), strongly suggesting that cellulose became more accessible and structurally altered toward an amorphous form (Bomfirm *et al.*, 2023).

The combined structural effects of alkaline pre-treatment which including lignin removal, hemicellulose solubilisation, increased porosity, and reduced cellulose crystallinity can greatly enhance enzymatic digestibility of SCG and improve fermentable sugar recovery (Park and Kim, 2012). Studies have shown that alkaline pre-treatment produces fewer inhibitory compounds compared to acid treatment while achieving higher fermentable sugar yields (Kim *et al.*, 2018; Jin *et al.*, 2020). For example, Wongsiridetchai *et al.* (2018) applied alkaline pre-treatment using 0.5 N NaOH at 50 °C for 6 h with a 1:2 SCG-to-solution ratio, followed by enzymatic hydrolysis with mannanase, producing 520 ± 0.01 µg/mL of reducing sugars. Jin *et al.* (2020) optimised NaOH pre-treatment (0.5% v/v NaOH, 100 °C, 5% SCG) and reported a sugar concentration of 57.02 mg/mL. Likewise, Lee *et al.* (2021) used KOH pre-treatment (3% w/w KOH, 75 °C, 2.8 h), combined with cellulase, cellobiase, and mannanase, obtaining 42% enzymatic efficiency and a 1.6-fold increase in fermentable sugars compared to untreated SCG. Optimised alkaline pre-treatment using KOH has also enabled downstream applications such as lactic acid production of up to 101.2 g per 1000 g SCG (Kim *et al.*, 2021).

The optimisation of alkaline pre-treatment is strongly influenced by factors such as alkali concentration, temperature, incubation time, and solid-to-liquid ratio. Statistical tools like RSM and ANOVA have been employed to optimise these parameters, reducing experimental costs and enhancing predictive accuracy (Rajewski and Dobrzyńska-Inger, 2021). Recent studies suggest that defatted SCG, obtained through lipid extraction, further improves sugar recovery since lipid removal facilitates better lignin hydrolysis (Trinh *et al.*,

2017; Trinh *et al.*, 2022). Additionally, structural analysis has shown that hemicellulosic polysaccharides such as galactomannan are largely preserved but more exposed after pre-treatment, contributing to efficient hydrolysis (Simões *et al.*, 2014; Ribeiro *et al.*, 2023). These findings support the role of alkaline pre-treatment in improving enzyme accessibility while maintaining carbohydrate integrity.

Despite its advantages, research gaps remain regarding comparative studies of different alkaline agents, such as NaOH versus KOH, and the effects of one-step versus two-step pre-treatment strategies on SCG. While most studies focus on single-step alkaline pre-treatment, evidence suggests that combining steps or integrating alkaline treatment with other methods can yield higher delignification and sugar recovery (Ravindran *et al.*, 2018; Trinh *et al.*, 2022). Addressing these gaps could further advance the valorisation of SCG into bio-based products. Overall, alkaline pre-treatment is widely recognised as one of the most reliable and efficient methods for modifying SCG's lignocellulosic structure, maximising enzymatic digestibility, and enhancing its bio-refinery potential.

2.6.2 Acid Pre-treatment

Acid pre-treatment is a widely applied strategy to enhance the hydrolytic efficiency of lignocellulosic biomass, including defatted SCG (Burniol-Figols *et al.*, 2016; Juarez *et al.*, 2017; López-Linares *et al.*, 2021). In this approach, dilute mineral acids such as H₂SO₄ or HCl are typically employed to hydrolyse and solubilize hemicellulose, thereby increasing cellulose accessibility to enzymatic attack (Zhang *et al.*, 2019; Broda *et al.*, 2022). For defatted SCG, acid pre-treatment is particularly useful because the removal of lipids improves the penetration of the acid into the lignocellulosic matrix, leading to more efficient disruption of hemicellulosic fractions (Kim and Yeom, 2020). This makes acid pre-treatment

a reliable option for defatted SCG as it enhances the release of pentose sugars from hemicellulose, while simultaneously exposing cellulose for subsequent enzymatic hydrolysis (Huang *et al.*, 2021; Huang *et al.*, 2024). For example, Juarez *et al.* (2017) performed direct dilute sulfuric acid hydrolysis on SCG, obtaining 33.4 g/L of sugars but also producing 0.056 g/L 5-HMF and 0.012 g/L furfural. Sequential acid-based methods have been explored to improve sugar yield. Ravindran *et al.* (2017) applied a combination of phosphoric acid–acetone pre-treatment followed by ammonia fiber explosion, achieving a 1.7-fold increase in sugar yield. Similar improvements were observed when combining KMnO₄ treatment with ultrasound. Chiyanzu *et al.* (2014a, 2014b) employed steam explosion, recovering 10.7% sugars from SCG.

While acid pre-treatment is effective in solubilising hemicellulose, the associated generation of inhibitors makes it less desirable than alkaline methods for SCG valorisation (Almeida *et al.*, 2019). The main drawback lies in the generation of inhibitors such as furfural, HMF, acetic acid, and phenolic derivatives, which result from the degradation of sugars and lignin during acid hydrolysis (Wang *et al.*, 2016; Ravindran *et al.*, 2018). These inhibitory compounds can significantly hinder microbial fermentation, reducing overall sugar to bio-based products conversion efficiency (Wang *et al.*, 2016; Huang *et al.*, 2021). Additionally, acid pre-treatment is less effective than alkaline pre-treatment in delignification, which is a critical step for SCG since LCC forms a compact and recalcitrant structure that restricts enzymatic access (Broda *et al.*, 2022; Trinh *et al.*, 2022).

Nevertheless, acid pre-treatment can play a complementary role when combined with alkaline pre-treatment in a sequential process. The acid step primarily solubilises hemicellulose and partially opens up the cell wall matrix, while the subsequent alkaline step effectively removes lignin and disrupts cellulose crystallinity (Wang *et al.*, 2016; Trinh *et al.*, 2022). The dual-stage strategy maximizes carbohydrate recovery by targeting different structural barriers of defatted SCG, leading to higher reducing sugar yields compared to either method alone (Ravindran *et al.*, 2017; Huang *et al.*, 2021). In particular, the acid–alkaline combination can reduce the severity of each individual treatment through neutralisation, thereby lowering the extent of inhibitor formation while still ensuring sufficient accessibility of cellulose for enzymatic hydrolysis.

2.6.3 Enzymatic Hydrolysis

Following chemical pre-treatment, enzymatic hydrolysis was performed to break down the complex polysaccharides in SCG into fermentable sugars that can be utilized for downstream bioconversion (Chen *et al.*, 2012). The main enzymes employed in this process such as cellulase, hemicellulase, and mannanase, specifically act on cellulose, hemicellulose, and mannans, respectively (Jin *et al.*, 2020; Lee *et al.*, 2021). These enzymes hydrolyse the β -glycosidic bonds within the carbohydrate polymers, releasing monomeric sugars such as glucose (Baruah *et al.*, 2018). Compared to harsh chemical hydrolysis, enzyme-assisted hydrolysis offers significant advantages, as it operates under milder conditions and minimises the formation of inhibitory compounds that can hinder microbial fermentation (Bomfim *et al.*, 2022; Pereira *et al.*, 2022).

Nevertheless, the overall efficiency of enzymatic hydrolysis is strongly influenced by the effectiveness of the prior pre-treatment step. A sufficiently disruptive pre-treatment increases enzyme accessibility by reducing lignin barriers, loosening the crystalline structure of cellulose, and exposing the hemicellulosic fractions, thereby enhancing hydrolysis yields (Jooste *et al.*, 2023; Trinh *et al.*, 2022). Conversely, inadequate pre-treatment can limit enzyme penetration and reduce sugar recovery, highlighting the interdependence between pre-treatment and enzymatic hydrolysis in SCG valorisation. For instance, Wongsiridetchai *et al.* (2018) demonstrated enhanced sugar recovery when combining alkaline pre-treatment with mannanase hydrolysis. Similarly, Lee *et al.* (2021) achieved substantial glucan and mannan recovery through enzymatic hydrolysis after KOH pre-treatment.

Several researchers have proposed the use of extracted sugars from SCG in the food industry. Certain extracted nutraceuticals, such as manno-oligosaccharides, can be utilised as food additives to enhance health benefits (Chiyanzu *et al.*, 2014a; Ballesteros *et al.*, 2015). One of the most extensively researched product from SCG bioconversion in the recent years is the production of bioethanol through fermentation process. Ethanol produced from SCG employed *S. cerevisiae* to convert the sugars into ethanol (Mussatto *et al.*, 2012; Kwon *et al.*, 2013; Burniol-Figols *et al.*, 2016). To illustrate, Grammatikos *et al.* (2021) performed an ethanolic fermentation of raw SCG using *S. cerevisiae*, producing ethanol yield of 19.9%. Meanwhile, sequential co-production of SCG produced bioethanol and biodiesel. However, fatty acids and triglycerides also present as the by-products of the reaction that hindered the hydrolysis of polysaccharide (Kwon *et al.*, 2013). Overall, enzymatic hydrolysis is an indispensable step for maximising fermentable sugar yields and enabling the bioconversion of SCG into valuable bio-based products.

CHAPTER 3

MATERIALS AND METHODS

The overview of this research is illustrated in Figure 3.1.

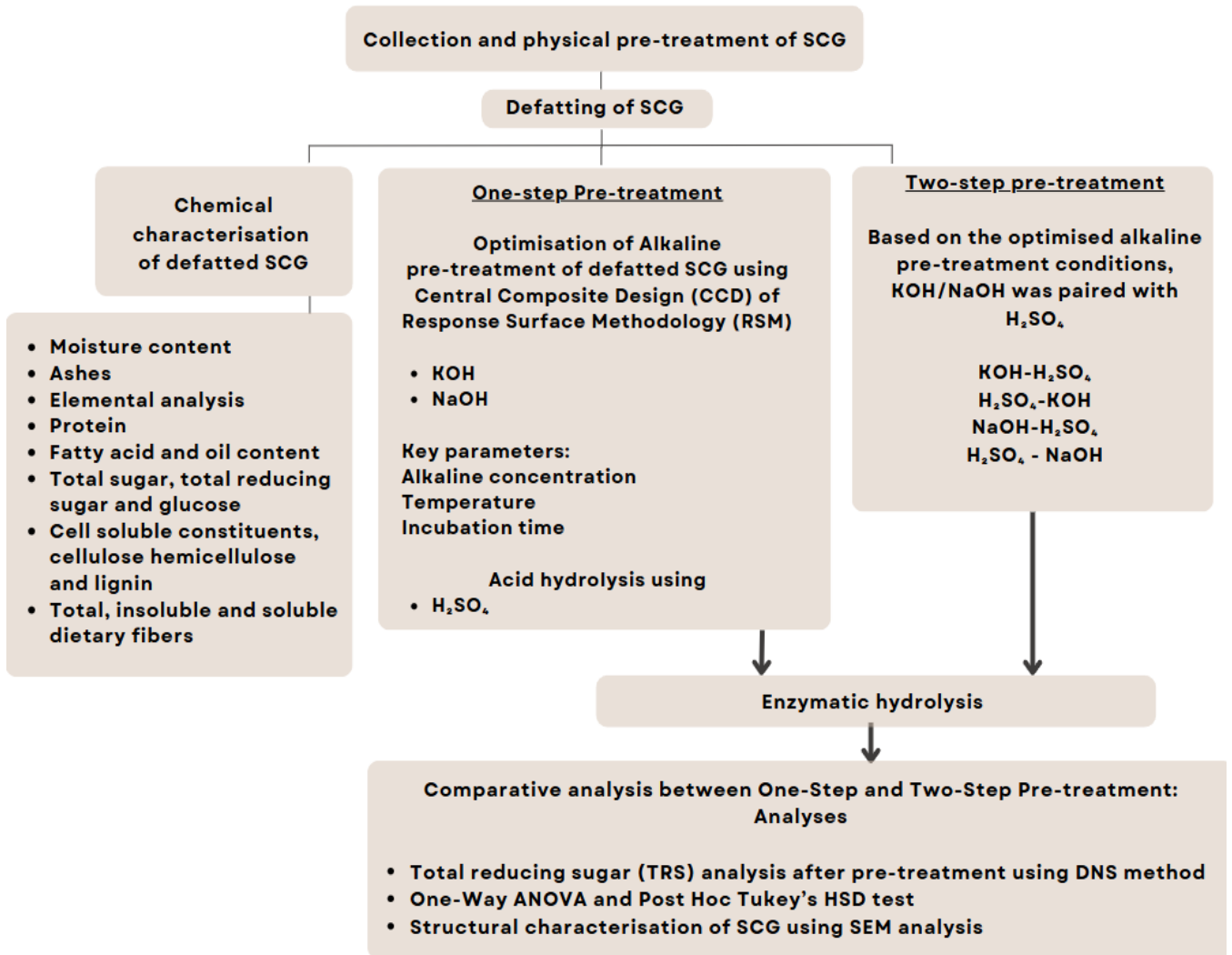


Figure 3.1: The flow diagram of this study.

3.1 Collection and Physical Pre-treatment of SCG

The spent coffee grounds (SCG) used in this study were collected from Richiamo Cafe, located within the Universiti Malaysia Sarawak (UNIMAS) campus in Kota Samarahan, Sarawak. After collection, the SCG were subjected to a drying process in a laboratory oven set at 85 °C for a duration of seven days to ensure complete removal of moisture. This drying step was essential to prevent microbial growth and degradation during storage. Once dried, the SCG were ground into finer particles using a mechanical grinder to increase surface area, facilitating more efficient subsequent processing. The ground material was then passed through a sieve to obtain a uniform particle size distribution. The sieved SCG were stored in an airtight container to preserve their quality and prevent contamination or moisture absorption prior to experimental use. Figure 3.2 shows the sieved dried SCG.



Figure 3.2: The sieved and dried SCG

3.2 Chemical Composition

3.2.1 Moisture Content

The moisture content (MC) of SCG was determined by a gravimetric method as outlined in the Official Methods of Analysis (AOAC, 1990) 923.03 with modification. The procedure involved drying of SCG in an oven for 85 °C until a constant weight was achieved. The results were expressed in % .based on the following formula:

$$\text{MC \%} = \frac{W_z - W_x}{W_y - W_x} \times 100 \quad \text{Equation 3.1}$$

where,

W_x – The weight of the empty crucible (g)

W_y – The weight of the sample before oven drying (g)

W_z – The weight of the crucible and dried SCG sample (g)

3.2.2 Ashes

The quantification of ashes was determined by gravimetric method as described in the Official Methods of Analysis (AOAC, 1990) 923.03 based in complete incineration of organic matter at 550 °C using a furnace for 4 hours. The results were expressed in % based on the following formula:

$$\text{Ash (dry basis) \%} = \frac{W_c - W_a}{W_b - W_a} \times 100 \quad \text{Equation 3.2}$$

where,

W_a – The weight of the empty crucible (g)

W_b – The weight of the sample before ashing (g)

W_c – The weight of the crucible and ash sample (g)

3.2.3 Protein

The protein content was also determined by the method by Ballesteros *et al.*, (2014). Thermo Scientific Flash 2000 Elemental Analyzer was used to determine the nitrogen content by combustion before the protein content was subsequently estimated by using the formula of $N_2 \times 6.25$ conversion factor. The result were expressed in percentage (%).

3.2.4 Fatty Acid Content

Fatty acid content was determined by a method by Juarez *et al.* (2017) with some modifications. About 10 g of dried SCG was extracted using Soxhlet extractor with n-hexane as solvent for 4 hours. Then, the n-hexane was evaporated in a fume hood to obtain crude lipid. The crude lipid samples were then used to analyze free fatty acid (FFA) using a methodology described by AOCS (1990) with slight modifications. About 0.05 g was dissolved in 10 ml pre-heated ethanol added with phenolphthalein as an indicator and titrated with 0.1 N NaOH solution. The delipidated SCG was stored for further analysis.

3.2.5 Oil Content

Total oil content was quantified by Soxhlet extraction with n-hexane as described in American Oil Chemist Society (1990) 945.16 with some modifications. About 10 g of SCG was transferred into a thimble using spatula and placed the thimble in the extraction chamber. A round-bottom with 300 mL hexane and was placed on top of a heating mantle. Extraction chamber containing thimble and sample was fitted on round-bottom flask and the apparatus was held using a clamp connected to a retort stand. A condenser was connected to the extraction chamber to complete the extraction unit.

One end of a tube was attached to the lowest side of the condenser and while the other side of the same tube was connected to the source of distilled water forming a water inlet. A second tube was attached to the highest side of the condenser while the other side of the same tube was placed inside a sink forming water outlet. The source of distilled water was turned on to allow the water rise to the top of the condenser through the water inlet. As water filled the condenser, it exited through the water outlet and was discharged into the sink. The heating mantle was set to 95 °C and the extraction was carried out for 4 h.

After the extraction process was completed, the heating mantle and the distilled water supply was switched off and the system was allowed to cool down. Defatted dried samples were further dried in an oven for the analysis of total, insoluble, and soluble dietary fibers. The extracted oil was collected and poured into a pre-weighed beaker. The beaker containing hexane and extracted SCG was boiled under low temperature and stirred continuously as the temperature was monitored using a thermometer until the mixture was boiled at 70 °C. The mixture was allowed to cool down at room temperature as hexane was evaporated into the surrounding. The results were expressed in percentage based on the following formula:

$$\text{Oil content:} = \frac{W_r - W_p}{W_q - W_p} \times 100 \quad \text{Equation 3.3}$$

where,

W_p – The pre-weighed beaker (g)

W_q – The initial weight of SCG (g)

W_r – The beaker and extracted SCG oil (g)

3.2.6 Glucose Analysis

The sugar composition of the SCGs was determined by the NREL laboratory analytical procedure (TP-510-42618), in which 0.3 g of SCGs was soaked in 30 mL of 72% (v/v) H_2SO_4 at 30 °C for 2 h. The mixture was diluted with 84 mL distilled water to a concentration of 4% and autoclaved at 121 °C for 1 h. After the reaction, the mixture was neutralised using CaCO_3 powder until the pH reached 7. Then, the supernatant was filtered with a 0.22 μm syringe filter for high-performance liquid chromatography (HPLC) analysis. The concentration of glucose was determined by HPLC, which was equipped with a refractive index detector and column. The HPLC analysis conditions were as follows: mobile phase of 0.05 M H_2SO_4 , flow rate of 0.6 mL/min with temperature of column maintained at 60 °C.

3.2.7 Carbohydrate Content

The carbohydrate content was determined (expressed in glucose equivalent, GE) through phenol–sulfuric acid (PSA) method and dinitrosalicylic acid (DNS) method for total sugar (TS) and total reducing sugar (TRS) respectively based on the procedure described by Juarez *et al.* (2017) with some modifications. The sample was prepared by employing acid hydrolysis method of SCG. The method required 2.5 N of 50 mL HCL as hydrolysing medium for 100 mg of dried SCG. The hydrolysis process was carried out in a conical flask covered with aluminium foil and incubated at 95 °C for 3 hours. The hydrolysate was then cooled down at room temperature and neutralised with Na_2CO_3 before it was filtered using tea filter.

3.2.7.1 Total Sugar (TS) Analysis

Phenol-sulfuric acid (PSA) method proposed by Juarez *et al.* (2017) was used to analyse total sugar (TS). About 1 ml of the sample was transferred into test tubes. Each test tube containing the sample was added with 1 mL of 5% phenol solution and 5 mL of 96 % H₂SO₄ before the tubes were left at room temperature for 10 minutes. The mixtures were then mixed vigorously using a vortex mixer and placed in a water bath at 25 - 30 °C for 20 minutes. The absorbance of the reaction mixture was then analysed using a spectrophotometer at 490 nm. The TS yield and TS recovery were calculated using the following equation:

$$\text{Total sugar yield, TS (\%)} = \frac{C_{TS}V_H}{M_{initial}} \times 100 \quad \text{Equation 3.4.1}$$

$$\text{TS recovery, } \eta_{TS} (\%) = \frac{Y_{TS}}{Y_{TS, \max}} \times 100 \quad \text{Equation 3.4.2}$$

where,

$C_{TS}V_H$ – The total sugar concentration obtained from the calibration curve

$M_{initial}$ – The weight of the initial dry lipid-free biomass before hydrolysis

3.2.7.2 Total Reducing Sugar (TRS) Analysis

Dinitrosalicylic acid (DNS) method proposed by Juarez *et al.* (2017) was used to analyse total reducing sugar (TRS). About 1 ml of sample was pipetted into a test tube and it was then diluted with 3 mL of distilled water. Next, 1 mL of DNS reagent was added into the mixture and boiled for 10 minutes before adding 1 mL of 40% Rochelle salt solution. The absorbance of the suspension was measured using a spectrophotometer at 540 nm. The TRS yield and TRS recovery were calculated using the following equations:

$$\text{Total reducing sugar yield, TRS (\%)} = \frac{C_{TRS}V_H}{M_{initial}} \times 100 \quad \text{Equation 3.5.1}$$

$$\text{TRS recovery, } \eta_{TRS} (\%) = \frac{Y_{TRS}}{Y_{TRS, \max}} \times 100 \quad \text{Equation 3.5.2}$$

where,

$C_{TRS}V_H$ – Total reducing sugar concentration obtained from the calibration curve

$M_{initial}$ – The weight of the initial dry lipid-free biomass before hydrolysis

3.2.8 Cell Soluble Constituents, Cellulose, Hemicellulose, and Lignin

Fibre analysis of animal feed including neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) was used to quantify the amount of cell soluble constituents, cellulose, hemicellulose and lignin in the SCG. NDF consists of hemicellulose, cellulose and lignin. ADF consists of cellulose and lignin while ADL consists of lignin only. Hence, hemicellulose content can be determined by subtracting ADF from NDF whereas cellulose content can be quantified by subtracting ADL from ADF.

3.2.8.1 Neutral Detergent Fiber (NDF)

The procedure was based on Van Soest *et al.* (1991) with some modifications. Neutral detergent solution consisting of 18.6 g EDTA, 6.8 g Na₂B₄O₇·10H₂O, 30 g sodium lauryl sulphate and 10 mL of ethylene glycol monoethyl ether was prepared. About 1 g of SCG was weighed and transferred carefully into a tall form beaker without sprout containing 100 mL of neutral detergent solution and 0.5 g Na₂SO₃. The solution was boiled for 1 h on a heating mantle while being stirred with a magnetic bar. A round-bottom flask containing 300 mL of boiling distilled water was fitted with a condenser. Next, the sample was filtered using a filtration assembly. The filtrate was rinsed using hot water and acetone for 2-3 times. The filtrate was transferred into a crucible and allowed to dry in the oven until a constant weight was achieved. NDF was calculated using the following equation where the results were expressed in percentage (%). Calculations:

$$\text{Filtrate (\%)} = \frac{W_k - W_i}{W_j - W_i} \times 100 \quad \text{Equation 3.6.1}$$

where,

W_i – The pre-weighed beaker (g)

W_j – The initial weight of SCG (g)

W_k – The beaker and SCG filtrate (g)

$$\text{Cell soluble constituents (as \% of dry matter)} = 100 - \text{NDF} \quad \text{Equation 3.6.2}$$

3.2.8.2 Acid Detergent Fiber (ADF)

The procedure was based on the Official Methods of Analysis (AOAC, 1990) 923.03 with some modifications. Acid detergent solution consisted of 36 g of CTAB and 50 mL of 1 N H₂SO₄ in a 1000 mL distilled water. Next, 1 g of SCG was weighed and transferred into a beaker containing 100 mL of ADF solution and boiled on heating mantle for 1 h while stirred using magnetic bar. A round-bottom flask containing 300 mL of hot distilled water was fitted as condenser. The filtrate was then filtered using a filtration assembly. The filtrate was rinsed using boiling hot water and acetone for 2-3 times. Subsequently, the filtrate was transferred into a crucible and dried in an oven until constant weight was achieved. ADF was calculated using the same equation to find NDF while hemicellulose content was determined by the difference between NDF and ADF values. Results were expressed as %.

$$\text{Hemicellulose (\%)} = \text{NDF} - \text{ADF} \quad \text{Equation 3.6.3}$$

3.2.8.3 Acid Detergent Lignin (ADL)

The procedure was determined by the gravimetric assay used for the direct quantification of lignin. It was based on the Official Methods of Analysis (AOAC, 1990) 923.03 with some modifications. Firstly, the sample was prepared by repeating the ADF procedure to obtain ADF residue. Next, the ADF residue was treated with 72 % H₂SO₄ and stirred with a glass rod as the solution dried. The solution was replenished continuously for 3 hours. The filtrate was then washed with hot distilled water for 2-3 times before it was dried in an oven until a constant weight was achieved. Lignin was determined using the same equation to calculate NDF. Cellulose content was determined by subtracting the ADL values from the ADF values. The results were expressed in percentage (%).

$$\text{Cellulose (\%)} = \text{ADF} - \text{ADL} \quad \text{Equation 3.6.4}$$

3.2.9 Total, Insoluble and Soluble Dietary Fibers

The total dietary fibre (TDF) of SCG sample was determined by enzymatic gravimetric method based on Official Methods of Analysis (AOAC, 1990) 923.03 standard procedure with some modifications. About 1 g of defatted SCG sample from Soxhlet extraction for fat content analysis was mixed with 50 mL of 0.08 M pH 6 phosphate buffer in a conical flask. Then, about 0.1 mL of α -amylase was added into the mixture and the flask was covered with aluminium foil and left for 15 minutes in a boiling water before the pH was adjusted to 7.5 using 2.0 M of NaOH solution. Protease solution was prepared by adding 50 mg of protease powder into 1 mL phosphate buffer.

Next, 0.1 mL of protease solution was added to the sample before the suspension was heated in a water bath at 60 °C for 30 minutes. The sample was left to cool down at room temperature before the pH was adjusted to 4.0 using 2 M HCL. Subsequently, 0.3 mL amyloglucosidase was added into the sample and the suspension was incubated in a water bath at 60 °C for another 30 minutes. Additionally, 280 mL of 98 % preheated ethanol at 60 °C was added to the sample and the suspension was left at room temperature for 1 hour. The sample was then filtered and washed consecutively with 20 mL of 70% ethanol, twice with 10 mL of 95% ethanol and once with 10 mL of acetone.

Finally, the solid residue was dried until a constant weight was achieved. This method was performed in triplicate: one sample was used to determine protein content, while another sample was used to determine ash content. Distilled water was used as a blank to replace phosphate buffer. The TDF percentage was calculated by using the following equation:

$$\text{TDF \%} = \frac{W_2 - W_3 - W_4 - W_5}{W_1} \times 100 \quad \text{Equation 3.7}$$

where,

W_1 = Average weight of the sample taken (mg)

W_2 = Average final weight of the sample (mg)

W_3 = Average weight of protein (mg)

W_4 = Average weight of ash (mg)

W_5 = Blank weight

Insoluble dietary fiber (IDF) were determined using Equation 3.7, but without adding alcohol in the precipitation stage while the soluble dietary fiber (SDF) percentage was obtained by the difference between TDF and IDF values.

3.3 Defatting of SCG

The defatting process of the SCG was carried out using a sonication-assisted extraction technique, adapted from the method described by Trinh *et al.* (2022), with slight modifications to suit the experimental conditions. In this procedure, hexane was used as the solvent for lipid extraction, applied to the SCG in a solvent-to-solid ratio of 5:1 (v/w). The mixture was subjected to ultrasonic treatment to enhance solvent penetration and facilitate the breakdown of cell structures, thereby improving the efficiency of oil removal from the biomass. Following the sonication process, the defatted SCG were thoroughly dried in a laboratory oven at 85 °C to eliminate any residual solvent and moisture. Once completely dried, the treated biomass was stored in an airtight container to maintain its integrity and prevent rehydration or contamination prior to further analysis.

3.4 Alkaline Pre-treatment of SCG

Alkaline pre-treatment of SCG was performed using KOH and NaOH. The reaction was carried out in 250 mL Erlenmeyer flasks with 50 mL working volume in a water bath. Based on the method outlined by Lee *et al.* (2021) and Jin *et al.* (2020), about 10 % (w/v) SCG was used by reacting 5 g of dried SCG with KOH and NaOH of various concentrations, temperatures and incubation times. Following alkaline pre-treatment using KOH and NaOH, enzymatic hydrolysis using were conducted for the pre-treatments samples across all levels. Enzymatic hydrolysis was performed at the substrate loading of 3% (w/v) of pre-treated defatted SCG with 50 mL of 50 mM citric buffer (pH 4.8) in 50 °C shaking water bath at 100 rpm for 72 h. Thereafter, the SCG sample was supplemented with cellulase (95 FPU/ml) from *Trichoderma reesei* (Sigma Aldrich) with 5% ratio of enzyme to SCG based on procedure by Trinh *et al.* (2022) (refer 3.7 for full description of enzymatic hydrolysis).

In order to optimise the alkaline pre-treatment of SCGs using KOH and NaOH respectively, two separate works were conducted using central composite design (CCD) of response surface methodology (RSM) performed using Design-Expert 13 software (Stat-Ease Inc., USA). The three factors affecting the alkali pre-treatment of SCGs were divided into five levels (-2, -1, 0, 1 and 2; see Table 3.1(a)). The factors and their ranges were as follows: concentration (X_a ; 0.33–3.67%), temperature (X_b ; 50.00–100.00 °C) and time (X_c ; 0.33–3.67 h). The experimental results were statistically analysed by Analysis of Variance (ANOVA). The effects of the experimental factors and their interactions were explained using the following higher order polynomial quadratic equation:

$$Y = \beta_0 + \beta_a X_a + \beta_b X_b + \beta_c X_c + \beta_{ab} X_a X_b + \beta_{ac} X_a X_c + \beta_{bc} X_b X_c + \beta_{aa} X_a^2 + \beta_{bb} X_b^2 + \beta_{cc} X_c^2 \quad \text{Eq. 3.8}$$

where Y refers to the predicted surface function whereas β_0 is the model constant. Meanwhile, β_a , β_b , and β_c are linear coefficient, β_{ab} , β_{ac} and β_{bc} cross product coefficient and β_{aa} , β_{bb} and β_{cc} are quadratic coefficient (Kobya *et al.*, 2013).

Table 3.1 shows the independent variables that were studied for these two models and their levels in the RSM. The experimental independent variables were KOH and NaOH concentrations (0.33, 1.00, 2.00, 3.00 and 3.67% w/v) at different temperatures (50.00, 60.00, 75.00, 90.00 and 100.00 °C) for various incubation times (0.33, 1.00, 2.00, 3.00 and 3.67 h). Each levels coded as -1 (low), 0 (middle) and +1 (high) concentrations. As for the experimental points, eight star points (± 1), six axial points (± 2) and six replicates at the centre point (0) were chosen.

Table 3.1: Factors and their levels in the Response Surface Methodology (RSM)

Levels	Concentration of KOH or NaOH (% w/v)	Temperature (°C)	Incubation Time (h)
-2	0.33	50.00	0.33
-1	1.00	60.00	1.00
0	2.00	75.00	2.00
1	3.00	90.00	3.00
2	3.67	100.00	3.67

3.5 Experimental Design

The data of defatted SCG pre-treatment using KOH and NaOH under various concentration, temperature and incubation following enzymatic hydrolysis using cellulase were tabulated in Table 3.2(a) and Table 3.2(b). These data outline the CCD design in the form of a 2^3 full factorial design for SCG pre-treatment using KOH and NaOH respectively with six additional experimental trials (run numbers 1, 3, 9, 10, 14, and 17) as replicates of the central point to find the influencing parameters. A total of 20 experiments were performed. All experiments were performed in triplicate.

The central point results were utilised to verify the consistency of outcomes according to CCD. ANOVA was employed to visually analyse the data and determine the relationship between the process variables and responses. The accuracy of the polynomial model fit was assessed using the coefficient of determination (R^2), and its statistical significance was evaluated using the Fisher *F-test* within the same framework. The significance of model terms was determined by their corresponding p-values with a confidence level of 95 %.

Table 3.2(a): Central composite design for 5-level-3-factor response surface analysis of
KOH pre-treatment

Run	Factors		
	Concentration of KOH (% w/v)	Temperature (°C)	Incubation Time (h)
1	2.00	75.00	2.00
2	3.67	75.00	2.00
3	2.00	75.00	2.00
4	2.00	75.00	0.33
5	3.00	60.00	1.00
6	1.00	60.00	1.00
7	1.00	60.00	3.00
8	0.33	75.00	2.00
9	2.00	75.00	2.00
10	2.00	75.00	2.00
11	2.00	75.00	3.67
12	2.00	100.00	2.00
13	3.00	90.00	1.00
14	2.00	75.00	2.00
15	3.00	90.00	3.00
16	1.00	90.00	3.00
17	2.00	75.00	2.00
18	2.00	50.00	2.00
19	1.00	90.00	1.00
20	3.00	60.00	3.00

Table 3.2(b): Central composite design for 5-level-3-factor response surface analysis of
NaOH pre-treatment

Run	Factors		
	Concentration of NaOH (% w/v)	Temperature (°C)	Incubation Time (h)
1	2.00	75.00	2.00
2	3.67	75.00	2.00
3	2.00	75.00	2.00
4	2.00	75.00	0.33
5	3.00	60.00	1.00
6	1.00	60.00	1.00
7	1.00	60.00	3.00
8	0.33	75.00	2.00
9	2.00	75.00	2.00
10	2.00	75.00	2.00
11	2.00	75.00	3.67
12	2.00	100.00	2.00
13	3.00	90.00	1.00
14	2.00	75.00	2.00
15	3.00	90.00	3.00
16	1.00	90.00	3.00
17	2.00	75.00	2.00
18	2.00	50.00	2.00
19	1.00	90.00	1.00
20	3.00	60.00	3.00

3.6 Two-Step Pre-treatment of SCG

Further analysis was conducted to compare the effects between one-step and two-step pre-treatments. For this study, the one-step alkaline pre-treatment was based on the optimised KOH and NaOH conditions obtained in Section 3.5. In addition, acid hydrolysis was incorporated using H_2SO_4 since it is a strong dibasic acid that can effectively solubilise hemicellulose and disrupt the lignocellulosic matrix. The specific condition for acid pre-treatment was selected based on the methodology outlined by Lee *et al.* (2021). These parameters were chosen because dilute acid concentrations (1 %) are effective in hydrolysing hemicellulose into soluble sugars without generating excessive inhibitory by-products. Operating at a moderate temperature (60 °C) and reaction time (1 hour) ensures sufficient breakdown of hemicellulose and partial disruption of lignin–carbohydrate linkages while minimising sugar degradation and inhibitor formation. This balance makes the selected condition reliable for integrating into a two-step pre-treatment strategy.

In this study, a two-step pre-treatment strategy was investigated by combining acid and alkaline pre-treatments in different sequences, namely acid–alkaline and alkaline–acid. The rationale for applying both methods sequentially was to maximise the breakdown of the lignocellulosic matrix SCG, as each treatment targets different structural components of the biomass. For the acid–alkaline sequence, dilute sulfuric acid pre-treatment was carried out first to hydrolyse hemicellulose and disrupt lignin–carbohydrate linkages. This step increased porosity and improved cellulose exposure. The subsequent alkaline pre-treatment further enhanced delignification by removing residual lignin, while also reducing cellulose crystallinity and promoting biomass swelling.

Conversely, in the alkaline–acid sequence, alkaline pre-treatment was performed first to target lignin removal and loosen the compact lignin–carbohydrate structure. This was followed by acid pre-treatment, which further facilitated hemicellulose solubilisation and improved accessibility of the cellulose fraction. By assessing both acid–alkaline and alkaline–acid sequences, the study aimed to determine the most effective order for achieving maximum reducing sugar recovery and structural deconstruction efficiency. Table 3.3 outlines the experimental conditions for one-step and two-step SCG pre-treatments. Then, One Way ANOVA was conducted to find any significant difference between the one-step chemical pre-treatment using KOH, NaOH and H₂SO₄ as well as two-step chemical pre-treatment of alkaline–acid and acid–base sequences.

Table 3.3: Different conditions of SCG pre-treatments

Pre-treatments	Conditions
Control (Untreated defatted SCG)	No treatment, only enzymatic hydrolysis
H ₂ SO ₄	1% (v/v) H ₂ SO ₄ ; 60 °C ; 1 hour
KOH	3% (w/v) KOH ; 90 °C; 2 hour
NaOH	3% (w/v) NaOH ; 85 °C; 1 hour
KOH-H ₂ SO ₄	3% (w/v) KOH ; 90 °C; 2 hours 1% (v/v) H ₂ SO ₄ ; 60 °C ; 1 hour
H ₂ SO ₄ -KOH	1% (v/v) H ₂ SO ₄ ; 60 °C ; 1 hour 3% (w/v) KOH ; 90 °C; 2 hours
NaOH-H ₂ SO ₄	3% (w/v) NaOH ; 85 °C; 1 hour 1% (v/v) H ₂ SO ₄ ; 60 °C ; 1 hour
H ₂ SO ₄ -NaOH	1% (v/v) H ₂ SO ₄ ; 60 °C ; 1 hour 3% (w/v) NaOH ; 85 °C; 1 hour

3.7 Enzymatic Hydrolysis of SCG after Pre-treatment

The pre-treated SCG samples were further filtered using tea filter and rinsed using distilled water to remove the excess alkali before the samples were dried in an oven at 50 °C for 6 hours. Enzymatic hydrolysis was performed at the substrate loading of 3% (w/v) of pre-treated defatted SCG with 50 mL of 50 mM citric buffer (pH 4.8) in 50 °C shaking water bath at 100 rpm for 72 h. Thereafter, the SCG sample was supplemented with cellulase (95 FPU/ml) from *Trichoderma reesei* (Sigma Aldrich) with 5% ratio of enzyme to SCG (Trinh *et al.*, 2022). Cellulase activity was determined by International Union of Pure and Applied Chemistry (IUPAC) (Miller, 1959; Ghose, 1987). About 0.5 ml of 0.05% sodium azide was introduced to avoid any microbial contamination and 0.5 mL of 1.0% (v/v) Tween 80 was added to facilitate the enzymatic action (Igbojionu *et al.*, 2020).

3.8 Total Reducing Sugar (TRS) Analysis after Pre-treatment

The analysis of reducing sugar were carried out after the enzymatic hydrolysis step of the chemically pre-treated SCG samples. TRS assay using dinitrosalicylic acid (DNS) was carried out based on the procedure outlined by Juarez *et al.* (2017) with some modifications. About 100 µL of sample was pipetted into a test tube and then diluted with 2, 900 µL of distilled water to make up 1:30 dilution before adding 1 mL of DNS reagent with Rochelle salt. The mixture was boiled for 10 minutes. The absorbance of the resulting mixture was then measured using a spectrophotometer at the wavelength of 540 nm. The calibration curve was prepared by using glucose as a standard.

3.9 Structural Characterisation of SCG using SEM Analysis

SCG were analysed by scanning electron microscopy (SEM) using JSM-IT800 Schottky Field Emission Scanning Electron Microscope from JEOL Company. Based on the method by Wongsiridetchai *et al.* (2018) with some modification, the sample was sprinkled on a black tap and coated with platinum using autocoater. The images were obtained by applying an acceleration voltage of 10 kV, at 500-fold, 2, 000-fold and 5, 000-fold magnifications.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Physical and Chemical Composition of Spent Coffee Ground (SCG)

4.1.1 Moisture, Ashes, Protein, Fatty Acid and Oil content

The initial part of this study focused on the chemical composition of SCG. Table 4.1 shows the results of present studies of moisture, ashes, protein and extractable crude lipid content of SCG measured in terms of oil content and fatty acid content compared to the previous works reported in the literature. The experimental result for moisture content of SCG used in this study, which is 56.25%, falls within the range of 40.50% and 63.43% whereas the ash content (1.95%) also within the range of 1.1% to 2.8%, based on the reports by several literatures.

Otherwise, the protein content in SCG is notably higher, with an experimental value of 15.56%, closely aligning with the findings of Vardon *et al.* (2013). Recent reviews have also highlighted the high protein content of SCG were between 13% to 17% (w/w) of dry mass (Battista *et al.* 2020; Coelho *et al.*, 2020). Thus, SCG can be a reliable nutrient source in the food industry and valuable substrates in biotechnological and fermentative processes, as recent studies demonstrated their potential in producing bioactive peptides and exhibiting antihypertensive and antioxidant activities (Kovalcik *et al.* 2018; Valdes *et al.*, 2020; Ribeiro *et al.*, 2021).

Table 4.1: Moisture, ashes, protein and extractable crude lipid content of SCG

No.	Characteristics	Present study (% dry materials)	Previous works reported in the literature
1.	Moisture Content	56.25% $\pm$ 0.36	53.0-69.8% (Cruz <i>et al.</i> , 2012) 51.0% (Jooste <i>et al.</i> 2013) 63.43 $\pm$ 0.44% (Juarez <i>et al.</i> , 2017) 58.98 $\pm$ 0.32% (Hussein <i>et al.</i> , 2019)
2.	Ashes	1.95% $\pm$ 0.54	1.1% (Ballesteros <i>et al.</i> , 2014) 1.71% (Silva <i>et al.</i> 2018) 1.6% (Mussatto <i>et al.</i> 2011) 2.20% $\pm$ 0.06 (Juarez <i>et al.</i> , 2017) 1.8% (Vardon <i>et al.</i> , 2013) 2.77% (Hussein <i>et al.</i> , 2019)
3.	Protein	15.56% $\pm$ 0.98	17.44% (Ballesteros <i>et al.</i> , 2014) 13.6% (Mussatto <i>et al.</i> , 2011) 12.9% (Grammatikos <i>et al.</i> , 2021) 10.03% (Silva <i>et al.</i> 2018) 11.20% (Martinez-Saez <i>et al.</i> , 2017) 16.9% (Cruz <i>et al.</i> , 2012) 15.4% (Vardon <i>et al.</i> , 2013)
4.	Oil content	14.0% $\pm$ 0.04	16.9% (Grammatikos <i>et al.</i> , 2021) 10-15% (Jenkins <i>et al.</i> , 2014) 9.3-16.2% (Cruz <i>et al.</i> , 2012) 14.7% $\pm$ 0.56 (Silva <i>et al.</i> 2018) 16.53% (Juarez <i>et al.</i> 2017) 16.2% (Vardon <i>et al.</i> , 2013) 13.89 $\pm$ 0.05% (Hussein <i>et al.</i> , 2019)
5.	Free Fatty Acid	5.08% $\pm$ 0.72	3.34% $\pm$ 0.038 (Juarez <i>et al.</i> , 2017) 2.29 g/100g $\pm$ 0.30 (Ballesteros <i>et al.</i> , 2014)

Besides, many studies have investigated the extraction of value-added products from the lipid fractions of SCG, which makes up 7–21% of the total dry mass of SCG based on the reports by Kovalcik *et al.* (2018), and Araújo *et al.* (2019), Toda *et al.* (2021). The experimental result for the extractable crude lipid of SCG showed an oil content of 14.0%, which is closely aligned with the findings of Silva *et al.* (2018) and Hussein *et al.* (2019), who reported 14.7% and 13.89%, respectively. However, this result still falls within the range reported by Jenkins *et al.* (2014) and Cruz *et al.* (2012), which is between 10–15% and 9.3–16.2%, respectively. Additionally, the free fatty acid content was calculated at 5.08%, which is higher compared to the results reported by Juarez *et al.* (2017) and Ballesteros *et al.* (2014). This oil has applications in producing biopolymers, biofuels, antioxidants, and composites (Karmee, 2018; Pereira *et al.*, 2020; Bomfim *et al.*, 2023).

Since lipids constitute a significant proportion of SCG, defatting is an essential preliminary step before subjecting the biomass to further valorization processes. The removal of oils not only allows for the recovery of high-value lipid-based products but also prevents interference during subsequent chemical or enzymatic hydrolysis. Residual lipids can hinder enzyme accessibility, reduce hydrolysis efficiency, and lead to the formation of inhibitory compounds during fermentation (Bouhlal *et al.*, 2020; Ribeiro *et al.*, 2021). Thus, defatting improves both the efficiency of downstream processing and the overall economic feasibility of SCG biorefinery. Once the lipid fraction is removed, the remaining lignocellulosic biomass requires further structural modification to overcome its inherent recalcitrance, which is typically achieved through chemical pre-treatment.

4.1.2 Sugar Components and Carbohydrate Contents of SCG

Table 4.2 shows that the total sugar content in SCG used in this study was found to be 35.28% while the total reducing sugar content was 29.56% respectively which is within the range of values reported in the literature. According to several literatures by Ballesteros *et al.* (2014), Campos-Vega *et al.* (2015), and Mussatto *et al.* (2011), carbohydrates are the most abundant constituents in SCG. For this study, Juarez and co-workers (2014) who reported that polysaccharides can be extracted from SCGs using acid treatment, yielding 33.76% of total sugar and 27.61 % of total reducing sugar, based the quantification of total sugar and total reducing sugar on the same procedure. In another study conducted by Ballesteros and co-workers (2015), polysaccharides extracted from SCGs using alkali treatment produces 39.0% of total sugar which is higher than the extraction of polysaccharides using acid treatment. In term of sugar composition of SCG, several studies reported that SCG consist of 11-24% of glucose, 37-57% of mannose, 26-31.9% of galactose and 3.6-6% of arabinose (Simões *et al.*, 2009; Mussatto *et al.*, 2011; Ballesteros *et al.*, 2014).

The SCG used in this study mainly consisted of 51.4% NDF, which includes hemicellulose, cellulose, and lignin. Besides, ADF that represents the cellulose and lignin, was also found in significant amounts (27.8%), indicating a substantial presence of hemicellulose while the lignin content of SCG was measured by the amount of ADL. Hence, the SCG used for this study consists of 38.6% cell soluble constituents based on dry matter basis as well as 33.6% hemicellulose, 9.3% cellulose, and 18.5% lignin. These results were summarized in Table 4.2 that comprises of sugar and carbohydrate contents of SCG. Both NDF and ADF results of this study are considerably higher in comparison to the 45.2% of NDF and 29.8% of ADF values reported by Vardon *et al.* (2013). In another study conducted

by Vítěz *et al.* (2016), the NDF value was reported to be within the range of 57.68 - 58.44% while the ADF value was between 42.14 - 43.03%.

Table 4.2: Sugar and carbohydrate contents of SCG

No.	Characteristics	Present study (% dry materials)	Previous works reported in the literature
1.	Total Sugar	35.28% $\pm$ 0.28	33.76% $\pm$ 2.98 (Juarez <i>et al.</i> , 2017) 39.0% (Ballesteros <i>et al.</i> , 2015)
2.	Total Reducing Sugar	29.56% $\pm$ 0.46	27.61% $\pm$ 0.96 (Juarez <i>et al.</i> , 2017)
3.	Glucose	15.50% $\pm$ 0.62	24.08% (Ballesteros <i>et al.</i> , 2014) 19.0% (Mussatto <i>et al.</i> , 2011) 11.0% Simões <i>et al.</i> (2009)
4.	Mannose	-	37.03% (Ballesteros <i>et al.</i> , 2014) 46.8% (Mussatto <i>et al.</i> , 2011) 57.0% Simões <i>et al.</i> (2009)
5.	Galactose	-	31.90% (Ballesteros <i>et al.</i> , 2014) 30.4% (Mussatto <i>et al.</i> , 2011) 26.0% Simões <i>et al.</i> (2009)
6.	Arabinose	-	3.6% (Ballesteros <i>et al.</i> , 2014) 3.8% (Mussatto <i>et al.</i> , 2011) 6.0% Simões <i>et al.</i> (2009)
7.	NDF	51.4% $\pm$ 0.26	45.2% (Vardon <i>et al.</i> , 2013)
8.	ADF	27.8% $\pm$ 0.32	29.8% (Vardon <i>et al.</i> , 2013)
9.	Lignin (ADL)	18.5% $\pm$ 0.68	23.9% (Ballesteros <i>et al.</i> , 2014) 19.6 g/100g (Ravindran <i>et al.</i> 2017)
10.	Cellulose	9.3% $\pm$ 0.04	12.4% (Ballesteros <i>et al.</i> , 2014) 8.6% (Mussatto <i>et al.</i> , 2011) 10.8% (Kondamudi <i>et al.</i> , 2008) 7.6% (Grammatikos <i>et al.</i> , 2021) 8.6 mg/100 g (Ravindran <i>et al.</i> 2017)

Table 4.2 continued

11.	Hemicellulose	33.6% $\pm$ 0.54	39.1% (Ballesteros <i>et al.</i> , 2014) arabinose - 3.6% mannose - 19.07% galactose - 16.45% xylose - n.d 36.7% (Mussatto <i>et al.</i> , 2011) arabinose - 1.7% mannose - 21.2% galactose - 13.8% xylose - n.d 28.4% (Kondamudi <i>et al.</i> , 2008) 42.0% (Grammatikos <i>et al.</i> , 2021) 33.5 g/100g (Ravindran <i>et al.</i> 2017)
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In addition to lipids, SCG is primarily composed of polysaccharides (cellulose and hemicellulose), proteins, and lignin, which together make up the structural lignocellulosic matrix (Caetano *et al.*, 2022). Typically, cellulose content ranges between 8–15%, hemicellulose between 30–40%, and lignin between 20–30%, depending on the roasting degree and extraction method (Mussatto *et al.*, 2011; Ballesteros *et al.*, 2014). SCG also contain high sugars that can be polymerized into cellulose and hemicellulose structures, making up to 42.9% (w/w) of SCG dry weight. Hemicellulose is a heteropolymer consisting mainly of mannose, followed by galactose and arabinose, and it forms the backbone of lignocellulosic structures such as galactomannan, arabinogalactan, and cellulose (Arya and Rao, 2007; Mussatto *et al.*, 2011; Ballesteros *et al.*, 2014; Bevilacqua *et al.*, 2023; Bomfim *et al.*, 2023). Meanwhile, cellulose is a linear homopolymer of repeated glucose units, suitable for pulp and paper production and convertible into glucose for biofuel generation, including ethanol and butanol (Battista *et al.*, 2020; Bomfim *et al.*, 2023).

However, this study indicates that the lignin content in SCG is higher than the cellulose content, consistent with report from other studies (Kondamudi *et al.*, 2008; Mussatto *et al.*, 2011; Ballesteros *et al.*, 2014; Ravindran *et al.*, 2017; Pereira *et al.*, 2020; Grammatikos *et al.*, 2021). There are two types of lignin, namely soluble and insoluble lignin. Ballesteros *et al.* (2014) reported that SCG contain 23.90% lignin, with 17.59% insoluble and 6.31% soluble lignin, whereas Lee *et al.* (2019) found that SCG contain 28.0% total lignin. According to Lee *et al.* (2019), lignin contains functional groups such as aliphatic hydroxyl, carboxyl, and phenol groups, making it a precursor for products like vanillin, carbon fiber, phenol, and ethylene, which can be further processed into paints, fuels, resins, and emulsifiers.

The high lignin fraction makes SCG a recalcitrant substrate that limits direct enzymatic hydrolysis (Jooste *et al.*, 2023; Zhang *et al.*, 2021). To overcome this barrier, chemical pre-treatment is necessary to disrupt the rigid lignocellulosic matrix, enhance porosity, and increase the accessibility of cellulose and hemicellulose for subsequent enzymatic or microbial conversion. Acid pre-treatment is commonly applied to solubilize hemicellulose and release fermentable sugars, while alkaline pre-treatment is more effective at delignification and breaking ester linkages between lignin and carbohydrates (Broda *et al.*, 2022; Kim *et al.*, 2023). However, each method alone has limitations whereby acid pre-treatment can generate fermentation inhibitors, whereas alkaline pre-treatment may not fully hydrolyse hemicellulose.

Therefore, a two-step pre-treatment approach is proposed, combining the strengths of both acid and alkaline processes. Acid pre-treatment can be applied first to solubilize hemicellulose and partially disrupt the biomass, followed by alkaline treatment to remove

lignin and improve cellulose accessibility. Alternatively, an alkaline–acid sequence may be employed depending on the desired sugar profile and downstream process requirements. This sequential strategy not only maximizes carbohydrate recovery but also reduces structural recalcitrance, leading to higher enzymatic digestibility and overall conversion efficiency of SCG into value-added products.

4.1.3 Total, Soluble and Insoluble Dietary Fibers of SCG

The high fiber content is primarily represented by the total dietary fiber (TDF) in SCG (64.25% w/w), that comprises of 56.72% of insoluble dietary fiber (IDF) and 7.53% of soluble dietary fibers (SDF). According to Vázquez-Sánchez *et al.* (2018), dietary fiber such as hemicellulose, cellulose, lignin as well as pectin, mucilage and gum, constitutes the edible parts of coffee beans. These fibers are resistant to digestion and absorption in the human small intestine and undergo partial or complete fermentation in the large intestine. Based on Table 4.3, the amount of IDF is higher than the SDF. The trend of these findings are similar to the results reported by several literatures (Muthy and Naidu, 2012; Ballesteros *et al.*, 2014; Vilela *et al.*, 2016; Montemurro *et al.*, 2024).

High content of IDF and SDF in SCG is justifiable as hemicellulose, cellulose and lignin are major components of insoluble fibers. Each type of fiber possesses specific properties. For example, SDF retains a large amount of water, supports the growth of bacterial flora, and reduces the absorption of fats and sugars. Conversely, IDF has low water retention, speeds up food transit through the digestive system, and promotes regular bowel movements (Ballesteros *et al.*, 2014). Since the SCG are materials containing high levels of SDF and IDF, and therefore, they have great potential to be used as raw material in the development of food products.

In this sense, recent studies by Papageorgiou *et al.* (2024) have highlighted the significant amount of TDF content in SCG which mainly composed of insoluble fibers such as lignin, hemicellulose and cellulose, alongside polyphenols like chlorogenic and neochlorogenic acids. The research has also explored the usage of SCG in enriching bakery products by enhancing their nutritional value and extending their shelf life. SCG-enriched cookies, for instance, demonstrated high fiber content and antioxidant properties, which prove the potential of SCG in functional food development.

Table 4.3: Total, Soluble and Insoluble Dietary Fibers of SCG

No.	Characteristics	Present study (% dry materials)	Previous works reported in the literature
1.	Total Dietary Fibers	64.25% $\pm$ 0.46	60.46% $\pm$ 2.19 (Ballesteros <i>et al.</i> , 2014) 55.70% (Montemurro <i>et al.</i> , 2024) 43% $\pm$ 0.8 (Muthy and Naidu, 2012) 50.62% $\pm$ 0.72 (Vilela <i>et al.</i> , 2016)
2.	Insoluble Dietary Fibers	56.72% $\pm$ 0.72	50.78% $\pm$ 1.58 (Ballesteros <i>et al.</i> , 2014) 50.24% $\pm$ 2.27 (Montemurro <i>et al.</i> , 2024) 35% $\pm$ 0.6 (Muthy and Naidu, 2012) 48.35% $\pm$ 0.55 (Vilela <i>et al.</i> , 2016)
3.	Soluble Dietary Fibers	7.53% $\pm$ 0.28	9.68% $\pm$ 2.70 (Ballesteros <i>et al.</i> , 2014) 5.46% $\pm$ 0.36 (Montemurro <i>et al.</i> , 2024) 8% $\pm$ 0.7 (Muthy and Naidu, 2012) 2.27% $\pm$ 0.16 (Vilela <i>et al.</i> , 2016)

4.2 Optimisation of Alkaline Pre-treatment

Previous studies have focused on raw SCG, with limited attention given to defatted SCG (Juarez *et al.*, 2017; Wongsiridetchai *et al.*, 2018; Jin *et al.*, 2020; Lee *et al.*, 2021). However, studying defatted SCG is crucial as the removal of lipids can enhance the efficiency of subsequent pre-treatment processes by improving solvent penetration and increasing the accessibility to cellulose and hemicellulose (Igarashi *et al.*, 2011; Battista *et al.*, 2020; Zhang *et al.*, 2021). Raw SCG retains significant amounts of oils, which can interfere with enzymatic or chemical treatments, leading to lower efficiency in structural breakdown (Kovalcik *et al.*, 2018). Defatting SCG can also reduce potential inhibitors that may affect further processing steps, making it a more suitable substrate for applications requiring high carbohydrate availability (Campos-Vega *et al.*, 2015).

In this study, alkaline pre-treatment was chosen for optimisation over acid pre-treatment or enzymatic hydrolysis because of its effectiveness in selectively removing lignin, which is the main barrier limiting carbohydrate accessibility in SCG. Alkaline treatment enhances delignification while preserving the carbohydrate fraction, thereby improving enzymatic digestibility in subsequent steps. Acid pre-treatment, while effective at solubilising hemicellulose, often generates inhibitory by-products such as furfural and HMF, which can hinder downstream processing (Wang *et al.*, 2016; López-Linares *et al.*, 2021).

Meanwhile, enzymatic hydrolysis is highly substrate-specific and its efficiency is significantly reduced without prior structural disruption by chemical pre-treatment (Igarashi *et al.*, 2011; Zhang *et al.*, 2019). This is because enzymes such as cellulases require direct access to the polysaccharide chains, which are embedded within the LCC in SCG (Kim *et al.*, 2016). The compact lignocellulosic matrix, reinforced by lignin, limits enzyme

accessibility and results in low sugar yields when hydrolysis is attempted without pre-treatment (Battista *et al.*, 2020). Moreover, the adsorption of enzymes onto lignin can lead to enzyme deactivation and increased processing costs due to higher enzyme loading requirements (Limayem and Ricke, 2017). Therefore, chemical pre-treatment, particularly alkaline processes, is essential to disrupt the rigid biomass structure, reduce lignin content, and enhance the overall efficiency of enzymatic hydrolysis.

For these experiments, an experimental design matrix called central composite design or CCD was utilised in the process of optimizing and investigating the best possible product from the analysis of interaction between the studied parameters and their effect on sugar recovery from the experimental batches. The parameters that have the greatest influence on the sugar recovery from alkaline pre-treatment of SCG were identified based on several recent studies by Jin *et al.* (2020) and Lee *et al.* (2021) which demonstrated the use of quadratic model to analysed sugar recovery after alkaline hydrolysis using SCG as substrate.

4.2.1 Regression Model Fitting and Statistical Analysis

In the present study, two types of alkali were selected and the relationship between three independent variables and a response were established using a quadratic model. The selected three factors consisted of concentration, $X_a = 0.33, 1.00, 2.00, 3.00, 3.67$ (% w/v); temperature, $X_b = 50.00, 60.00, 75.00, 90.00, 100.00$ °C and incubation time, $X_c = 0.33, 1.00, 2.00, 3.00, 3.67$ h. The CCD results were estimated using the second-order polynomial equation by analysing a quadratic regression ANOVA for the experimental response:

Equation 4.1

$$Y_{HY(KOH)} (\%) = 2.15 + 0.1447x_a + 0.2728x_b - 0.0914x_c + 0.0003x_ax_b + 0.0948x_ax_c - 0.0662x_bx_c - 0.0418x_a^2 - 0.0642x_b^2 - 0.0682x_c^2$$

Equation 4.2

$$Y_{HY(NaOH)} (\%) = 2.53 + 0.0572x_a + 0.0957x_b - 0.1735x_c + 0.0330x_ax_b + 0.0004x_ax_c - 0.0528x_bx_c - 0.0484x_a^2 - 0.1425x_b^2 - 0.0396x_c^2$$

where x_a , x_b and x_c are the coded values of tested variables. A positive signs in front of the terms refer to a synergistic effect while the negative signs in front of the terms represent an antagonistic effect respectively (Ashari *et al.*, 2009; Salam *et al.*, 2015).

Table 4.4(a) and Table 4.4(b) summarize the central composite design (CCD) of 5-level, 3-factor response surface analysis for KOH and NaOH pre-treatments, respectively. The variation of concentration (0.33–3.67% w/v), temperature (50–100 °C), and incubation time (0.33–3.67 h) revealed clear differences in their influence on sugar release. The response, expressed as sugar concentration, demonstrates how each factor and their interactions contribute to treatment efficiency, thereby providing critical insights into the conditions most favorable for maximising sugar recovery. The experimental data generated from these runs formed the basis for developing the response surface model that enabled the prediction and optimisation of sugar yields under varying conditions for this study.

Table 4.4 (a): The experimental data of central composite design 5-level-3-factor response surface analysis for KOH pre-treatment

Run	Factors			Sugar concentration (mg/mL)
	Concentration of KOH (% w/v)	Temperature (°C)	Incubation Time (h)	
1	2.00	75.00	2.00	2.20
2	3.67	75.00	2.00	2.28
3	2.00	75.00	2.00	2.17
4	2.00	75.00	0.33	2.09
5	3.00	60.00	1.00	1.83
6	1.00	60.00	1.00	1.66
7	1.00	60.00	3.00	1.53
8	0.33	75.00	2.00	1.73
9	2.00	75.00	2.00	2.06
10	2.00	75.00	2.00	2.12
11	2.00	75.00	3.67	1.77
12	2.00	100.00	2.00	2.38
13	3.00	90.00	1.00	2.43
14	2.00	75.00	2.00	2.17
15	3.00	90.00	3.00	2.40
16	1.00	90.00	3.00	1.85
17	2.00	75.00	2.00	2.22
18	2.00	50.00	2.00	1.50
19	1.00	90.00	1.00	2.45
20	3.00	60.00	3.00	1.88

Table 4.4 (b): The experimental data of central composite design 5-level-3-factor response surface analysis for NaOH pre-treatment

Run	Factors			Sugar concentration (mg/mL)
	Concentration of NaOH (% w/v)	Temperature (°C)	Incubation Time (h)	
1	2.00	75.00	2.00	2.48
2	3.67	75.00	2.00	2.43
3	2.00	75.00	2.00	2.59
4	2.00	75.00	0.33	2.67
5	3.00	60.00	1.00	2.40
6	1.00	60.00	1.00	2.29
7	1.00	60.00	3.00	2.01
8	0.33	75.00	2.00	2.37
9	2.00	75.00	2.00	2.56
10	2.00	75.00	2.00	2.53
11	2.00	75.00	3.67	2.19
12	2.00	100.00	2.00	2.30
13	3.00	90.00	1.00	2.76
14	2.00	75.00	2.00	2.49
15	3.00	90.00	3.00	2.27
16	1.00	90.00	3.00	2.03
17	2.00	75.00	2.00	2.56
18	2.00	50.00	2.00	1.98
19	1.00	90.00	1.00	2.53
20	3.00	60.00	3.00	2.11

The fit quality of the model was confirmed through ANOVA in Table 4.5(a) and Table 4.5(b). For this regression analysis, each coefficient (slope) in the model is associated with the probability of error or p-value. According to Kobya *et al.* (2013), low p-value suggests that the predictor variable is likely a meaningful addition to the model because changes in the predictor's value are related to changes in the response variable. If a p-value is high, it suggests the changes in the predictor are not associated with changes in the response. In model responses for KOH pre-treatment, a, b, c, ac, bc, b^2 and c^2 were significant model terms while a, b, c, bc, a^2 , b^2 and c^2 were significant model terms for NaOH pre-treatment. The rest of the model terms apart from the aforementioned were insignificant. Moreover, to accept the significance of each terms in the model, the p-values should be less than 0.05 at 95% confidence level.

For the optimisation of KOH pre-treatment and NaOH pre-treatment, the model F-value for the percentage of reducing sugar concentration produced after enzymatic hydrolysis were 35.19 and 31.28 respectively which implies that these models were significant and there were only 0.01% chance that the large F-value could occur due to noise in consonance with Amosa (2015). Besides, KOH pre-treatment exhibits the Lack of Fit for F-value of 1.99 for sugar concentration implies that the Lack of Fit was not significant. This shows that there were 23.36 % chance to obtain a large Lack of Fit F-value. In contrast, the value of 2.51 for the percentage of sugar yield were produced after NaOH pre-treatment suggesting that the Lack of Fit F-value was also non-significant and the chances for the Lack of Fit F-value was large due to noise was 16.73%. As stated by Kobya *et al.* (2013), the insignificant lack of fit value that was higher than 0.05 interpreted that the quadratic model was valid for the present work as the effect of each responsive variables was the combination of coefficient, variable values and joint variables that cannot be observed through conventional experimentation means.

Table 4.5 (a): Analysis of variance (ANOVA) for the response surface of quadratic model
of yield percentage for KOH concentration

Source	Coefficient Estimate	Sum of Squares	df	Mean Square	F-value	p-value	Result
Model	2.15	1.64	9	0.1822	35.19	< 0.0001	significant
a-KOH concentration	0.1447	0.2842	1	0.2842	54.87	< 0.0001	
b-Temperature	0.2728	1.01	1	1.01	194.83	< 0.0001	
c-Incubation Time	-0.0914	0.1134	1	0.1134	21.90	0.0009	
ab	0.0003	8.928E-07	1	8.928E-07	0.0002	0.9898 ^a	
ac	0.0948	0.0720	1	0.0720	13.89	0.0039	
bc	-0.0662	0.0350	1	0.0350	6.77	0.0264	
a ²	-0.0418	0.0247	1	0.0247	4.77	0.0538	
b ²	-0.0642	0.0578	1	0.0578	11.16	0.0075	
c ²	-0.0682	0.0657	1	0.0657	12.68	0.0052	
Residual		0.0518	10	0.0052			not significant
Lack of Fit		0.0345	5	0.0069	1.99	0.2336	
Pure Error		0.0173	5	0.0035			
Cor Total		1.69	19				
Std. Dev.	0.0720			R²		0.9694	
Mean	2.04			Adjusted R²		0.9418	
C.V. %	3.54			Predicted R²		0.8126	
				Adequate Precision		20.0009	

DF: Degree of freedom; Prob > F < 0.500 indicate that the model terms are significant; Not significant at $p \geq 0.05^a$

Table 4.5 (b): Analysis of variance (ANOVA) for the response surface of quadratic model
of yield percentage for NaOH concentration

Source	Coefficient Estimate	Sum of Squares	df	Mean Square	F-value	p-value	Result
Model	2.53	0.9187	9	0.1021	31.28	< 0.0001	significant
a-NaOH concentration	0.0572	0.0445	1	0.0445	13.63	0.0042	
b-Temperature	0.0957	0.1240	1	0.1240	38.00	0.0001	
c-Incubation Time	-0.1735	0.4086	1	0.4086	125.20	< 0.0001	
ab	0.0330	0.0087	1	0.0087	2.68	0.1330 ^a	
ac	0.0004	1.102E-06	1	1.102E-06	0.0003	0.9857 ^a	
bc	-0.0528	0.0223	1	0.0223	6.83	0.0259	
a ²	-0.0484	0.0331	1	0.0331	10.13	0.0098	
b ²	-0.1425	0.2849	1	0.2849	87.28	< 0.0001	
c ²	-0.0396	0.0221	1	0.0221	6.77	0.0264	
Residual		0.0326	10	0.0033			not significant
Lack of Fit		0.0233	5	0.0047	2.51	0.1673	
Pure Error		0.0093	5	0.0019			
Cor Total		0.9514	19				
Std. Dev.	0.0571			R²		0.9657	
Mean	2.38			Adjusted R²		0.9348	
C.V. %	2.40			Predicted R²		0.7995	
				Adequate Precision		18.2352	
C.V. %	2.40			Predicted R²		0.7995	
				Adequate Precision		18.2352	

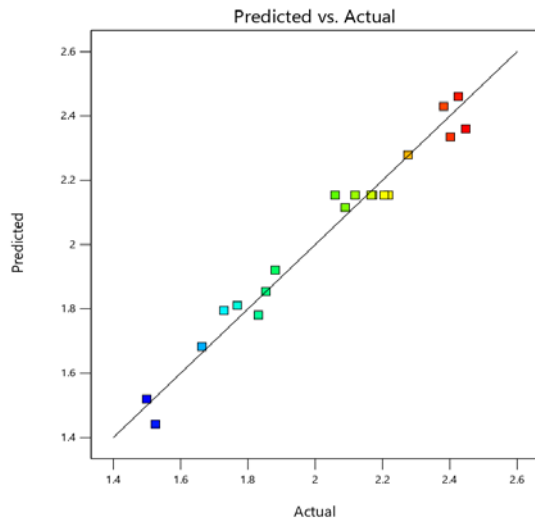
DF: Degree of freedom; Prob > F < 0.500 indicate that the model terms are significant; Not significant at $p \geq 0.05^a$

Furthermore, the coefficient of variation (CV) refers to the statistical measurement that expresses the relative variability of a set of data points in relation to the mean. It is often expressed as a percentage and considered to be reproducible when the CV is shown to be less than 10% (Kim and Yeom, 2020). The CV of each model responses for KOH pre-treatment and NaOH pre-treatment were found to be 3.54 and 2.40 for percentage of sugar yield respectively which signified that the result is reliable and accurate. Apart from that, the adequate precision determines the signal to noise ratio where a ratio greater than 4 is desirable to be used to navigate the design space (Behera *et al.*, 2020). Based on the regression model for the percentage of sugar recovery, the adequate precision of KOH pre-treatment model was 20.00 while NaOH pre-treatment was 18.24 which signified an adequate signal for the predicted model to be used for investigating the design space.

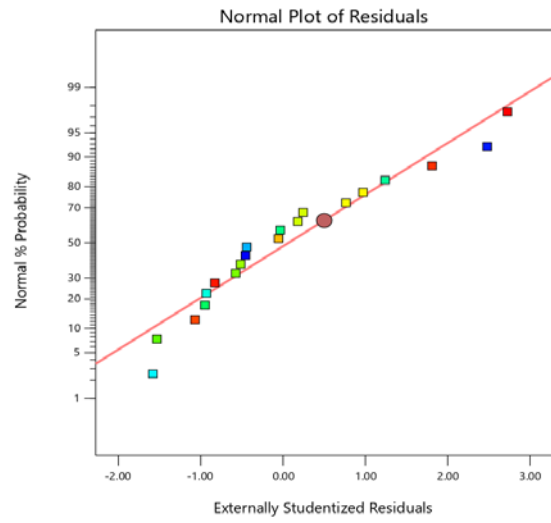
The goodness of fit for the predicted models includes the coefficient of determination (R^2), adjusted coefficient of determination (adjusted R^2) as well as the probability of error value of the model terms (p -value). Based on the results in Table 4.5(a) and Table 4.5(b), the R^2 values for these response variables exceeded 0.80 for both KOH pre-treatment (0.9694) and NaOH pre-treatment (0.9657) which indicating that the regression models exhibit a satisfactory fit with the experimental data (Karim *et al.*, 2014). The difference between R^2 and adjusted R^2 must be less than 0.2 while the adjusted R^2 must be in reasonable agreement with and closer to the value of predicted R^2 (Ying *et al.*, 2020). The results depicted that the adjusted R^2 was 0.9418 and predicted R^2 was 0.8126 for KOH pre-treatment. As for NaOH pre-treatment, the adjusted R^2 was 0.9348 and the predicted R^2 was 0.7995 respectively. The values were approximately close to 1 which shows a relatively good correlation between the predicted and experimental values of the sugar recovery after SCG pre-treatment using KOH and NaOH because the residual values of $\pm 20\%$ were acquired for

most of the predicted and actual values of the response indicating a relatively small discrepancies between these two values (Salam *et al.*, 2015).

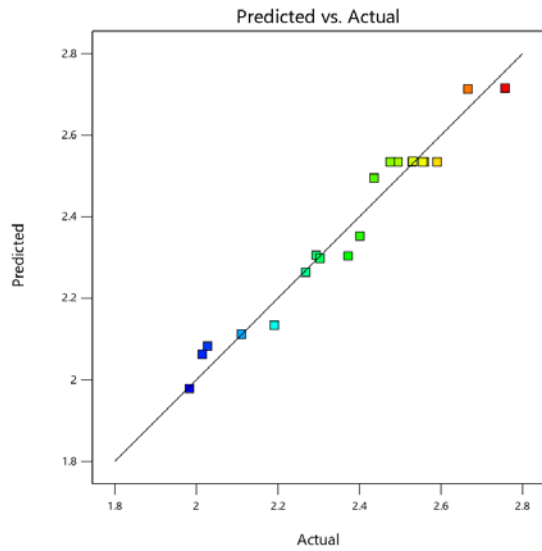
The actual and predicted values of sugar concentration after KOH and NaOH pre-treatment actual are portrayed in Figure 4.1(a) and Figure 4.1(c) respectively. The actual values represent the measured response data for specific runs, while the predicted values are derived from the model using approximation functions. As depicted in both figures, the data points closely align with the diagonal line, indicating that the developed models are adequate to predict each response. Meanwhile, Figure 4.1(b) and Figure 4.1(d) display the normal probability and studentized residuals plot. These figures illustrated the extent to which the model fulfils the ANOVA assumption, with studentized residuals indicating the number of standard deviations between the actual and predicted values.



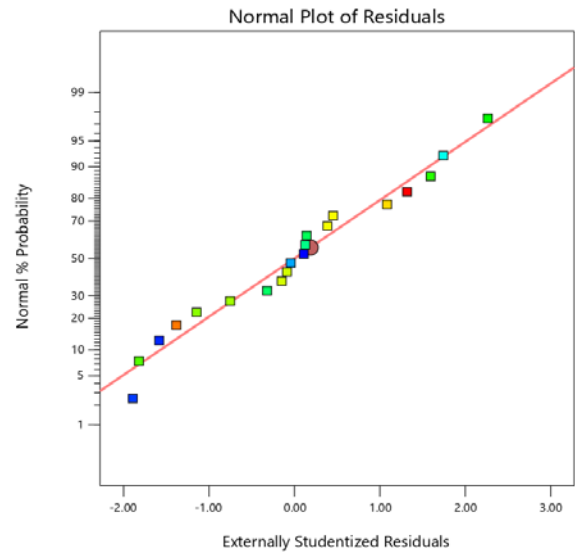
(a)



(b)



(c)



(d)

Figure 4.1: (a) Cross plot between the experimental and predicted values of sugar concentration after KOH pre-treatment and (b) Normal plot of the residuals for the model in equation (4.1). (c) Cross plot between the experimental and predicted values of sugar concentration for NaOH pre-treatment and (d) Normal plot of the residuals for the model in equation (4.2).

4.2.2 One Factor Plot

The behaviour of the effect of each three variables considered individually on the sugar concentration produced after KOH pre-treatment is presented in Figure 4.2. Figure 4.2(a) shows the effect of KOH concentration on the sugar concentration at the temperature of 75 °C and incubation time of 2 hours. When the KOH concentration increases from 1% (w/v) to 3% (w/v), there was a significant increase in the sugar concentration from 1.97 mg/mL to 2.26 mg/mL. Besides, Figure 4.2(b) portrays the relationship between the sugar concentration and the temperature with constant values of 2% (w/v) KOH concentration and 2 hours incubation time. When the temperature increase from 50°C to 90 °C, the sugar concentration increase linearly, producing around 1.19 mg/ml to 2.36 mg/mL. Next, Figure 4.2(c) exhibits the incubation time from 1 h to 3 h at constant 2% (w/v) KOH concentration and 75 °C temperature. The sugar concentration decline steadily generating sugar concentration of 2.18 mg/mL at 1 h and further drop to 1.99 mg/mL at the end of 3 h.

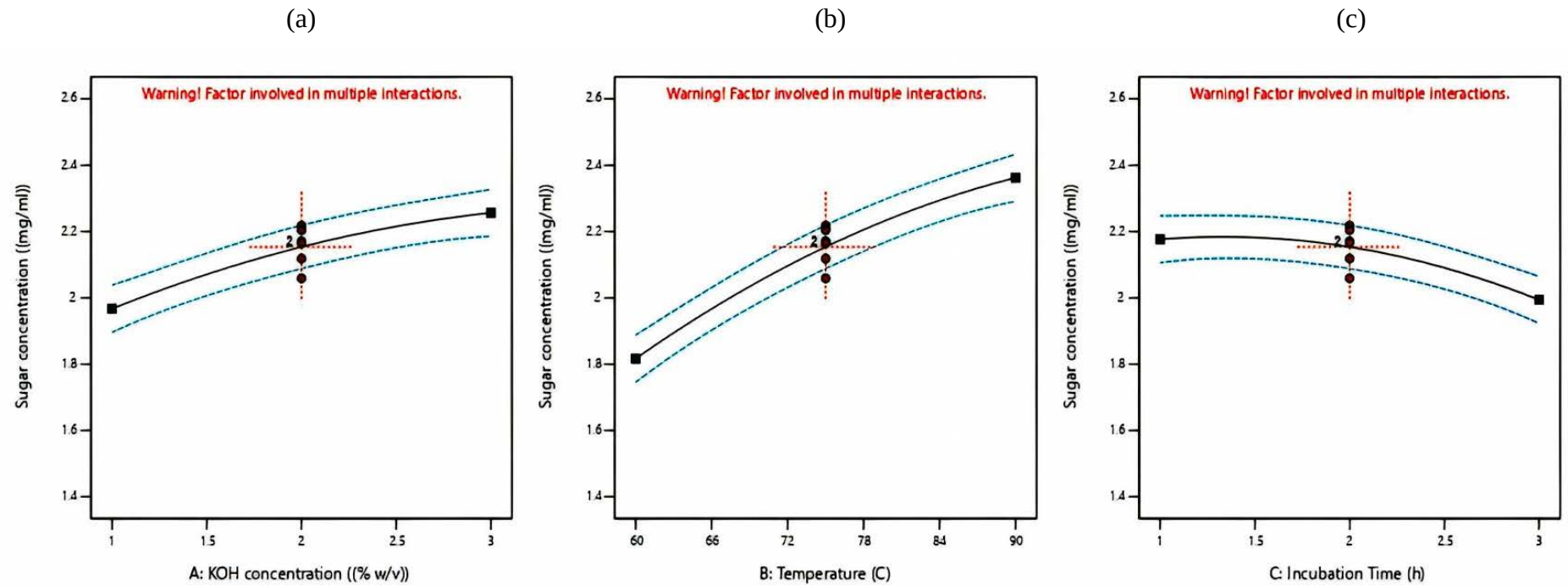


Figure 4.2: (a) One-factor effect of sugar concentration against KOH concentration.

(b) One-factor effect of sugar concentration against temperature.

(c) One-factor effect of sugar concentration against incubation time.

On the other hand, Figure 4.3 depicts the individual response of each three variables on the sugar concentration produced after NaOH pre-treatment. Figure 4.3 (a) illustrates the effect of NaOH concentration on the production of sugar. The sugar concentration increase slightly from 2.43 mg/mL to 2.54 mg/mL at the temperature of 75 °C during 2 h incubation time. Meanwhile, Figure 4.3 (b) represent the correlation between sugar concentration and temperature at NaOH concentration of 2% (w/v) and 2 h incubation time. When the temperature rise from 60 °C to 75 °C, the sugar concentration also increase from 2.29 mg/mL to 2.53 mg/mL. However, the sugar concentration gradually decrease as the temperature continue to increase generating 2.49 mg/mL at the temperature of 90 °C. Furthermore, Figure 4.3 (c) shows the relationship between the sugar concentration and time of incubation at 2 % (w/v) NaOH concentration and temperature of 75 °C. The increment of incubation time from 1 h to 3 h lead to the declining in the production of reducing sugar from 2.67 mg/mL to 2.32 mg/mL respectively.

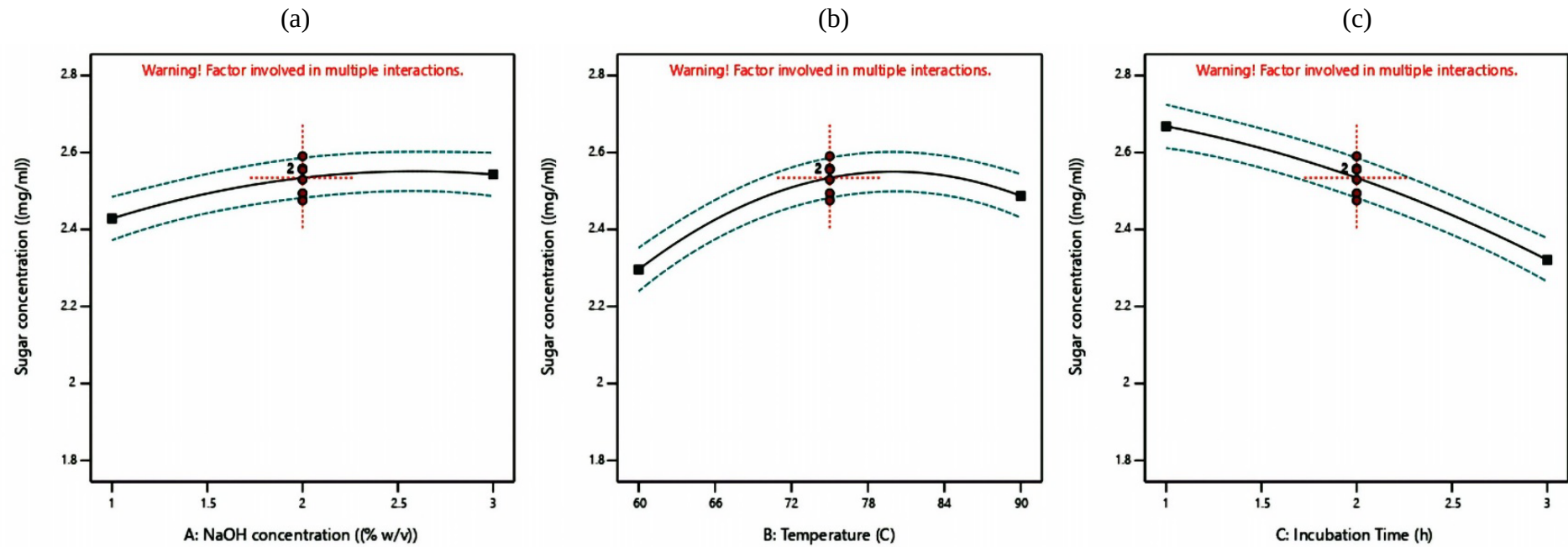


Figure 4.3: (a) One-factor effect of sugar concentration against NaOH concentration

(b) One-factor effect of sugar concentration against temperature

(c) One-factor effect of sugar concentration against incubation time

4.2.3 3D Response Surface Graphs and Contour Plots

Three-dimensional (3D) response surface plots as shown in Figure 4.4 (a) illustrate the interactive effects between two variables on sugar concentration produced after KOH pre-treatment while contour plots in Figure 4.4 (b) shows the optimised region between two factors which gives a better understanding on the concentration of sugar produced that were represented by the quadratic equation 4.1. Each 3D response graph and contour plot provide the analysis on the interaction between the experimental variable of each factor to maximise the response. In order to determine the optimal level of each experimental variables, 3D response surface plots were constructed by plotting the percentage of sugar yield (response) on the Z-axis against any two dependent variables while the other variable was kept at constant value (Ashari *et al.*, 2009). The optimised value was indicated by the symmetrical surface with flat regions that can be found near the optimum side of the graph. The contour plots describe the behaviour of optimised interactions between the variables that signified by the number of lines as well as the degree of curves and curls.

Figure 4.4 (a) shows the surface graphs of combined effects of KOH concentration-temperature, KOH concentration-incubation time and incubation time-temperature on the concentration of sugar produced after KOH pre-treatment. The KOH concentration-temperature surface graph described a linear relationship with the sugar concentration where, as the concentration of KOH and temperature increase, more sugar was recovered at the end of the enzymatic hydrolysis. The incubation time was fixed at centre point (2 h). The model predicted that the highest sugar concentration (2.47 mg/mL) can be obtained at the KOH concentration of 3% (w/v) and 90 °C temperature. Besides, KOH concentration-incubation surface graph illustrates that, at constant temperature of 75 °C, low KOH concentration (1% w/v) and the high incubation time (3 h) produce low sugar yield (1.71 mg/mL).

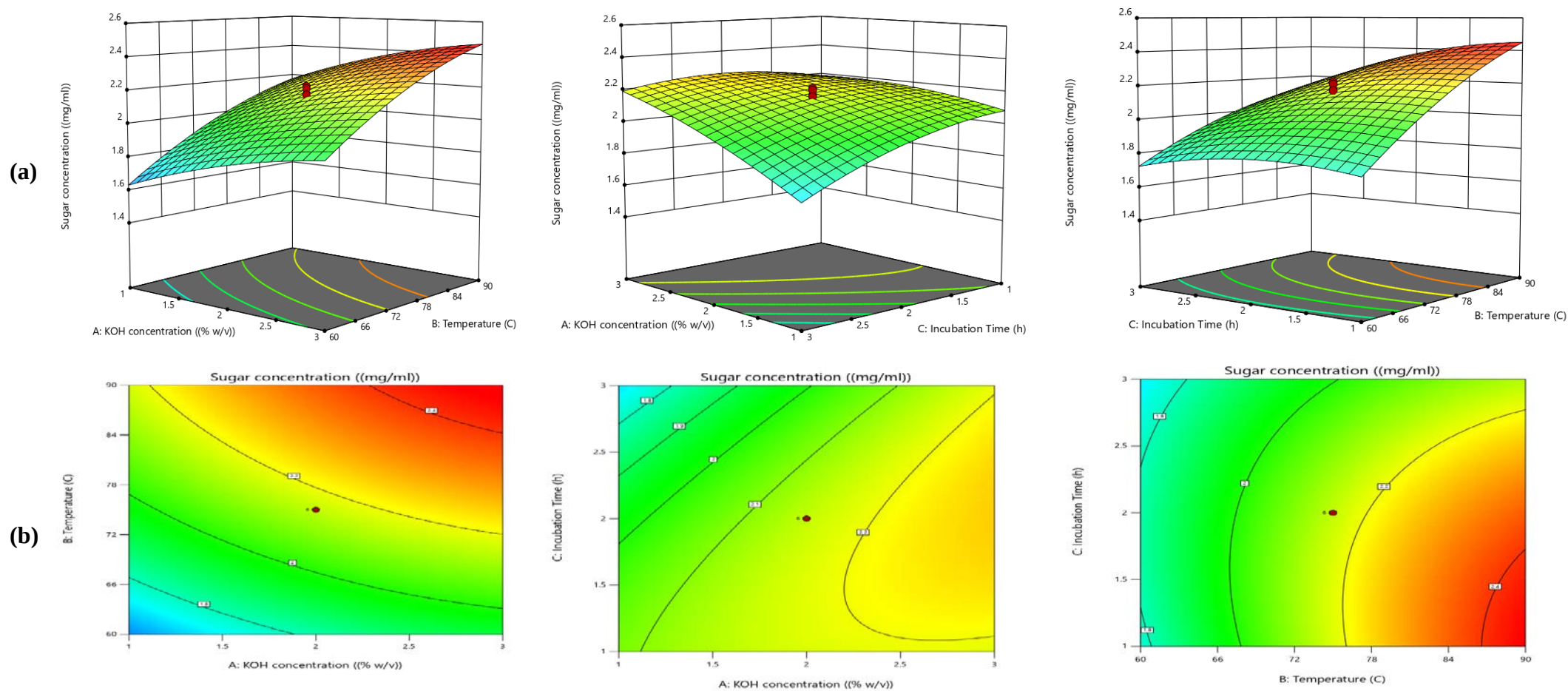
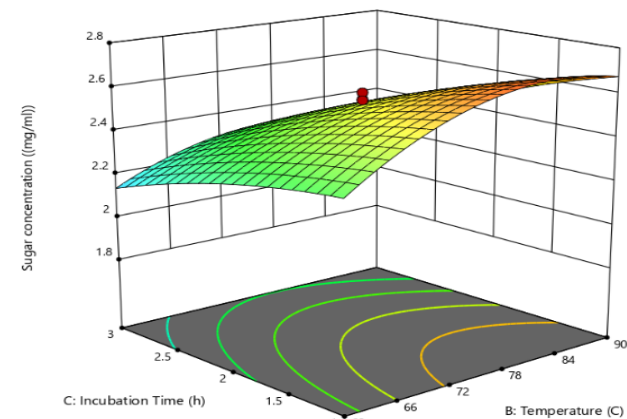
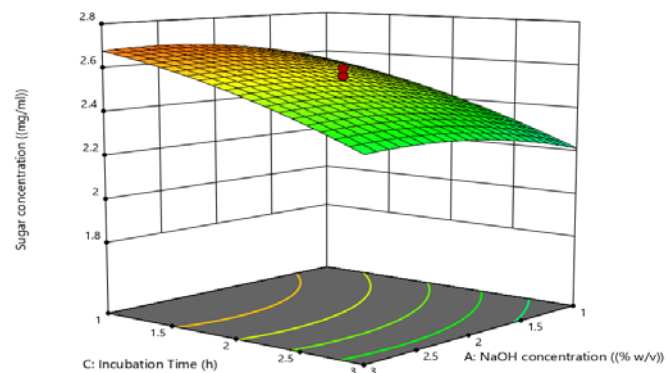
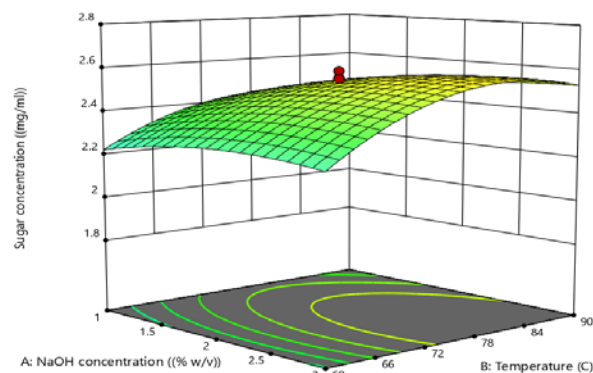


Figure 4.4: (a) 3D response surface graphs illustrates the combined effects of KOH concentration-temperature, KOH concentration-incubation time and incubation time-temperature on sugar concentration obtained after KOH pre-treatment and (b) contour plots of sugar concentration representing temperature-KOH concentration, incubation time-KOH concentration and incubation time-temperature.

Moreover, incubation time-temperature graphs show the reduction of incubation time in surge of temperature generated higher sugar concentration. This indicates that, regardless of the time level, the highest sugar yield was achieved at 90°C. This finding partially aligns with Lee *et al.* (2021), who used raw SCG instead of defatted SCG. Both studies show that glucose-equivalent sugar yield was lowest at 50 °C but increased with higher KOH concentration and temperature. Unlike the literature that reported a directly proportional relationship between incubation time and temperature in regard to glucan content, the result of the response in regard to this study observed the decline of sugar concentration in longer incubation time as temperature was decrease. At a constant KOH concentration of 2% (w/v), the sugar concentration generated was predicted to be around 1.72 mg/mL at the temperature of 60 °C and incubation time of 3 h.

The outcome above might stem from a heightened concentration of alkali and elevated temperature during extended incubation periods have harsh effects on cellulose and hemicellulose of the defatted SCG. Since the most of the lignin has been removed during the sonification step, alkali pre-treatment under high temperature in extended duration not only degrade the lignin and hemicellulose, but also potentially causing the dissolution of cellulose components (Asghar *et al.*, 2015). This is supported by Chen *et al.* (2012) who reported that high temperatures led to an increase in the breakdown of carbohydrates through random chain cleavage that can diminish the overall sugar output from the process, which prove to be detrimental as the time increases in relation to the current study. Figure 4.5 (a) shows the three-dimensional (3D) response surface plots that demonstrates the interactive effects between two variables on the sugar concentration produced for NaOH pre-treatment while contour plots in Figure 4.5 (b) depicts the optimised region between two factors.

(a)



(b)

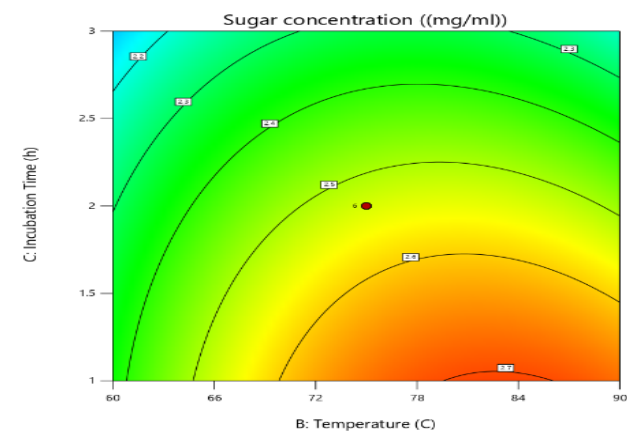
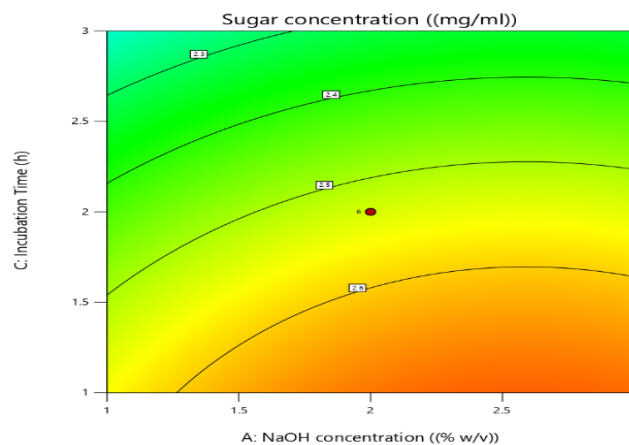
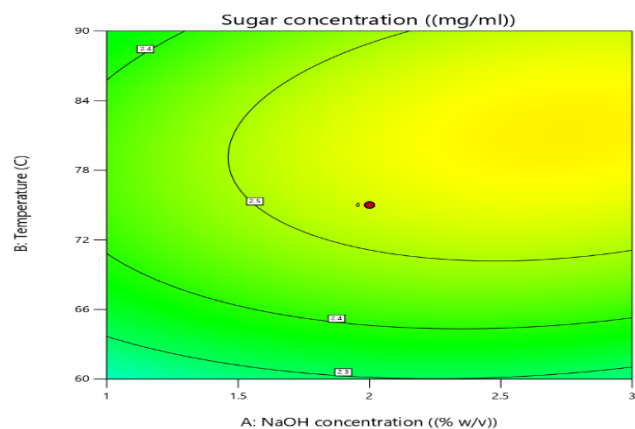


Figure 4.5: (a) 3D response surface graphs of factor interactions that shows the combined effects of NaOH concentration-temperature, incubation time-NaOH concentration and incubation time-temperature on concentration of sugar produced after NaOH pre-treatment and (b) contour plots of sugar concentration represents temperature-NaOH concentration, incubation time-NaOH concentration and incubation-temperature.

The illustration of 3D surface graphs in Figure 4.5(a) and contour plots in Figure 4.5(b) shows the concentration of sugar produced for NaOH pre-treatment that represented by the quadratic equations 4.2. The greatest sugar concentration generated after NaOH pre-treatment was 2.76 mg/mL which was obtained by Run 13 (3% w/v; 90 °C; 1 h) whereas Run 18 (2% w/v; 50 °C; 2 h) attaining 1.98 mg/mL of sugar concentration was the lowest response for NaOH pre-treatment. Figure 4.5 (a) depicts the combined effects of temperature-NaOH concentration, incubation time-NaOH concentration and incubation time-temperature on the sugar concentration produced after NaOH pre-treatment. The temperature-NaOH concentration surface plot illustrates the relationship between temperature and NaOH concentration at the fixed incubation time of 2 h. Based on the graph, sugar concentration increased when NaOH concentration and temperature increased. The most sugar concentration (2.53 mg/mL) was obtained at 3 % (w/v) NaOH concentration and 90 °C temperature.

Meanwhile, the interaction between incubation time-NaOH concentrations at fixed temperature of 75 °C observed that more reducing sugar was generated as the NaOH concentration increases from 1% (w/v) to 3% (w/v) while the incubation time decreases from 3 h to 1 h. The sugar concentration was at its peak (2.68 mg/mL) at NaOH concentration of 3% (w/v) and incubation time of 1 h. Similarly, when the NaOH concentration was fixed at 2% (w/v), the sugar increases as the temperature escalated up to 90 °C while the incubation time continued to decline based on the incubation time-temperature surface plot, yielding the highest sugar concentration of 2.67 mg/mL. This occurred because high concentration of alkali increases the solubilisation of lignin while temperature enhances the removal of lignin in accordance to the report by Tao *et al.* (2013) and Jin *et al.* (2020). However, the prolonged exposure of lignocellulosic biomass of SCG during treatment, potentially causing adverse

effects on the hemicellulose structure as well as leading to the solubilisation of cellulose components (Asghar *et al.*, 2015; Wongsiridetchai *et al.*, 2018).

4.3 Model Validation

Numerical optimisation incorporated all factors (concentration = X_a ; temperature = X_b ; incubation time = X_c) as each one exerted a significant impact on the response (sugar concentration) for KOH and NaOH pre-treatment. The sugar concentration was derived from SCG hydrolysate generated after enzymatic hydrolysis and was selected as response in order to determine the enzymatic digestibility of various experiments. The optimised conditions for KOH pre-treatment was 3% (w/v) KOH concentration, temperature of 90 °C and incubation time of 2 h. On the other hand, the ideal conditions for NaOH pre-treatment was 3% (w/v) NaOH concentration, 85 °C temperature and 1 h incubation time.

Under optimum environment, the sugar concentration obtained after KOH pre-treatment was predicted to be 2.47 mg/ml while the sugar concentration generated after NaOH pre-treatment was predicted to be 2.73 mg/ml. To confirm the reliability of the predicted model, the pre-treatment of SCGs was conducted in triplicate using the optimal conditions. The results were shown in Table 4.6 (a) and Table 4.6 (b). The average of actual sugar concentration were found to be 2.53 mg/ml with approximately 2.43% error for KOH pre-treatment whereas the average actual value of sugar concentration acquired for NaOH pre-treatment was 2.79 mg/ml, with 3.18% error. Both experimental values were closer to the predicted value from the model while the error is less than 5%.

Table 4.6(a): Validation of the model for the optimisation of KOH pre-treatment based on regression model analysis

Factors	NaOH concentration	Temperature	Incubation Time	Sugar concentration (mg/mL)		Error (%)
				Predicted	Actual	
Experimental	3.000	85.000	1.000			
				2.47	2.56	3.64
				2.47	2.50	1.21
				2.47	2.53	2.43
Average				2.47	2.53	2.43

Table 4.6(b): Validation of the model for the optimisation of NaOH pre-treatment based on regression model analysis

Factors	NaOH concentration	Temperature	Incubation Time	Sugar concentration (mg/mL)		Error (%)
				Predicted	Actual	
Experimental	3.000	85.000	1.000			
				2.73	2.69	1.47
				2.73	2.82	3.30
				2.73	2.86	4.76
Average				2.73	2.79	3.18

4.4 Effect of Defatting, Alkali Pre-treatment and Enzymatic Hydrolysis on Reducing Sugar Production

The removal of lipids from spent coffee grounds (SCG) proved to be a critical preparatory step prior to chemical pre-treatment. Lipids were shown to hinder enzyme accessibility, particularly cellulase, by forming hydrophobic barriers that restrict contact with cellulose surfaces. Sonication-assisted defatting not only enhanced lipid removal but also facilitated lignin solubilization, thereby creating a more porous structure for subsequent pre-treatment. These findings highlight the importance of defatting as an enabling step in the valorisation of SCG, as it directly influenced the efficiency of later saccharification processes by improving substrate accessibility (Trinh *et al.*, 2018).

In this study, alkaline pre-treatment was selected as the main strategy, employing KOH and NaOH. Alkaline pre-treatment was favoured over acid-based methods such as H₂SO₄ due to its lower causticity and demonstrated effectiveness in delignification (Kim *et al.*, 2016; Lee *et al.*, 2021). NaOH pre-treatment was particularly effective at attacking the bonds in lignin-carbohydrate complexes (LCC), primarily the linkage between lignin and hemicellulose. During this process, sodium hydroxide undergoes dissociation into sodium ions (Na⁺) and hydroxide ions (OH⁻). As the concentration of hydroxide ions increases, the hydrolysis reaction rate also correspondingly rises (Modenbach, 2013). Meanwhile, KOH was shown to be highly feasible, with studies reporting its superior performance in eliminating lignin and extractives (Paixão *et al.*, 2016; Lee *et al.*, 2021). The results suggest that both alkaline reagents are viable, though their effectiveness may vary depending on the composition of SCG and processing conditions.

The experimental results indicated that both NaOH and KOH pre-treatments increased sugar concentration significantly, particularly under elevated temperatures. The combination of alkaline reagents and heat exhibited a synergistic effect on biomass disruption, loosening the macromolecular structure and enhancing enzyme penetration. This was in alignment with the positive sign appear in both equations (2) and (3) generated from these models as well as in agreement with the study by Kim *et al.* (2018) and Tyagi and Lo (2012), that showed chemical and thermal factors act complementarily to degrade lignocellulosic structures. However, high temperatures and prolonged incubation also posed a risk of generating inhibitory by-products such as 5-HMF, furfural, and phenolic compounds derived from lignin, which reduce reducing sugar yields (Getachew & Chun, 2017; Pereira *et al.*, 2020). This was why longer incubation time yield lower sugar concentration especially when the pre-treatment subjected defatted SCG to high alkaline concentration and high temperature but more sugar was generated when the period of incubation reduced.

Following pre-treatment, all SCG samples were subjected to enzymatic hydrolysis using cellulase as the primary enzyme. The results demonstrated that alkali pre-treatment markedly enhanced sugar yields as KOH-treated samples achieved a 2.97-fold increase under the condition of 3% KOH concentration under 90 °C for 2 h, while NaOH-treated samples showed a 3.28-fold increase under the condition of 3% NaOH concentration within the temperature of 85 °C for 1 h, compared to the untreated control. The significant enhancement was attributed to the structural modifications caused by alkali treatment, which increased enzyme-binding sites and reduced steric hindrance from lignin, thereby enhancing cellulase access to cellulose and boosting conversion efficiency. The enzymatic hydrolysis

outcomes were validated by DNS analysis, where the glucose standard curve confirmed the increase in reducing sugars following treatment (Zhang *et al.*, 2021).

The mechanism of enzymatic hydrolysis involves multiple steps, including enzyme diffusion, adsorption, and catalytic reaction on cellulose surfaces. Cellulase molecules diffused from the solution to SCG by penetrating through the pore structure, adsorb onto the adsorption sites where an enzymatic hydrolysis reaction takes place on the surface, and bind with the active site of the cellulose. Previous studies have highlighted enzyme diffusion as a key rate-limiting factor in enzymatic hydrolysis (Kumar & Wyman, 2009; Igarashi *et al.*, 2011; Zhang *et al.*, 2016). Based on Zhang *et al.* (2021), the rate and degree of hydrolysis was dictated by the rate at which the enzyme molecules move from the liquid phase to the biomass particles while the rate of diffusion was influenced by the structure of the pore, the force of the fluid and the enzyme to fluid gradient. These enzyme diffusion-based constraints explain why structural modifications during pre-treatment are critical to achieving higher hydrolysis efficiency.

Despite the significant improvements, it is important to note that cellulase targets only cellulose by converting the carbohydrate molecule into glucose equivalents. Given that SCG contains a higher proportion of hemicellulose, particularly mannose-rich polymers, a substantial fraction of potential reducing sugars remained untapped. This limitation underscores the need to investigate enzyme cocktails or alternative enzymes such as hemicellulases or mannanases to complement cellulase activity and to maximise reducing sugar recovery from SCG. Such an approach could enhance the recovery of mannose and other hemicellulosic sugars, thereby improving the overall bioconversion efficiency of SCG as well as its suitability as a substrate for fermentation and other bio-based product synthesis. Thus, future studies should therefore focus on integrating multi-enzyme systems with

optimised alkaline pre-treatment to unlock the full sugar potential of SCG. However, it is also worth exploring whether multi-enzyme systems alone, without prior alkaline treatment, could achieve comparable hydrolytic efficiency. This approach would represent a cleaner and more environmentally sustainable strategy.

The results of this study demonstrated that defatting was a necessary preliminary step to improve substrate accessibility, while alkaline pre-treatments using NaOH and KOH were effective in lignin removal and structural modification of SCG. The enhanced enzymatic hydrolysis performance confirmed the strong influence of these treatments, with NaOH yielding slightly higher sugar recovery than KOH under the tested conditions. However, the persistence of unutilized hemicellulosic sugars suggests that further process refinement is required. Overall, the combination of defatting, optimized alkaline pre-treatment and targeted enzyme application represents a promising pathway for maximizing the conversion efficiency of SCG into reducing sugars.

4.5 Comparative Analysis between One-Step and Two-Step Chemical Pre-treatment

In the subsequent study, the efficacy of one-step alkali pre-treatment using KOH and NaOH and acid pre-treatment using H₂SO₄, as well as two-step pre-treatments by combining acid–alkali and alkali–acid sequences, were investigated. The procedures for these methods were based on the optimised conditions of KOH and NaOH pre-treatment respectively while the condition for acid pre-treatment was selected based on the method by Lee *et al.* (2021). An effective bioconversion of a lignocellulosic substrate requires adequate exposure of cellulose fibres and the partial removal of hemicellulose and lignin (Ravindran *et al.*, 2017). However, the results from one-step pre-treatments indicated that sugar release remained relatively low, suggesting that a single alkaline pre-treatment was insufficient to overcome the recalcitrant nature of SCG lignocellulosic structures.

To overcome the limitations observed in one-step alkaline pre-treatment methods using KOH and NaOH, two-step pre-treatment was investigated as a strategy to enhance sugar recovery. This approach was based on the complementary mechanisms of acid and alkali treatments, each targeting different components of the SCG lignocellulosic matrix. In the acid–alkaline sequence, H₂SO₄ pre-treatment was first applied to hydrolyse hemicellulose and disrupt lignin–carbohydrate linkages, thereby improving porosity and cellulose exposure (Zhang *et al.*, 2021; Kim *et al.*, 2023; Ribeiro *et al.*, 2023). The subsequent alkaline treatment then promoted further delignification by solubilising residual lignin, while simultaneously reducing cellulose crystallinity and inducing biomass swelling (Das *et al.*, 2020; Awogbemi and Kallon, 2022). This sequence created a more open structure, allowing enzymes to access cellulose more effectively during hydrolysis.

Conversely, the alkaline–acid sequence was designed to reverse the order of action, with alkaline pre-treatment initially targeting lignin removal and loosening the compact lignin–carbohydrate network. This disruption facilitated the penetration of the subsequent acid treatment, which then enhanced hemicellulose solubilisation and exposed additional cellulose regions (Park and Kim, 2012; Juarez *et al.*, 2017). The sequential application of both treatments therefore provided a synergistic effect, addressing the structural recalcitrance of SCG more effectively than either method alone. Pre-treated defatted SCG samples were subjected to sugar analysis at each stage of pre-treatment following enzymatic hydrolysis. The summarised data in Figure 4.6 shows the total concentration of reducing sugar produced after enzymatic hydrolysis of SCG following various pre-treatments, compared to the control.

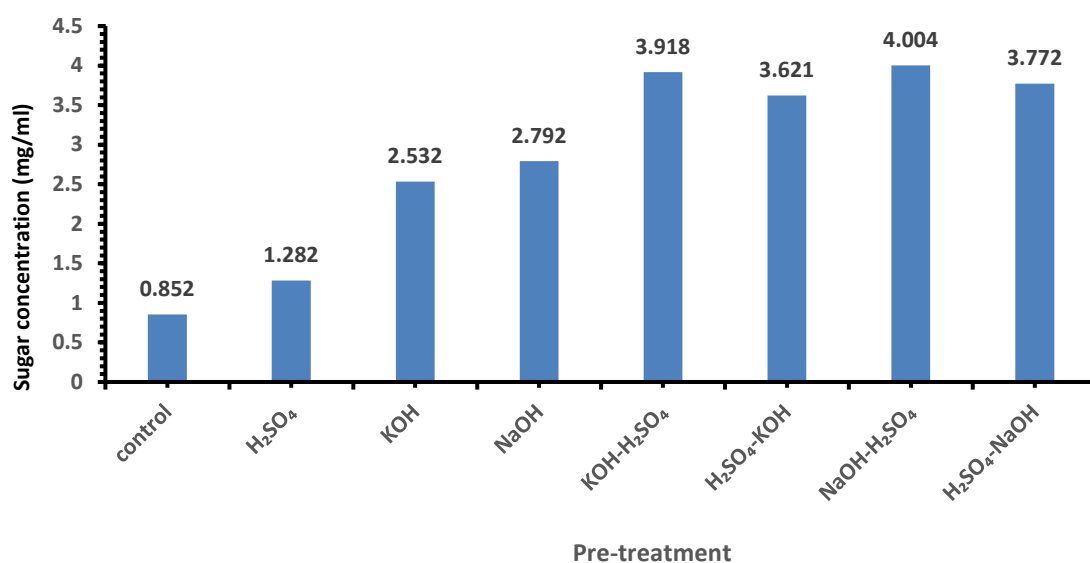


Figure 4.6: Total reducing sugar released after enzymatic hydrolysis of pre-treated SCG waste under various pre-treatment conditions

4.5.1 One-Way ANOVA and Post Hoc Comparison Test: Tukey's HSD

Based on reducing sugar yield, the most effective pre-treatment strategy for defatted SCG was a two-step process consisting of 3% (w/v) NaOH at 85 °C for 1 h, followed by 1% (v/v) H₂SO₄ at 60 °C for 1 h, which produced the highest reducing sugar concentration of 4.00 mg/mL, representing a 4.7-fold increase compared to the control. This sequential chemical pre-treatment effectively removed substantial fractions of hemicellulose and enhanced cellulose conversion by improving substrate accessibility for enzymatic hydrolysis (Trinh, 2022). One-Way ANOVA was conducted to find any significant difference among the chemical pre-treatments. The results was attested in Table 4.7 revealed a p-value below 0.01, providing strong evidence of significant differences between untreated and chemically treated samples. Additionally, the F-statistic (1347.936) exceeded the F-critical value (2.657), which indicate significant differences among one or more treatments.

Table 4.7: One-Way ANOVA for the pre-treatment of defatted SCG

Groups	Count	Sum	Average	Variance		
(A) Control	3	2.556	0.852	0.001137		
(B) H ₂ SO ₄	3	3.846	1.282	0.000907		
(C) KOH	3	7.596	2.532	0.000931		
(D) NaOH	3	8.376	2.792	0.000496		
(E) KOH-H ₂ SO ₄	3	11.754	3.918	0.000849		
(F) H ₂ SO ₄ -KOH	3	10.863	3.621	0.021619		
(G) NaOH-H ₂ SO ₄	3	12.012	4.004	0.000201		
(H) H ₂ SO ₄ -NaOH	3	11.316	3.772	0.000496		
Source of Variation	SS	df	MS	F	P-value	F critical
Between Groups	31.41567	7	4.487953	1347.936	5.97E-21	2.657197
Within Groups	0.053272	16	0.00333			
Total	31.46894	23				

Further analysis was conducted by performing a post hoc test. The result of the post hoc test is depicted in Table 4.8. Post hoc tests, also known as multiple comparison tests, assess the statistical significance of differences between group means after conducting an ANOVA that indicates an overall difference (Nanda *et al.*, 2021). These methods focus on comparing specific pairs of means, providing researchers with the most relevant insights into the data. One way to address the multiple comparison problem is to analyse each comparison separately using an appropriate statistical method. In this study, Tukey's Honestly Significant Difference (HSD) test was applied to compare each pair of means.

This method was used in this study to minimise Type 1 errors during statistical analysis. The Type I error rate (false positive rate) occurs because each additional test increases the chance of incorrectly rejecting a true null hypothesis (Ostertagova and Ostertag, 2013; Nanda *et al.*, 2021). Tukey's HSD test is widely accepted as an effective technique for controlling Type 1 errors. Tukey's HSD test was conducted on 28 pairs among the $k = 8$ treatments to determine which pairs show a statistically significant difference. This is performed by comparing Tukey HSD Q-statistic to Q-critical from the Studentized Range. The value of Q-critical (6.0796) was compared to the calculated Tukey HSD Q-statistic. If the value of the calculated Q-statistic is larger than the critical value, then there is a significant difference between the compared pre-treatment at 99% confidence level.

Table 4.8: Result for Post Hoc Tukey's HSD test

Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD conclusion
A vs B	12.9074	0.0010053	** p<0.01
A vs C	50.4290	0.0010053	** p<0.01
A vs D	58.2335	0.0010053	** p<0.01
A vs E	92.0329	0.0010053	** p<0.01
A vs F	83.1178	0.0010053	** p<0.01
A vs G	94.6144	0.0010053	** p<0.01
A vs H	87.6504	0.0010053	** p<0.01
B vs C	37.5216	0.0010053	** p<0.01
B vs D	45.3261	0.0010053	** p<0.01
B vs E	79.1255	0.0010053	** p<0.01
B vs F	70.2104	0.0010053	** p<0.01
B vs G	81.7070	0.0010053	** p<0.01
B vs H	74.7430	0.0010053	** p<0.01
C vs D	7.8045	0.0010053	** p<0.01
C vs E	41.6039	0.0010053	** p<0.01
C vs F	32.6888	0.0010053	** p<0.01
C vs G	44.1854	0.0010053	** p<0.01
C vs H	37.2214	0.0010053	** p<0.01
D vs E	33.7994	0.0010053	** p<0.01
D vs F	24.8843	0.0010053	** p<0.01
D vs G	36.3809	0.0010053	** p<0.01
D vs H	29.4169	0.0010053	** p<0.01
E vs F	8.9151	0.0010053	** p<0.01
E vs G	2.5815	0.6019860	insignificant
E vs H	4.3825	0.0971818	insignificant
F vs G	11.4966	0.0010053	** p<0.01
F vs H	4.5326	0.0805022	insignificant
G vs H	6.9640	0.0029636	** p<0.01

$k = 8$; $\nu = 16$; Q-critical = 6.0796; $\alpha = 0.01$

Based on the results, there is a significant difference between raw and defatted SCG samples compared to all treated SCG samples. Additionally, a significant difference is observed between acid-treated and alkaline-treated samples in terms of reducing sugar yield. Based on the amount of reducing sugar recovered, this indicate that alkaline is relatively a more effective treatment than acid. Meanwhile, there is a significant difference observed between NaOH- and KOH-treated samples as the Q-statistic shown to be 7.8045. This indicate that although both KOH and NaOH can be utilised for defatted SCG pre-treatment, using NaOH in the pre-treatment can result in higher reducing sugar concentration. It also has lower requirement of temperature by 5 °C than KOH pre-treatment and more time effective as the incubation time is 1 hour shorter despite both alkalis having the same optimal concentration (3%).

Besides, there is significant difference between NaOH-H₂SO₄ treated samples with H₂SO₄-NaOH and H₂SO₄-KOH treated samples where the Q-statistic were shown to be 6.9640 and 11.4966 respectively. Furthermore, there is a significant difference between KOH-H₂SO₄ and H₂SO₄-KOH treated samples (8.9151) but no significant difference between KOH-H₂SO₄, and H₂SO₄-NaOH treated samples (4.3825) whereas H₂SO₄-KOH and H₂SO₄-NaOH (4.5326) treated samples also showed no significant difference, suggesting they may have a similar effect on the results. However, KOH-H₂SO₄ treated samples may be the better treatment, as it produces relatively higher sugar concentrations than the other two. Interestingly, there is no significant difference between KOH-H₂SO₄ and NaOH-H₂SO₄ treated samples as the Q-statistic was found to be 2.5815, suggesting that either sequence may produce similar results. However, since NaOH-H₂SO₄ treated samples consistently produce more reducing sugar than KOH-H₂SO₄ treated samples, the result suggest that NaOH-H₂SO₄ treated samples is the overall more effective treatment.

4.5.2 Statistical Analysis between One-Step and Two-Step Pre-treatment

The statistical analysis of Tukey's HSD test prove that there is a significant different between one-step and two-step pre-treatment of defatted SCG. One-step pre-treatment using H_2SO_4 produced 1.50 folds of reducing sugar while one-step alkaline pre-treatment using KOH and NaOH produced 2.97 to 3.28 folds of reducing sugar in comparison to control which according to the multiple comparison test were significantly less compared to two-step pre-treatment that combines acid and alkaline that produces 4.25 to 4.70 folds of reducing sugar. Table 4.9 shows the conversion yield reducing sugar recovered from defatted SCG after different pre-treatment methods. The conversion yield was calculated based on the concentration of reducing sugar relative to the initial substrate loading (50 mg/mL).

Table 4.9: The conversion yield reducing sugar recovered from defatted SCG after different pre-treatment methods.

Treatment	Reducing sugar (mg/mL)	Conversion Yield (%)
Control	0.852	1.70
H_2SO_4	1.282	2.56
KOH	2.532	5.06
NaOH	2.792	5.58
KOH- H_2SO_4	3.918	7.84
H_2SO_4 -KOH	3.621	7.24
NaOH- H_2SO_4	4.004	8.01
H_2SO_4 -NaOH	3.772	7.54

The results demonstrated that pre-treatment significantly enhanced the release of reducing sugars from defatted SCG compared to the untreated control. Among the one-step methods, alkaline pre-treatments (KOH and NaOH) were more effective than acid pre-treatment alone, producing yields of 5.06% and 5.58%, respectively, compared to only 2.56% from H₂SO₄. This outcome is consistent with previous reports that alkali pre-treatment is particularly efficient in disrupting LCC, thereby improving enzyme accessibility to polysaccharides (Zhang *et al.* 2021; Park and Kim, 2012). More notably, sequential two-step acid–alkali or alkali–acid treatments further increased sugar yields, with NaOH–H₂SO₄ achieving the highest at 8.01%. The improvement from two-step pre-treatment supports the synergistic effect of acid in solubilising hemicellulose and alkali in delignification, as reported in other lignocellulosic biomass studies (Das *et al.*, 2020; Awogbemi and Kallon, 2022). These findings highlight that combining alkali and acid steps maximizes structural deconstruction of SCG, thereby unlocking higher sugar recovery and making it a promising strategy for bioconversion applications.

The application of one-step alkaline pre-treatments using KOH and NaOH offers a simplified, straightforward and time-efficient approach that can effectively disrupt the lignocellulosic structure of SCGs. This include breaking down lignin and hemicellulose, leading to increased surface area and improved enzymatic digestibility by enhancing the accessibility of cellulose for subsequent enzymatic hydrolysis to facilitate the release of fermentable sugars (Zhang *et al.*, 2021; Jooste *et al.*, 2023). This method is generally cost-effective due to lower chemical requirements and shorter processing times. Studies have shown that one-step alkaline pre-treatment can achieve high delignification rates, making it suitable for bioethanol and biogas production (Huang *et al.*, 2021; Sayoud *et al.*, 2025).

However, one-step alkaline pre-treatments have certain limitations. The efficiency of sugar extraction may be lower compared to more intensive methods, and the high pH required for this process can cause corrosion of equipment as the process generate significant amounts of alkaline waste, posing environmental disposal and economic challenges. Moreover, the use of strong bases can lead to the formation of inhibitory compounds, such as phenolic derivatives, potentially hindering downstream bioconversion processes (Bomfim *et al.*, 2022; Pereira *et al.*, 2020). Additionally, the high alkalinity of the resulting slurry may require extensive neutralization, adding to processing costs. Furthermore, the structural complexity of SCGs may require more robust treatment to achieve optimal yields of desired products.

On the other hand, two-step acid-base pre-treatment process was developed in this study to address these challenges since this method offers a more nuanced approach. Based on the result, the more favourable approach was to involve an initial alkaline hydrolysis using NaOH to solubilize lignin, followed by an acid using H₂SO₄ treatment to break down hemicellulose, thereby enhancing the accessibility of cellulose for enzymatic hydrolysis. This sequential approach lead to higher overall sugar yields and reduced inhibitor formation compared to one-step alkaline pre-treatment, making it more suitable for downstream biological processes (Niglio *et al.*, 2019; Lee *et al.*, 2021). This result is coherent with multiple studies demonstrated that two-step pre-treatments can significantly increase the yield of fermentable sugars (Ravindran *et al.*, 2018; Sjulander and Kikas, 2022; Trinh, 2022).

Despite the improved efficiency, two-step pre-treatments come with their own set of drawbacks. The process is more complex, requiring more chemicals, increased energy consumption, time-consuming, and involve multiple stages. This translates to higher operational costs and potentially greater environmental impact. Moreover, the use of both acidic and alkaline chemicals necessitates careful handling and neutralization steps to mitigate environmental impacts. Therefore, while two-step pre-treatments can enhance product yields, the decision to implement such a process should consider the balance between the increased output and the associated economic and environmental costs.

Whether the added complexity of two-step pre-treatment is "worth it" depends largely on the specific application and economic considerations. One-step alkaline pre-treatment is advantageous for its simplicity and lower operational costs, making it appealing for large-scale applications where time and resource efficiency are critical. However, its environmental impact and potential for inhibitor formation may limit its suitability for certain applications. On the other hand, the two-step acid-base method, while more resource-intensive, offers superior sugar yields and reduced inhibitor formation, which can justify the additional investment in contexts where high efficiency and product quality are prioritized. Recent studies suggest that the choice of pre-treatment method should align with the intended end-use of the SCG, such as biofuel production, material synthesis, or chemical extraction (Cho *et al.*, 2022; Gu *et al.*, 2023).

If the primary goal is maximising sugar yields for biofuel production or other bioconversion processes (Hamedani *et al.*, 2022; Gu *et al.*, 2023), the higher yields achieved with two-step pre-treatment may justify the increased costs. While two-step pre-treatment can offer superior sugar yields and reduced inhibitor formations, its higher cost and complexity must be weighed against the simpler and more cost-effective one-step alkaline treatment. Further research focusing on minimising inhibitor formation and maximising sugar recovery in both methods is crucial for the economic viability of SCG valorisation. Meanwhile, for applications where lower yields are acceptable, such as composting or direct combustion (Santos *et al.*, 2017; Bomfim *et al.*, 2022), the simplicity and lower cost of one-step alkaline pre-treatment may be preferable.

A thorough techno-economic assessment, considering factors such as raw material costs, chemical inputs, energy consumption, and product yields, is essential for determining the most suitable pre-treatment strategy. Ultimately, both one-step and two-step chemical pre-treatment methods have their merits and drawbacks. The decision to adopt either approach depends on a careful evaluation of the specific requirements and goals of the valorisation process, such as cost-effectiveness, environmental impact, and desired product quality. Hence, the choice of pre-treatment should be guided by a thorough evaluation of technical, economic, and environmental factors to ensure sustainable and efficient utilization of SCGs.

4.6 Surface and Morphological Analysis of SCG Samples

The samples for raw, defatted, one-step acid or alkaline pre-treated as well as two-step acid-base pre-treated samples of SCGs following enzymatic hydrolysis are depicted below in Figure 4.8. The raw and defatted SCG samples (Figure 4.7 i-ii) exhibit a denser morphology compared to the particles of chemically pre-treated SCG samples (Figure 4.8 iii-ix) which consist of thin material sheets powder that resemble sawdust. The raw SCGs has a dark brown, soil-like and dense morphology primarily because of the high concentration of organic compounds according to Hu *et al.* (2022) and Waisarikit *et al.*, (2025). The raw SCG loses its deep brown colour after the defatting process due to delignification and decolourisation following pre-treatment and enzymatic hydrolysis process, which lead to the extraction of organic compounds according to Yang *et al.* (2023). Besides, the alkaline-treated SCG samples had an even lighter brown colour while the particulate of the samples were smaller compared to both raw and defatted SCGs.

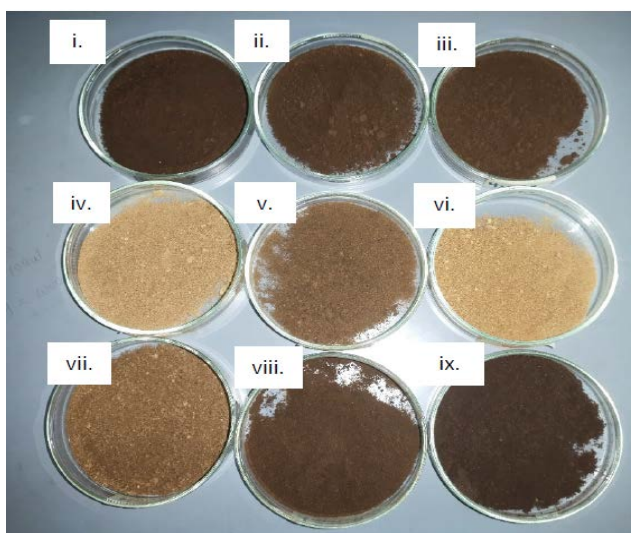


Figure 4.7: Samples of (i) raw SCG, (ii) defatted SCG, (iii) H_2SO_4 pre-treated SCG, (iv) KOH pre-treated SCG, (v) KOH- H_2SO_4 pre-treated SCG (vi) H_2SO_4 -KOH pre-treated SCG, (vii) NaOH pre-treated SCG, (viii) NaOH- H_2SO_4 pre-treated SCG, (ix) H_2SO_4 -NaOH pre-treated SCG

On the other hand, Figure 4.8 (a-i) depicted the micrographs of raw SCG, defatted SCG, acid-treated, alkaline-treated and acid-base treated SCG samples following enzymatic hydrolysis that were observed and analysed using SEM in order to understand the changes in the surface structure of SCG. SEM micrographs allows for the examination and analysis of the microstructural changes in SCG after one-step and two-step pre-treatment under different conditions. Prior to the analyses, the samples were coated with a thin layer of platinum to improve image quality. These images were obtained by applying an acceleration the voltage of 10 kV, at 500-fold, 2,000-fold and 5,000-fold magnifications. These SEM images highlighted the unique and distinctive surface morphologies of SCG when subjected under different conditions. As shown in Figure 4.8 (a), the raw SCG display a dense and irregular surface morphology, similar to the report in previous study by Afolabi *et al.* (2020).

Meanwhile, the defatted SCG also exhibit a noticeably irregular yet flatter and smoother surface compared to the raw SCG while featured improve pores and loosen structures with fewer gaps. When viewed closer in Figure 4.8 (b), the surface of defatted SCG appear to have many uneven, bowl-shaped structures, but the structure still relatively flat and compact. The acid-treated SCG sample using H_2SO_4 develops a clumpy texture with small chunks as tiny pores develop on its uneven surface (Figure 4.8 c). After one-step alkaline pre-treatment using KOH and NaOH, the SCG particles showed signs of broken components into smaller pieces and had many wrinkles or creases as shown in Figure 4.8 (d) and Figure 4.8 (g). Regardless of the pre-treatment conditions, the surface overview of alkaline-treated SCG was smooth with formation of many smaller pores in between the irregular structures. This indicated that the pre-treatment of SCG using KOH and NaOH had transformed the surface structures of the SCG. From these figures, an increment in both the number and size of folds as well as porous sites can be observed. These folds and pores

increased the surface area of the coffee grounds which make it easier for enzymes to reach the cellulose and hemicellulose.

Upon further magnification, the pores and tunnelling structures for SCG sample after NaOH pre-treatment was also hollower than the SCG samples treated with KOH. Despite the pre-treatment of SCG using alkaline shows a significant pores enlargement as the surface of the SCG became more uneven, the tunnelling structure of the SCG remain intact which is why alkaline pre-treatment mainly used for lignin removal since its results in less sugar degradation than acid pre-treatment (Hudeckova *et al.*, 2018; Titiri *et al.*, 2023). Furthermore, two-step acid-base pre-treated SCGs after enzymatic hydrolysis, depicted in Figure 4.8 (e-f) and Figure 4.8 (h-i) displayed an even more fractured and fragmented particles. This effect is caused by the dual effect of acid and alkaline pre-treatment as well as an ongoing degradation of cellulose by the enzyme. Enzymatically hydrolysed two-step alkaline-treated SCGs exhibited different morphological patterns that characterised by tunnelling features, pore formation and enlargement, as well as microstructural fragmentation that were significantly smaller and less chunky in comparison to SCGs after one-step acid or alkaline pre-treatment as well as raw and defatted SCGs.

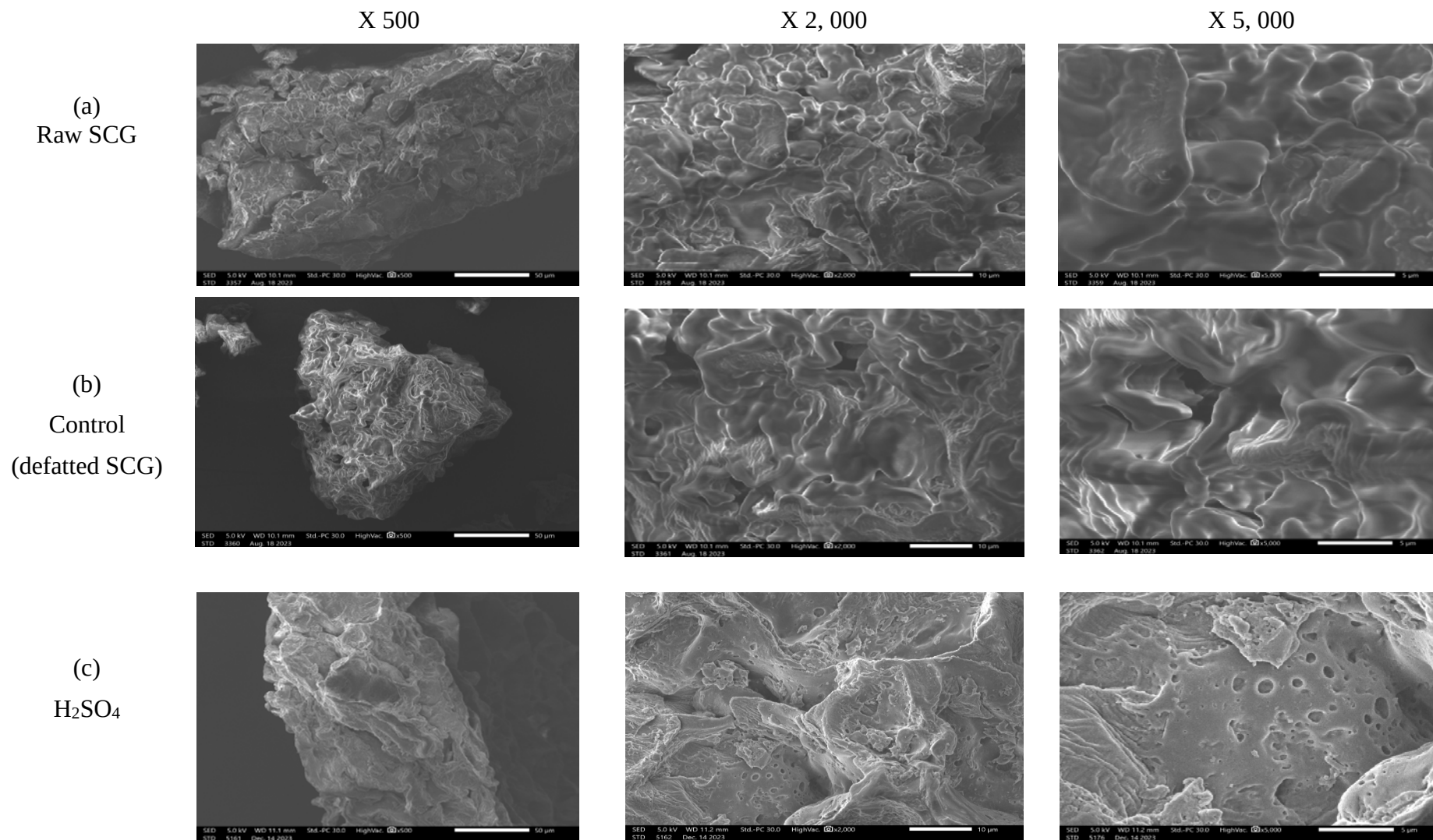


Figure 4.8: SEM micrographs of (a) raw SCG sample, (b) defatted SCG sample, and (c) H₂SO₄ pre-treated sample

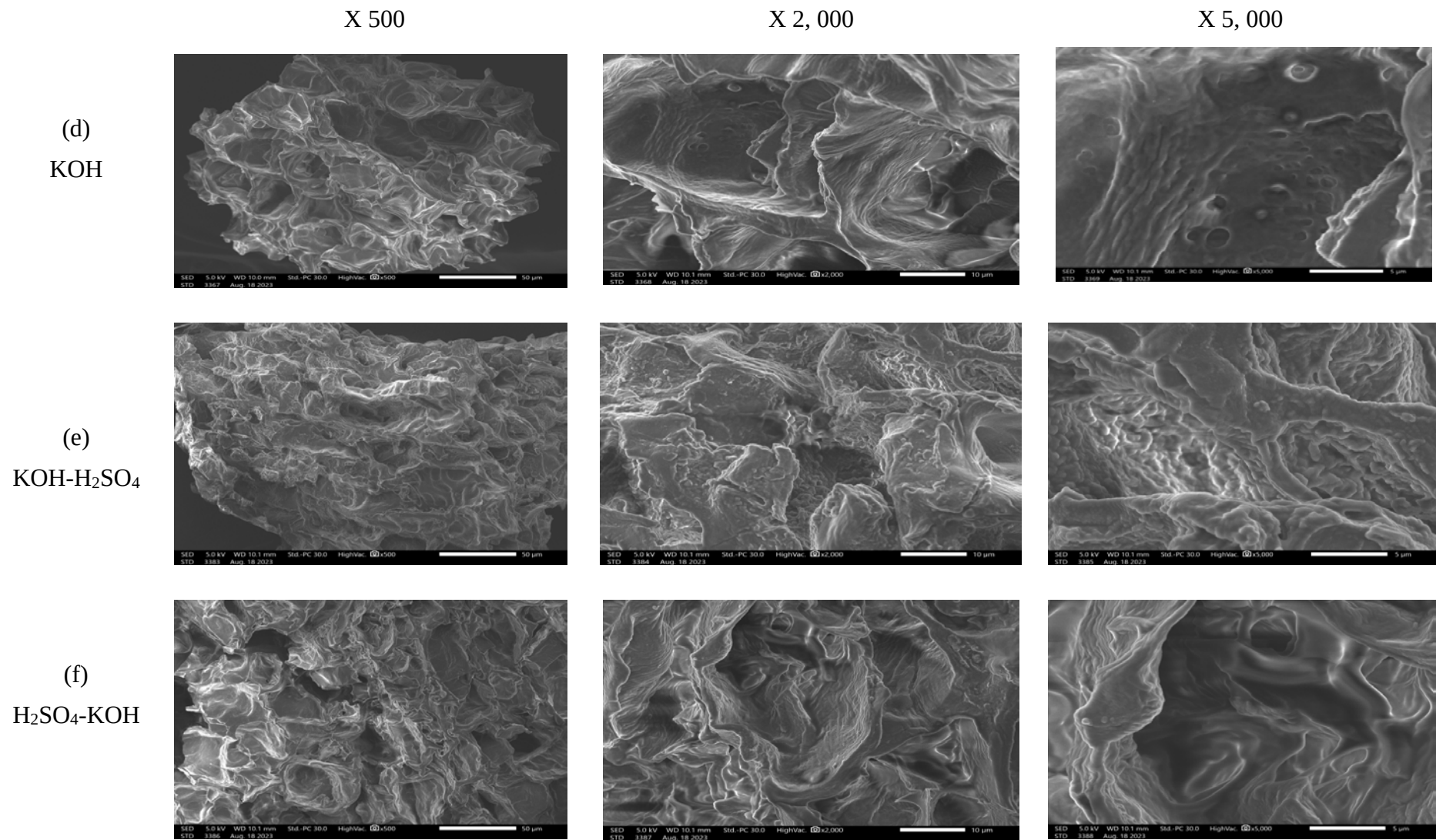


Figure 4.8: SEM micrographs of (d) KOH pre-treated sample, (e) KOH-H₂SO₄ pre-treated sample, and (f) H₂SO₄-KOH pre-treated sample

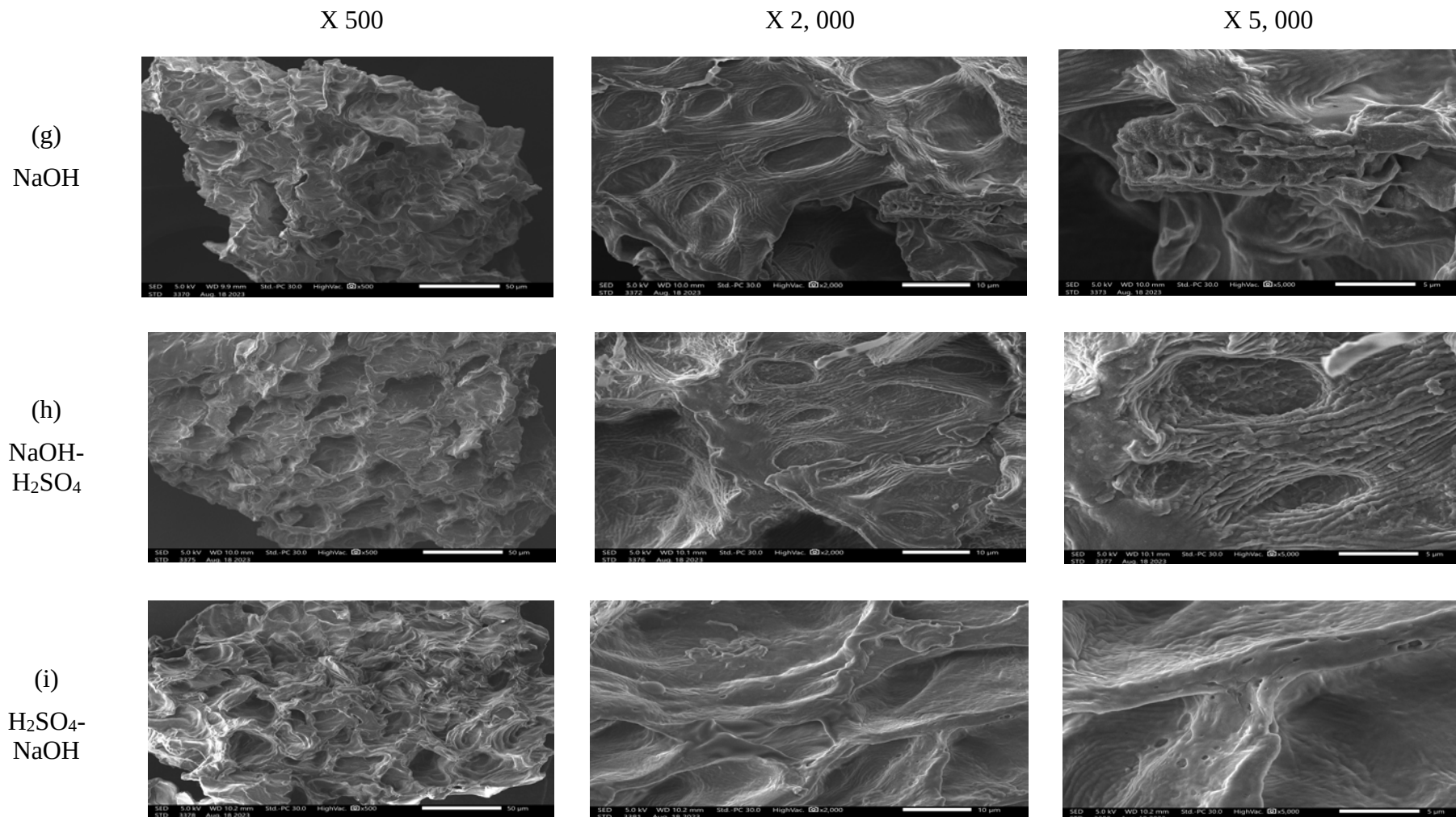


Figure 4.8: SEM micrographs of (g) NaOH pre-treated sample, (h) NaOH-H₂SO₄ pre-treated sample, and (i) H₂SO₄-NaOH pre-treated sample

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In conclusion, this study has successfully outlined the key findings of the optimisation of alkaline pre-treatment of defatted SCG. By applying RSM with CCD approach, two optimisation models were successfully established for KOH and NaOH pre-treatments of defatted SCG. The KOH model indicates that the best conditions were 3% KOH concentration, 90 °C, and an incubation period of 2 h, yielding 2.532 mg/ml of reducing sugar which equivalent to a 2.97-fold increase in total sugar recovery compared to the control. As for NaOH pre-treatment, the optimal conditions were 3% NaOH concentration, 85 °C, and an incubation period of 1 h, yielding 2.792 mg/ml of reducing sugar and resulting in a 3.28-fold increase in total sugar recovery. Both models demonstrated that all studied factors significantly influenced the pre-treatment process, confirming the effectiveness of these alkaline pre-treatments in enhancing the subsequent enzymatic hydrolysis of pre-treated defatted SCG. Although both alkalis can be used effectively for pre-treating defatted SCG, NaOH pre-treatment is more cost-effective than KOH pre-treatment due to its lower optimal temperature requirement and shorter incubation time.

This study also discovered that there is a significance difference between one-step and two-step pre-treatment of the defatted SCG. One-step acidic pre-treatment using H_2SO_4 boosted around 1.50 times reducing sugar production compared to control, obtaining 1.282 mg/ml of reducing sugar which equivalent to 2.56% conversion yield. Meanwhile, one-step alkaline pre-treatment of defatted SCG using KOH and NaOH yielded an increase of 2.97 to 3.28 times compared to the control, producing about 2.532 mg/ml and 2.792 mg/ml of reducing sugar as well as conversion yield of 5.06% and 5.58% respectively. However, single-step methods produced considerably lower reducing sugar yields compared to two-step acid-base pre-treatments, which achieved 4.25–4.70 times higher recovery. Specifically, H_2SO_4 –KOH yielded 3.621 mg/mL with conversion yield of 7.24%, H_2SO_4 –NaOH yielded 3.772 mg/mL with conversion yield of 7.54%, KOH– H_2SO_4 yielded 3.918 mg/mL with conversion yield of 7.84%, and NaOH– H_2SO_4 achieved the highest yield at 4.004 mg/mL with conversion yield of 8.01%. The two-step chemical process resulted in a slightly less than one unit increase in reducing sugar compared to the one-step chemical methods.

This shows that acid pre-treatment alone yielded low amounts of reducing sugars, but it was essential in enhancing substrate accessibility in two-step acid-base pre-treatment. When this approach was further coupled with enzymatic hydrolysis using cellulase, which converts cellulose into glucose equivalents, the overall reducing sugar recovery improved significantly. This demonstrates that combining two-step acid-base pre-treatment with enzymatic hydrolysis provides a synergistic strategy for maximising reducing sugar yield. One-step alkaline pre-treatments using KOH or NaOH offers a simpler, faster, and more direct approach compared to the two-step acid-base pre-treatment. The latter, while producing more reducing sugar, requires more chemicals, energy, and time leading to increased operational costs and a larger environmental footprint. The economic viability of

SCG valorisation hinges on whether the increased complexity of two-step pre-treatment justifies the added cost. This depends on the specific application and economic factors.

The structural analysis of SCG before and after pre-treatment provided further insights into the effectiveness of alkaline pre-treatment in enhancing sugar recovery. SEM analysis revealed that raw SCG exhibited a dense and irregular surface, while defatted SCG showed a smoother texture with small gaps. Upon alkaline treatment, significant morphological changes were observed, with the development of tunnel-like structures, enlarged hollow pores, and increased surface fragmentation. These modifications indicate successful disruption of the lignocellulosic matrix, making the substrate more accessible to enzymatic hydrolysis. The enhanced porosity and reduced recalcitrance of the pre-treated SCG contributed to the improved efficiency of cellulase action, ultimately leading to a higher sugar yield. These findings reaffirm the effectiveness of alkaline pre-treatment in breaking down the structural complexity of SCG, facilitating its potential for further bioconversion into value-added bio-based products.

5.2 Recommendations

Future research should prioritise the optimisation of enzymatic hydrolysis conditions to maximise reducing sugar recovery from SCG. Parameters such as pH, substrate concentration, and enzyme loading should be systematically investigated to enhance hydrolysis efficiency. In addition, the use of complementary enzymes such as hemicellulases, which can target the hemicellulosic fraction of SCG, may further improve sugar yields by expanding the range of polysaccharides converted into fermentable sugars. Since cellulase targets only cellulose, incorporating enzymes such as hemicellulases could enhance the hydrolysis of hemicellulosic and lignin-associated fractions, thereby increasing overall sugar recovery. Mannanase can also be used as mannan is the most abundant carbohydrate in SCG.

Another important area of investigation is the formation of inhibitory compounds during pre-treatment. By-products such as furfural, hydroxymethylfurfural (HMF), and phenolic compounds can reduce enzymatic efficiency and hinder subsequent bioprocesses. Future studies should therefore assess the conditions under which these compounds are generated and explore strategies to mitigate their impact. Advanced analytical techniques, including HPLC or GCMS, could provide valuable insights for optimising pre-treatment severity. Due to equipment constraints, sonication was used instead of Soxhlet extraction for SCG defatting. Future studies should employ Soxhlet or other effective lipid-removal methods for defatting SCG process. Finally, the current study was limited to laboratory-scale experiments, which may not reflect industrial feasibility. Pilot-scale studies should be conducted to evaluate process performance under realistic operating conditions, including enzyme reusability, reactor design, and process integration.

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