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The Influence of Processing Conditions on the Formation Potential of Type V Resistant Starch from Jackfruit Seed Starch

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Abstract: Resistant starch (RS) originates from various natural sources and brings health benefits. Through retrogradation, RS3, RS4, and RS5 are formed. This has been demonstrated in a study using starch from initial jackfruit seeds containing 63.48% resistant starch, to produce RS5. In this study, jackfruit seed starch was combined with peanut oil, sunflower oil, soybean oil, olive oil at a ratio of 10:2 (w/w), along with palmitic acid, stearic acid at a ratio of 10:2 (w/w), processed under hydrothermal and non-hydrothermal conditions to form RS5. The results showed that using peanut oil resulted in the highest RS5 ratio when hydrothermally treated (86.48%) and non-hydrothermally treated (84.47%), surpassing other types of fatty oils: 81.38%, 76.15%, 73.46% respectively. Furthermore, when hydrothermally treated, using palmitic acid produced higher RS5 (80.58%) compared to stearic acid (75.87%), and the non-hydrothermally treated samples were 71.58% and 67.12% respectively. SEM analysis of the formed starch samples showed that non-hydrothermally treated samples retained their original granular structure, while hydrothermally treated samples underwent modification and agglomeration, resulting in increased resistance to hydrolysis, thus significantly increasing the RS content. Although type 5 resistant starch is not widely known, the results from the jackfruit seed starch study demonstrate great potential for future applications.

Keywords: Resistant starch, jackfruit seed starch, type 5 resistant starch, fatty oil, fatty acid.

I. Introduction

Resistant starch (RS) is defined as starch and its degradation products that resist digestion as they pass through the small intestine and colon. There are five types of RS: (1) RS1 - physically inaccessible starch found in whole or partially milled grains; (2) RS2 - raw starch granules (such as those in bananas and potatoes) and starch with high amylose content (such as maize with high amylose content); (3) RS3 - retrograded starch (processed from unmodified starch or food through processing); (4) RS4 - chemically modified starch such as some starch ethers, starch esters, and cross-linked starch [1] and (5) RS5 - a complex of amylose and lipids capable of resisting enzymatic digestion [2]. Lipids form complexes between the hydrocarbon portions of lipids found in the helical cavity of amylose. This mechanism is governed by unbranched chains (straight-chain molecules with α-1,4-linked d-glucose units) of glucan [3], [4]. RS5 has been reported to reduce postprandial glycemic responses and is reported to have the potential to induce metabolic syndrome interventions such as type 2 diabetes, obesity, hypertension, and heart disease [5]. From a crystallization standpoint, the amylose-lipid complex (ALC), in which amylose helices are surrounded by lipid molecules, can be evaluated in two different forms: indefinite complex formation and crystalline complex formation. The crystalline complex is known to resist enzymatic hydrolysis more than the indefinite complex [2]. The formation of such ALCs alters other properties of starch, such as retrogradation, swelling, viscosity, retrogradation, and enzyme hydrolysis ability. RS5 is easier to produce and thermally more stable than other types of RS previously reported, making RS5 more suitable for food processing industries [6]. Several RS5 formation methods have been published: the classical method (1) involves starch and lipid contacting water at high temperatures without mechanical shearing [7], [8], [9]; enzyme method (2) involves starch synthesis in the presence of lipid compounds from simpler sugars such as sucrose and maltotetraose. Phosphorylase and amylosucrase are enzymes used to synthesize ALC [10], although the benefits such as the ability to form suitable amylose-lipid complexes but enzymes used in the synthesis process are expensive and generally unsuitable for large-scale production; steam method (3), starch and lipid are continuously pumped into a hydrothermal machine at high temperature and then immediately cooled to form a starch-lipid complex [11]; extrusion method (4) includes a mixture of starch and lipid being extruded at high moisture, pressure, and temperature in a short time, the mixture along the barrel surface is retrograded and forms a glassy phase, the synthesis effects of shear force, along with high heat and pressure, lead to the formation of ALC [12]; spray drying method (5) is starch being retrograded with a lipid solution by the classical method in water or alkaline environment and then this mixture passes through a spray

drying chamber to form fine powder. The separation process almost completely separates amylose from amylopectin and the interaction between amylose and lipid molecules is not hindered by any granular starch. The obtained powder is indefinite due to rapid water removal, with no time for crystallization [13], [14].

The purpose of this study is to create ALCs from jackfruit seed starch with different fats and fatty acids. The ALCs formed serve as a direction for further application studies in processing food products from this raw material source.

II. Materials and Methods

2.1. Materials

Raw materials: Fresh jackfruit seeds of the Thai jackfruit variety (Artocarpus heterophyllus) when collected can be refrigerated to facilitate the research process because this material depends on the harvest season. Fatty oils such as peanut oil, sunflower oil, soybean oil and olive oil. Fatty acids such as palmitic acid and stearic acid.

Chemicals: Sodium Hydroxide >97% (AR, Fisher, Cas 1310-73-2); HCl ≥99% by HPLC (Merck); Resistant starch Assay Kit (Product code K-RSTAR of Megazyme); C_2H_5OH 99.99%; Potassium hydroxide 85% (Merck)and some other common chemicals.

Instruments: UV-VIS Spectrophotometer (Jasco V-630, Japan); Scanning Electron Microscope (SEM) JEOL JSM-5410LV (JEOL, USA); Hettich EBA20 centrifuge, Germany; STUART CB162 Hotplate Stirrer, UK; Vortex Mixer GEMMY-VM-300, Taiwan; EMM20K22B microwave oven, China; Thermostatic Water Tank Bluepard DK-8AD, China; Autoclave HYSC AC-100, Korea.

2.2. Methods

2.2.1. Preparation of jackfruit seed starch

The jackfruit starch was obtained using the chemical method outlined by the research group of Wong and colleagues, as depicted in Figure 1. After removing the tough outer skin, the jackfruit seeds were ground into a paste and diluted in distilled water (1:2 ratio). This mixture was continuously stirred (500 revolutions per minute) for 3 hours at room temperature. Subsequently, the residue was separated using a sieve or filter cloth. The supernatant obtained was centrifuged at 8000g for 5 minutes, and the upper layer was decanted. The sediment at the bottom was treated with 0.1M sodium hydroxide to dissolve the remaining proteins. The supernatant was kept at room temperature for 18 hours with continuous stirring. Then, it was centrifuged at 8000g for 10 minutes (25°C) and washed twice with 0.1M sodium hydroxide. The upper layer was decanted, and the remaining brown layer was also removed. The sample was thoroughly washed with water and neutralized using 0.1M hydrochloric acid until reaching a pH of about 6.5 to 7.0. The starch sample was further washed three times with distilled water to remove excess salts and then centrifuged at 8000g for 10 minutes (25°C). The moisture content of the starch supernatant was adjusted to 70% before being dried in an oven at 40°C for 18 hours. The dried starch sample was powdered using a grinder. The starch recovery efficiency from jackfruit seeds was calculated by dividing the final starch mass by the mass of jackfruit seeds used in the extraction process[15].

2.2.2. Investigating the effect of different fatty oils on the ability to create RS5.

- **Gelatinization process:**

Prepare 4 samples of jackfruit seed starch (each sample 4g), mix each with the following fatty oils: peanut oil, sunflower oil, soybean oil, and olive oil in a ratio of (10:2 w/w). Cook the samples with ion-deionized water (3x, w/w) for 8 minutes in a double-boiler pot boiling (approximately 100°C), continuously stirring manually until the samples are completely gelatinized. The samples are then dried in an air oven at 45°C, ground into fine powder, and sieved through a 0.3 mm sieve [16]. Then determine the amount of RS formed using the AOAC 2002.02 method[17].

- **Non-gelatinization process:**

Prepare 4 samples of jackfruit seed starch (each sample 4g), mix each with the following fatty oils: peanut oil, sunflower oil, soybean oil, and olive oil in a ratio of (10:2 w/w - calculated based on dry weight at room conditions). The samples are then dried in an air oven at 45°C, ground into fine powder, and sieved through a 0.3 mm sieve[16] . Then determine the amount of RS formed using the AOAC 2002.02 method[17].

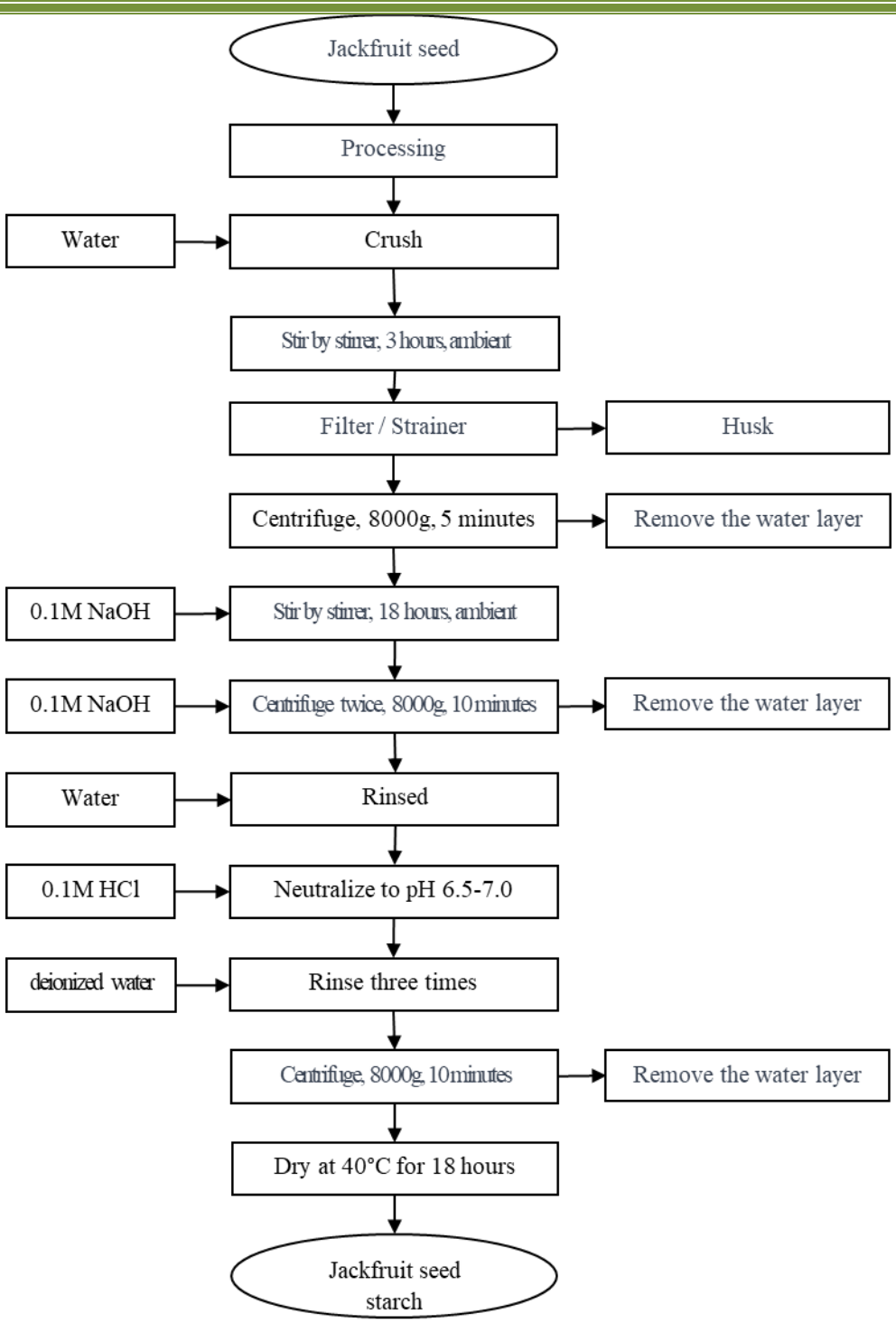

Figure 1. Process Flowchart for Extracting Jackfruit Seed Starch

2.2.3. Investigating the effect of fatty acid type on the ability to form RS5

- **Gelatinization process:**

Prepare 4 samples of jackfruit seed starch (each sample 4g), mix each with 2 types of fatty acids: palmitic and stearic acid in a ratio of (10:2, w/w), cook the samples with ion-deionized water (3x, w/w) for 8 minutes in a double-boiler pot boiling (approximately 100°C), continuously stirring manually until the samples are completely gelatinized. The samples are then dried in an air oven at 45°C, ground into fine powder, and sieved through a 0.3 mm sieve [16]. Then determine the amount of RS formed using the AOAC 2002.02 method[17].

- **Non-gelatinization process:**

Prepare 2 samples of jackfruit seed starch (each sample 4g), mix each with 2 types of fatty acids: palmitic and stearic acid in a ratio of (10:2, w/w - calculated based on dry weight at room conditions). The samples are then dried in an air oven at 45°C, ground into fine powder, and sieved through a 0.3 mm sieve [16]. Then determine the amount of RS formed using the AOAC 2002.02 method[17].

2.2.4. Determining the resistant starch content

The resistant starch content is analyzed using AOAC 2002.02 method. It involves weighing 0.1 grams of starch sample into a test tube with 0.1M sodium acetate buffer (pH=4.5), adding porcine pancreatic α-amylase and amyloglucosidase from Aspergillus Niger, vortex mixing, and enzymatic hydrolysis occurred in a shaking water bath maintained at 37°C for a duration of 16 hours. The reaction is stopped by adding ethanol, and the resistant starch fraction is recovered via centrifugation. Quantification of the combined liquid portions is conducted to ascertain the amount of digested starch (DS). The residue is dried, dissolved in 2M KOH in an ice-water bath, adjusted to pH ~ 4.5 with acetate buffer, and the undigested starch is quantified, considered as the quantity of resistant starch (RS) broken down by amyloglucosidase was measured in a shaking water bath maintained at 37°C for a duration of 30 minutes. This is further diluted to 100ml, and glucose absorption is measured using a glucose oxidase-peroxidase (GOPOD) assay at 510nm wavelength against a standard sample to determine the RS starch content, calculated as glucose content × 0.9. The total starch content of the sample is obtained by adding DS and RS [17].

2.2.5. Scanning electron microscopy (SEM) analysis.

Experimental SEM to analyze particle surface, shape and size using a JEOL JSM-5410LV instrument (JEOL, USA) equipped with a large field detector. The acceleration voltage is 15 kV in low vacuum mode (0.7-0.8 torr). The sample was mounted onto a copper stub using adhesive tape and subsequently coated with gold within a vacuum environment. Images were taken at 2000x magnification [18].

2.2.6. Statistical Analysis

The statistical analysis was conducted under the following conditions: The experimental results were carried out with 5 replicates and statistically processed using Microsoft Excel 2016 and Statgraphic Centurion 19.1.2 software. The numerical data represent the mean values of 3 replicates ± standard deviation with a significance level of $p \leq 5\%$. The data and optimal charts were processed using Design Expert 12 software.

III. Results and Discussion

3.1. Investigating the effect of different fatty oils on the ability to create RS5

During the formation of amylose-lipid complexes to create RS5, several factors influence the resistant starch content. These factors include the species' nature due to variations in their protein content, the amylose/amylopectin ratio [19], particle size [20], amylose retrogradation and crystallinity degree [19], lipid nature [20], [5], [21], [22]… However, within the scope of this study, only the influence of fatty oil type and gelatinization conditions on the resistant starch content in jackfruit seed starch was investigated, considered as the most significant factors in previous studies on RS5. Figure 2 presents the depicted outcomes.

The results show that the RS5 content of jackfruit seed starch increases compared to the control sample when using different types of fatty oils, and there is also a tendency for a greater increase with gelatinization. Specifically, among the fatty oils, peanut oil has the highest ability to increase RS5 content from 20.99% (non-gelatinized sample) to 23% (gelatinized sample) compared to the control sample. Samples treated with sunflower oil, soybean oil, and olive oil respectively have lower RS5 content.

The sensitivity of the enzyme to the amylose-lipid complex is proposed through two different mechanisms. According to the first mechanism, this complex reduces the extent of starch granule swelling, making it difficult for the enzyme to access the interior of the starch granule. On the other hand, the second mechanism suggests that the crystalline amylose-lipid complex is more resistant to enzyme digestion compared to the free complex [23].

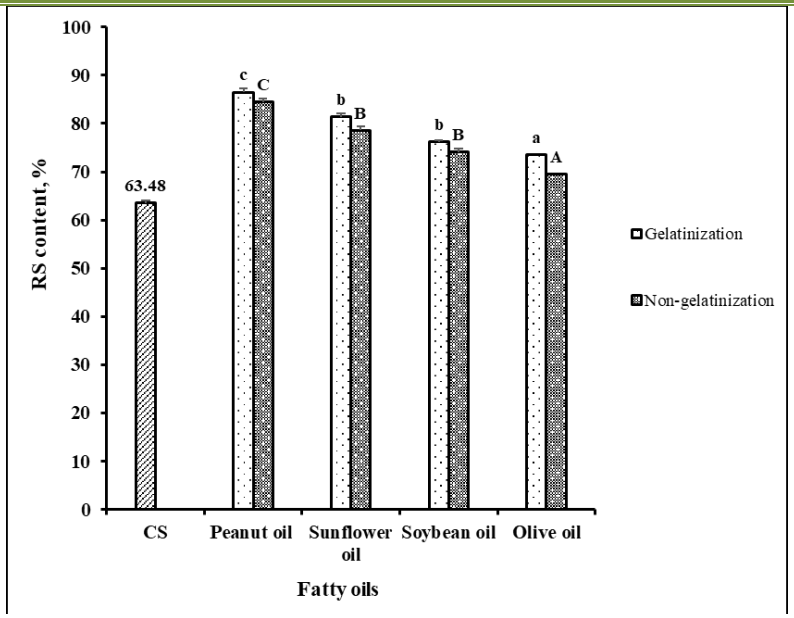

Figure 2. Influence of various types of fatty oils on the formation of RS5
A, B, C; a, b, c: Different letters represent statistically significant differences ($p < 0.05$) for the same monitored parameter.

These results are similar to the study by Okumus et al. on "Formation of resistant starch type 5 in brown lentil (Lens culinaris Medikus) starch with different fats/fatty acids." The study showed that the rapidly digestible starch (RDS) content in brown lentil starch (27.4%) decreased after lipid supplementation and significantly decreased ($p < 0.05$) after supplementation with hydrolyzed sunflower oil (reduced by approximately 46%) in the samples that were not fully cooked. The least reduction was observed with olive oil, approximately 4.8% in the uncooked sample and 5.5% in the cooked sample, with no statistical significance ($p > 0.05$). This is primarily explained by the decrease in the amount of amorphous amylose after disrupting starch granules during the gelatinization process. Additionally, the unstable physical and chemical structure of amorphous amylose and its presence in the amylose-lipid complex slows down enzyme hydrolysis. Thus, changes in starch hydrolysis rates are attributed to the formation of complexes between amylose and fatty acids [20].

Similar findings were also reported in the study by Zhang et al. on "Structural characteristics and digestibility of high amylose maize starches modified by branching enzymes complexed with lauric acid (LA)." The study partly elucidated the relationship between gelatinization and starch branching processes regarding the formation of RS5 from high-amylose maize starch and lauric acid. Maize starch, after cooking, was branched using pullulanase and then complexed with lipid. Monitoring the RS5 content showed that the pre-branching pretreatment and prolonged branching time (from 2 hours to 24 hours) could improve the formation of lipid-starch complexes, specifically achieving the highest resistant starch (RS) content (45.6%) when the branching time was extended to 24 hours [23].

Firstly, regarding the effect of branching time on RS formation: prolonged branching time significantly increased the RS content of lipid-starch complexes and reduced the RDS content due to the enzyme-resistant amylose-lipid complex and the stable double-helix re-crystallized amylose characterized by high thermal stability. Amylose produced from branching reactions is more flexible and free than amylopectin. Therefore, they enhance the kinetics of crystallization processes at later stages, forming both double-helix starch complexes and amylose-lipid complexes with higher enzyme resistance than free amylose [5].

Furthermore, after part of the starch is branched and allows retrogradation or complexation with the hydrophobic domain of fatty acids, the process of packing the helical loops between residual amylopectin and amylose or lipid can be hindered, resulting in imperfectly packed loops leading to the formation of less dense crystalline structures, which can be slowly digested. However, with prolonged branching time, more free amylose is produced, and the lipid-amylose or retrograded amylose complexes with perfect crystalline structures are formed. These dense structures contribute to the RS component. Thus, the RS content of starch containing amylose-lipid complexes is higher than that of only cooked starch due to the additional type 5 resistant starch component [5].

Next, during the cooking process, starch granules are ruptured by heating in excess water at a temperature of 100°C, also known as gelatinization, which allows molecules to fully access digestive enzymes, significantly reducing the RS content. However, upon cooling, the starch undergoes a relatively slow reassociation process called retrogradation. In this process, the starch granules lose their original shape, forming honeycomb structures through water molecule freezing and thawing, and the broken amylose molecules in the previous stage form hydroxyl bonds within themselves and between molecules, facilitating rapid double helix formation and the formation of recrystallization regions, thereby increasing the total RS content. This is more effective when there is water loss [24]. Therefore, the increase in RS is mainly due to the increased density of loops from amylose-lipid complexes, in addition to gelatinization, resulting in crystalline structures and recrystallized amylose due to the cooling process after gelatinization. Moreover, the cooked starch shows partially swollen and deformed granules leading to reduced swelling and solubility, hence reducing digestibility [23].

3.2. Investigate the effect of fatty acid type on the ability to create RS5

The survey results indicate that the resistant starch (RS) content of jackfruit seed starch increased in all samples compared to the control sample when using different types of fatty acids, and there was a tendency for greater increase with hydrogenation. Specifically, among the two types of fatty acids, palmitic acid showed the highest ability to increase the RS5 content, reaching 17.1% compared to the control sample. Samples treated with stearic acid exhibited lower RS5 content, increasing from 3.64% (non-hydrogenated sample) to 8.1% (hydrogenated sample), as shown in Figure 3.

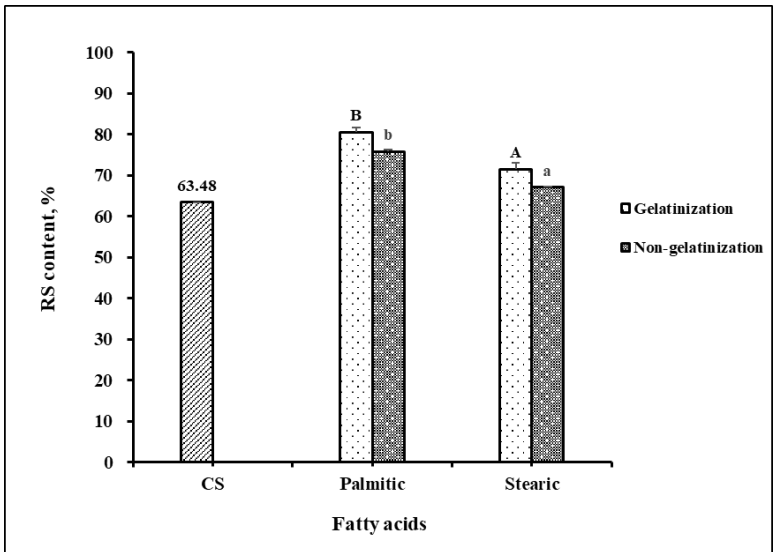

Figure 3. Effect of fatty acids on the ability to form RS5

A, B; a, b: Different letters represent statistically significant differences ($p < 0.05$) for the same monitored parameter.

Treating starch with acid is a method that has been extensively studied in the past regarding its ability to create resistant starch type 4. The fatty acids used in this study are of a vegetable oil origin, thus they are not as hazardous as other inorganic acids. Moreover, a low pH environment catalyzes the hydrolysis of glycosidic bonds, increasing amylose content, thereby enhancing crystallization potential. Therefore, the increase in RS content in this experiment could be attributed to the increase in resistant starch types 3 (gelatinization), 4 (acid), and 5 (lipid). Thus, compared to the method of creating resistant starch with fatty oils (which only yields RS3 and RS5), this method has the potential to produce higher RS content. This finding contradicts the results obtained, suggesting that fatty acids are significantly less effective in producing RS compared to fatty oils, possibly due to the high intensity of the method, which disrupts the structure of resistant starch, making it more susceptible to enzymatic breakdown [25].

This result is consistent with the study conducted by Kaur & Singh on the influence of rice starch and various fatty acids (palmitic, myristic, and stearic acids) on the formation of amylose-lipid complexes. According to their findings, myristic acid had the highest ability to form complexes, followed by palmitic and stearic acids with lower abilities. These scientists reported that increasing the concentration of fatty acids used

and the thermal processing method (i.e., hydrogenation) also increased the degree of amylose-lipid complexation [22].

Similarly, a study by Okumus et al. on "Resistant Starch V Formation in Brown Lentil Starch (Lens culinaris Medikus) with Various Lipids/Fatty Acids" demonstrated that rapidly digestible starch (RDS) content in brown lentil starch (27.4%) decreased after lipid supplementation and significantly decreased ($p<0.05$) after supplementation with fatty acids such as stearic and palmitic acids (decreasing by approximately 46%) in uncooked samples. The authors attributed this decrease to the indefinite amylose structure after starch granule disruption due to the hydrogenation process, and the unstable physical and chemical structure of indefinite amylose, making it more susceptible to hydrolysis, while its presence in amylose-lipid complexes also slowed down the enzyme hydrolysis rate. Therefore, the main change in starch hydrolysis rate is due to the formation of complexes between amylose and fatty acids, resulting in a higher RS5 content compared to the initial RS content [20].

The role of palmitic acid in RS5 formation is also confirmed by Hasjim et al. in their study on "Characteristics of a Novel Resistant Starch and Its Postprandial Effects on Blood Glucose and Insulin Response." The study discovered a new resistant starch (RS5) by creating a complex of high amylose VII corn starch (HA7) with palmitic acid (PA). The HA7 starch was heat-treated and debranched using isoamylase (ISO) and then complexed with PA (HA7+ISO+PA), resulting in an RS content of 52.7%, higher than that of the HA7 control (35.4%). The increase in RS content is attributed to the formation of starch-lipid complexes. It is concluded that starch treated with fatty acids with longer hydrocarbon chains has the ability to form more stable amylose-lipid complexes under the action of amylase enzymes[5].

3.3. Scanning Electron Micrograph of RS5

When observed under scanning electron microscopy (SEM), the size of jackfruit seed starch particles ranges from 5-12µm, exhibiting circular, convex, and polygonal shapes with smooth surfaces (Figure 4), consistent with the study by Mahanta and Kalita [26].

Clear differences can be observed between hydrogenated and non-hydrogenated samples, where the hydrogenated samples no longer show any traces of the original particle structures but instead display the presence of starch fragments, whereas the non-hydrogenated samples almost maintain their original particle structures with no visible alterations (Figure 4). This indicates that the impact of hydrogenation on the structure of starch particles is significant, causing decomposition and recrystallization, altering their original shapes.

For the hydrogenated samples, SEM images also show no significant differences in particle structure when using different fatty acid/fat agents; however, there appears to be a slight reduction in size compared to the original samples (Figure 5). This may be due to starch branching and complex formation resulting from reactions with lipids, making the amorphous regions more compact. Additionally, experimental conditions or magnification levels in our study might not reveal substantial differences.

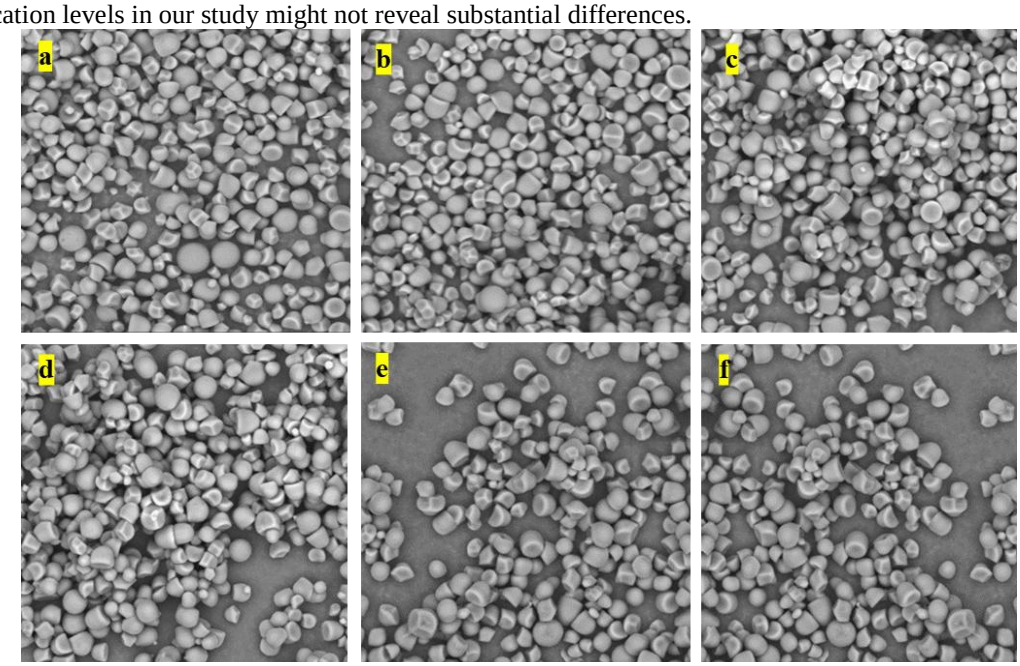

Figure 4. SEM images of non-hydrogenated RS5 samples: peanut oil (a), sunflower oil (b), soybean oil (c), olive oil (d), stearic acid (e), palmitic acid (f)

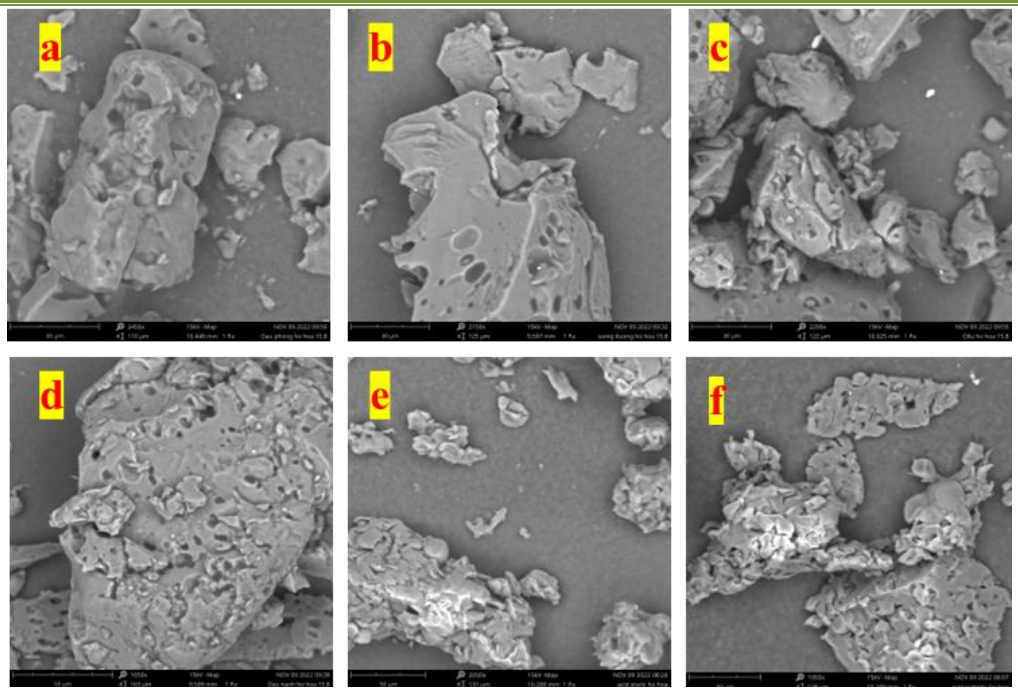

Figure 5. SEM images of hydrogenated RS5 samples: Peanut (a), sunflower (b), olive (c), soybean (d), stearic acid (e), palmitic acid (f)

Through SEM, it is easily noticeable that samples treated with fatty acids have rougher surfaces. This change in morphology could be attributed to the acidic action penetrating into the available pores on the starch surface and accessing the core (amorphous region) through channels connecting the pores to the core of the particle, thus causing erosion of the amorphous region and increasing the crystalline proportion. Specifically, SEM images of samples treated with palmitic acid exhibit rougher surfaces compared to stearic acid-treated samples, indicating a higher attacking and reacting capability of palmitic acid than stearic acid, leading to more erosion and increased resistant starch content (consistent with experimental results).

IV. Conclusion

When creating RS5 from jackfruit seed starch combined with fatty oils or fatty acids, it demonstrates a higher resistance to digestion compared to the original RS. Additionally, RS5 under hydrogenation conditions is more resistant to hydrolysis compared to non-hydrogenated RS5. Among the different types of fatty oils, peanut oil performs superiorly in both conditions, with the highest RS5 content formed. Regarding the two types of fatty acids, RS5 formed with palmitic acid shows higher levels compared to stearic acid. SEM analysis reveals that hydrogenated RS samples undergo structural changes, with particles becoming agglomerated and bonded together, making it difficult for enzymes to penetrate and hydrolyze them. Although the RS5 content formed in this study is very high for both fatty oils and acids, their applicability in food processing needs to be carefully considered, especially in cases of obesity and fat-restricted diets.

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