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Utilization of Waste Cashew Pseudo Fruit (*Anacardium occidentale* L.) as a Prebiotic Source

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ABSTRACT

Persea americana and *Bryophyllum pinnatum* are important plants with age-long application in Cashew pseudo fruit is rich in carbohydrates. These carbohydrates can be chemically hydrolyzed into simpler monomers such as oligosaccharides. The research aims to analyze the oligosaccharide contents in pseudo cashew fruit produced by chemical hydrolysis using sulphuric acid and citric acid so that they can be used as a prebiotic source. The powdered pseudo fruit of cashew was hydrolyzed to convert polysaccharides into oligosaccharides using citric and sulphuric acids as solvents. Oligosaccharide analysis was performed using thin layer chromatography (TLC), reducing and total sugar, and liquid chromatography-mass spectrometry (LC-MS) method. Furthermore, *in vitro* testing was carried out as a prebiotic source of LAB (Lactic Acid Bacteria). Analysis by TLC and the degree of polymerization from hydrolysis in sulphuric acid solvent shows an R*f* value of 0.43- 0.5 with the degree of polymerization of 2.3 and citric acid R*f* 0.38-0.5 with the degree of polymerization of 1.6. LC-MS analysis showed that hydrolysis with sulphuric acid resulted in 118.53 mg/L glucose and with citric acid resulted in 1565.10 mg/L glucose; lactose 848.4 mg/L and maltose 1242.60 mg/L. *In vivo* test showed the amount of LAB in sulphuric acid solvent as $1.5x10^6$ CFU/mL and in citric acid solvent as $1.4x10^6$ CFU/mL Based on the results, it can be concluded that the chemical hydrolysis of cashew pseudo fruit can produce oligosaccharides in the form of maltose and lactose which have the potential to be used as a prebiotic source because they can support the growth of probiotic bacteria *in vitro*.

Keywords: Cashew, Pseudo, Fruit, Oligosaccharide, Prebiotic.

Introduction

Indonesia has several cashew (*Anacardium occidentale* L.) plantations spread across several provinces including Southeast Sulawesi, Central Sulawesi, South Sulawesi, East Nusa Tenggara, West Nusa Tenggara, Maluku, Bali, East Java, Central Java and Yogyakarta provinces, Indonesia. Indonesia could produce cashew fruit reaching 139.968 tons with a plantation area of 510.113 ha. Southeast Sulawesi Province, located in Muna Regency is the second largest cashews (true fruit) production center after East Nusa Tenggara, the amount of pseudo cashew fruit waste is also high.[1]

Prebiotics in general can be obtained from undigested food and can improve the balance of bacteria in the intestine so that it is beneficial to the large intestine. This prebiotic will undergo a fermentation process to produce beneficial bacteria. Food is used for the breeding of good bacteria in the large intestine so that the good bacteria can multiply. These foods are very useful for the proliferation of good bacteria, so that these bacteria multiply. Meanwhile, bad bacteria, because they do not like this food, their development are hampered so that the number of good bacteria becomes more and dominates the bacterial population present in the intestine, prebiotics is known for their ability to nourish gut microbes present in the gastrointestinal tract (GIT) and substantially improve their metabolic activity, enhancing digestion, nutrient absorption ability, and the immune system while curbing the growth of pathogenic microbes.

These significant improvements show a positive effect on human health.[2] Prebiotics are non-digestible carbohydrate (CHO) molecules, including sugar polyols, poly and oligosaccharides, and resistant starches, as well as fibre that have a beneficial role in both the maintenance and progression of gut microflora.[3]

Prebiotic compounds that cannot be digested by the small intestine, will reach the large intestine and be degraded or fermented by intestinal bacteria, and stimulate the growth of LAB (Lactic Acid Bacteria).[4] Oligosaccharides can improve blood lipid metabolism, regulate gastrointestinal function, prevent and treat constipation, increase vitamin synthesis, and improve human immunity. Oligosaccharides can also be used as a protective agent when bacteria encounter changes in temperature, pH, and other growth conditions. The synergistic benefits of prebiotics and probiotics could enhance and extend the therapeutic and nutritional benefits of fermented dairy products.[5] Apart from acids, intestinal bacteria will also produce substances that are antimicrobial. Almost all substances produced by acidic bacteria are the result of the fermentation of oligosaccharide carbohydrates.[6]

Oligosaccharides are a source of prebiotics that can be used as nutrients for probiotic bacteria. However, information on the use of oligosaccharides from cashew fruit waste as a prebiotic source is still very limited. According to the analysis of the Directorate of Nutrition, Ministry of Health of the Republic of Indonesia, every 100 g of fake fruit contains 64 calories, 0.7 g of protein, 15.8 g of carbohydrates, 4.0 mg of calcium, 13.0 mg of phosphorus, 0.5 mg of iron, 82.6 g of water and fiber.[7]

This study aims to produce oligosaccharides through chemical hydrolysis so that the cashew pseudo fruit (Figure 1) waste can be utilized as a prebiotic source and this is expected to increase the economic value of the cashew pseudo fruit.

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Figure 1: Cashew Pseudo Fruit

Materials and Methods

Materials

The citric acid (Cap Gajah, Indonesia), Sulfuric acid (Merck, Germany), n-butanol (Lux Chemicals, Indonesia), Acetic acid (Merck, Germany), Glucose (Lux Chemicals, Indonesia), Raffinosa (Merck, Germany), α-diphenylamine (Merck, Germany), Acetone (Rocky Mountain Reagents, USA), Phosphoric acid (Lux Chemicals, Indonesia), Aniline (Merck, Germany), DNS reagent (Sigma Aldrich, USA), TLC Silica gel 60 GF 254 (Merck, Germany), Sodium Carbonate (Tata Chemicals, America), Methanol (Merck, Germany)

Preparation of Cashew Pseudo Fruit Powder

Cashew pseudo fruit was collected from Rasuna Said Cipete Plantation, Tangerang City, Banten, Indonesia in January 2020. The species was identified at Herbarium of the Indonesian Institute of Science, Research Center for Plant Conservation Botanical Gardens, Bogor, Indonesia and a voucher specimen was deposited (No. B-1640).

The cashew fruit was squeezed to separate the liquid. The solid waste was cut into thin slices of about 0.1 cm and dried in an oven at a temperature of 50^{o}C for 12 hours. Then it was ground and sieved using a 60 mesh sieve.[8]

Chemical Hydrolysis

Cashew fruit powder (20 g) was hydrolyzed with 100 mL of solvent (0.5 M citric and sulphuric acid) for 20 minutes at 700 rpm using a magnetic stirrer and then it was filtered using a Buchner funnel. Filtrate was neutralized to pH 7 using sodium carbonate.

Analysis of Reducing and Total Sugar

Reducing sugar analysis.

Samples that have been centrifuged were taken (100 µL) using a micropipette, then 900 µL of water was added and stirred using a vortex. Furthermore, 1 mL of the mixture was taken and then added with 3 mL of DNS and heated for 5 minutes then cooled. Then measured using a spectrophotometer to determine the absorbance.[9]

Total sugar analysis.

Samples that had been centrifuged were taken as much as 0.100 µL, then put into the test tube using a micropipette. Add 200 µL of HCl then heated for 20 minutes using a water bath with a temperature of 100^{o}C then cool to room temperature. Add 400 µL of NaOH and add 300 µL of water. So that the results obtained from the mixture of the parent sample as much as 1 mL. Then the sample was stirred using a vortex. Then added with 3 mL of DNS, heat for 5 minutes, and cooled to room temperature. Furthermore, it was measured using a spectrophotometer at a wavelength of 550 nm.[9]

Analysis by TLC

The oligosaccharide from the hydrolysis reaction was analyzed by thin-layer chromatography (TLC). The hydrolysis sample was placed on the TLC plate. The standards used are glucose (monosaccharide representation), maltose, lactose (disaccharide), and raffinose (trisaccharide). The TLC plate was then put in the TLC vessel with the eluent of n-butanol: acetic acid: aquades (12:6:6). The viewing reagent, consisting of 0.5 gram of α-diphenylamine, 25 mL of acetone, 2.5 mL of phosphoric acid, and 0.5 mL of aniline was sprayed to reveal the spots on the TLC plate. TLC plates were heated at 100^{o}C for 15 minutes.[10]

Oligosaccharide analysis using LC-MS

Oligosaccharide analysis used LC-MS. The mobile phase used is acetonitrile: water (80:20). The column used is Luna Sugar. The standards used are glucose, lactose, and maltose. The sample was dissolved with distilled water and then filtered using a 0.45 µL PTFE filter. The volume injected is 5 µL.[10]

In vitro oligosaccharide testing

Testing of the extracted oligosaccharides was carried out using lactic acid bacteria (LAB). The type of LAB used was *Lactobacillus casei*. The medium used for bacterial growth was MRS (deMann Rogosa and Sharpe) broth media (10 g of casein, 8 g of meat extract, 4 g of yeast extract, 20 g of D(+)-glucose, 2 g of dipotassium hydrogen phosphate, 1 mL of tween 80, 2 g of di-ammonium hydrogen citrate, 5 g of sodium acetate, 0.2 g of magnesium sulfate, 0.04 g of manganese sulfate) but the glucose medium was replaced with oligosaccharide sugar purified from the extract of cashew pseudo fruit and the medium used as control was MRS broth media without sugar components. The culture of each LAB was grown on media containing oligosaccharide sugar. To avoid contamination, each of them was made in duplicate. Incubation was carried out in an incubator at 37^{o}C for 24 hours. Control was carried out through the same steps, but no added source of sugar (oligosaccharides).[11]

Table 1: LC-MS Condition

LC-MS	Condition
LC-MS column	Luna Sugar
Mobile phase	Acetonitrile: Water (80:20)
Gradient Program	25 minutes
Flow rate	1 mL/min
Injection volume	5µL
MS Condition	MSDS2 341: Lactose and Maltose
	MSDS2 179 : Glucose

The calculation of the number of bacteria was carried out in the following manner: the sample suspension in a physiological solution of 0.85% NaCl (10^{-1} dilution) was pipetted as much as 1 mL and put into 9 mL of 0.85% NaCl physiological solution so that a 10^{-2} dilution was obtained, then in the same way, made dilution 10^{-3}, 10^{-4} and so on until the desired level of dilution (expected plating results obtained between 25-250 colonies). Calculation of the number of bacteria (CFU) of sample was carried out by dividing the number of colonies by the dilution factor. 1 mL of suspension from the appropriate dilution level (10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}) was pipetted and poured into a sterile petri dish. Then MRS base media was poured into the petri dish and mixed carefully until homogeneous, then left to freeze. The sample was incubated at 37^{o}C for 24 hours and the growing colonies were counted as the number of lactic acid bacteria.[11]

Statistical analysis

Each experiment was conducted in duplicate. Data were expressed as mean ± standard deviation. Statistical analysis was carried out using statistical package for social sciences (SPSS) version 18.0 software for Windows. Data analysis was carried out using a simple linear regression model with the formula Y = a +. bX to determine the total and reducing sugar contents. Statistical significance was evaluated at the significance level of ≤ 0.05.

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Results and Discussion

Chemical Hydrolysis of Cashew Pseudo Fruit

Hydrolysis is a chemical reaction that breaks a molecule into two parts by adding a water molecule (H_2O) to convert polysaccharides into simple monomers.[12] The hydrolysis of pseudo cashew fruit flour was carried out chemically using citric acid and sulfuric acid as a catalyst. As much as 5 grams of cashew pseudo fruit flour, each added with a solution of 25 mL of 0.5 M citric and sulfuric acid, then stirred for 20 minutes at 1000 rpm. The use of high pressure was because the acid used is in the form of dilute acid and the acid used has a low concentration.[13] Acid hydrolysis of polysaccharides will produce simpler sugars, but at high temperatures, it can cause further degradation so that furfural and hydroxymethylfurfural compounds (HMF) are formed.[12] The breakdown mechanism of cellulose into glucose in the acid hydrolysis reaction began with the H^+ ion from the acid which binds to H_2O to H_3O^+which broke the glycoside bonds in cellulose, so that simple monomers was formed.[9]

The hydrolyzed sample was filtered to separate the extract residue in the form of a solid from a solution containing dissolved oligosaccharides and neutralized using sodium carbonate (alkali salt) until pH 7 so that the hydrolysis process did not occur a chain reaction that could cause the obtained oligosaccharides to become monosaccharides. If hydrolysis by acid continues, oligosaccharides will also be hydrolyzed to produce monosaccharides.[9,14]

Analysis by Thin Layer Chromatography

Thin Layer Chromatography is a separation technique that uses a mobile phase and a stationary phase.[14] The purpose of using Thin Layer Chromatography was to qualitatively determine oligosaccharides, judging by the resulting Rf value. The mobile phase used was n-butanol: acetic acid: aquades (2:1:1),[9] because carbohydrates are highly hydrophilic compounds, so they could bind strongly to TLC plates, such as silica gel, alumina, and cellulose so that it took a very polar mobile phase in the development of TLC.[15] The stationary phase used was a TLC plate of silica gel 60 F_{254}. The sugar dye solution (visible solution) used was a mixture of several ingredients, namely: α-diphenylamine, phosphoric, aniline.[9] Sugar dye solution was used as an appearance solution which is sprayed on the TLC plate. Detection by chemical reaction using sighting reagents was possible, which means that any type of compound could be detected if an appropriate detection reagent was used. This visible reagent reacted chemically with all solutes containing certain functional groups so that the spots become colored.[15] For comparison, glucose, lactose, maltose, and raffinose were used. The results of identification with TLC can be seen in Table 2.

Samples with citric acid solvent had R*f* values in the range of 0.38; 0.4; 0.5. This Rf value was between the R*f* value of glucose, lactose, and maltose, so it was most likely a monosaccharide or disaccharide. For samples with sulfuric acid solvent, the Rf value was in the range of 0.43-0.5. The R*f* value was between the R*f* value of glucose, lactose, and maltose so it was most likely a monosaccharide or disaccharide. Oligosaccharides were generally defined as a carbohydrate molecule containing 2 to 10 units of monosaccharide molecules. The most common oligosaccharides were disaccharides composed of two monosaccharide molecular units, which were joined by glycoside bonds. Important disaccharides, which were abundant in nature were lactose and maltose.[16] So it could be concluded that the results of this qualitative analysis contained oligosaccharides, because the longer the oligosaccharide chain, the lower the resulting R*f* value. This was because long-chain oligosaccharides had more hydroxyl groups.[14]

Analysis of reducing and total Sugar

Reducing sugars were sugars that could reduce due to the presence of an aldehyde and a ketone group. Total sugar is the sugar contained in these foodstuffs.[17] The ratio of total sugar to reducing sugars could be used to predict the degree of polymerization (DP) resulting from hydrolysis. DP provides initial clues about how long the oligosaccharide chain was formed by hydrolysis. The purpose of this test was to find out how long the oligosaccharide chain was formed.[9]

Reducing sugar content in cashew nuts from hydrolysis with citric acid solvent was 0.14%. Reducing sugar indicated the formation of simple sugars such as oligosaccharides in cashew nuts. The number of reducing and non-reducing sugars in the sample was expressed in terms of the total sugar contained in the sample.[10] The total sugar content in the sample with citric acid solvent was 0.23%. The degree of polymerization (DP) is obtained from the ratio of total sugars and reducing sugars.[10] The DP in the sample with citric acid solvent is 1.64, meaning that the sample produces 1.6 polymers as a result of the degradation of polysaccharides into oligosaccharides or simple sugars. Meanwhile, the reducing sugar content in cashew nuts from hydrolysis with sulfuric acid solvent is 0.15%. The total sugar content in the cashew pseudo fruit sample was 0.32%. The degree of polymerization was obtained from the ratio of total sugars and reducing sugars. The DP in the cashew pseudo fruit sample with sulfuric acid as solvent is 2.13, meaning that the sample produces 2.13 polymers as a result of the degradation of polysaccharides into oligosaccharides or simple sugars. So it could be concluded that this type of oligosaccharide is a disaccharide. The DP value of sulfuric acid is 2.4 when compared to this test, the results are not too different, namely 2.1. For the DP value of citric acid, namely 4.9 when compared with this test the results are different, namely 1.6.[9]

The low value of the degree of polymerization shows that the polymer chains of the polysaccharides have broken down into monomers. The decreasing degree of polymerization value indicates that more polysaccharides are depolymerized into shorter-chain compounds, where the degree of polymerization is a dependent variable, depending on the total value of sugar and reducing sugars produced.[18]

Oligosaccharide analysis using LC-MS

Oligosaccharide analysis using LC-MS is a quantitative analysis to determine oligosaccharide levels in cashew pseudo fruit. The mobile phase used in this test is a solvent mixture, namely acetonitrile and water (H_2O) with a ratio of 80:20 with the flow rate used in this test is 1 mL/minute. This flow rate is used because the lower the flow rate, the higher the ability to separate the components and save the use of the mobile phase, and the time it takes will be longer and also to prevent the chromatography process from starting with a wide band.[19] The retention time of standard chromatogram were obtained a sharp peak in 5.966 minutes of glucose; lactose 12.121 minutes of lactose and 10.616 minutes of maltose.

Based on the results of the sample chromatogram with citric acid solvent, the retention time was 5.887; 12.010 and 10.283 minutes which respectively indicate the presence of glucose with a concentration of 156.510 mg/L; lactose 848.54 mg/L and maltose 1242.60 mg/L. The difference in retention time is due to differences in the molecular weight of the compounds in the sample. The retention time that is close between sample components with the standard indicates the components contained in the sample are the same as the standard. The molecular weights of the smallest to the largest are glucose, maltose, and lactose, respectively. Glucose ($C_6H_{12}O_6$) has a molecular weight of 180.116 g/mol, maltose and lactose ($C_{12}H_{22}O_{11}$) of 342.31 g/mol. Based on these differences, glucose retention time is faster and lactose retention time is longer.[9]

The results of quantitative analysis on cashew pseudo fruit samples hydrolyzed using sulphuric acid solvent obtained using the LC-MS (liquid chromatography-mass spectrometry) method showed that cashew pseudo fruit samples with sulphuric acid solvent only contained glucose with levels of 1565.10 mg/L.

Testing the ability of lactic acid bacteria to ferment oligosaccharides was carried out to determine whether the hydrolyzed oligosaccharide could be fermented by lactic acid bacteria (*Lactobacillus casei*), namely by modifying the microbial growth medium, where the commercial media containing glucose was replaced with oligosaccharide sugar from the hydrolysis of cashew pseudo fruit. The test results using oligosaccharide sugar as a substitute for glucose showed positive results. The test results can be seen in Table 4.

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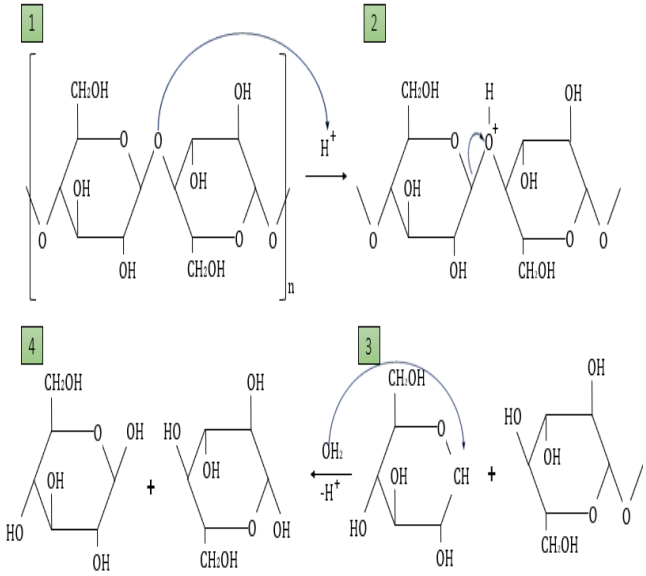

Figure 2: Polysaccharide Hydrolysis Mechanism

Table 2: Identification results with TLC of citric and sulfuric acid solvents

Sample	Spot	R*f*
Comparison	Glucose	0.5
	Lactose	0.38
	Raffinosa	0.7
	Maltose	0.4
Sample (Citric Acid Solvent)	Sample	0.38 – 0.5
Comparison	Glucose	0.5
	Lactose	0.41
	Raffinosa	0,81
	Maltose	0.43
Sample (Sulfuric Acid Solvent)	Sample	0.43 – 0.5

The results showed *Lactobacillus casei* bacteria could grow in media containing oligosaccharides, the highest was achieved at 24 hours of incubation period, namely, $1.4x10^6$ CFU/mL in citric acid solvent and $1.5x10^6$ in the sulphuric acid solvent, while in control media 1,18x0.17 log CFU/mL. Bacterial growth refers more to the increase in the number of cells, not to the development of individual cell organisms. Bacteria have the ability to multiply exponentially because their reproductive system is transverse binary fission, in which each cell divides into two cells. The time it takes for a cell to divide is called generation time. Each species of bacteria has a different generation time. If a single bacterium (such *as Bifidobacterium bifidum*) is inoculated in a medium and multiplies at a constant rate, then at a time, its growth will stop because the nutritional support in the environment is no longer sufficient, so that eventually the number of cells decreased because the many cells were no longer getting nutrition. The *in vitro* test results showed that the purified oligosaccharide sugar from the hydrolyzed extract of cashew pseudo fruit can be fermented and can support the growth of the tested lactic acid bacteria. Family of LAB are grame-positive bacteria that ferment carbohydrate into energy and lactic acid. In the processed fermented food, the number of probiotic bacteria is 10^6-10^7 CFU/mL daily food consumption in order to get the medicinal benefit.[19]

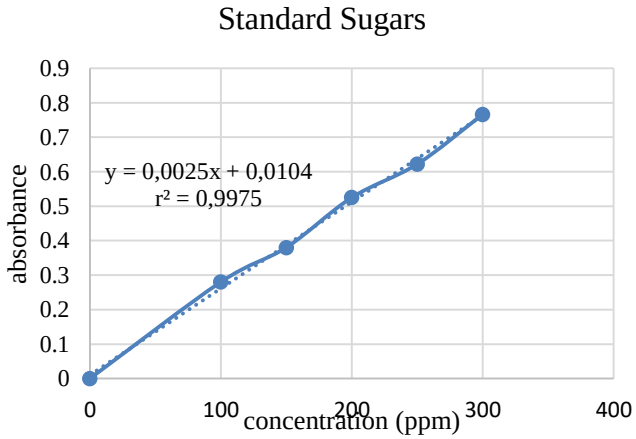

Figure 3: Sugar Standard Curve

Table 3: Results of the Analysis of Reducing and Total Sugars

No	Sample	Parameter	Absorbance	Concentration (ppm)	Concentration (%)	Average± SD	DP
1	Citric acid solvent	Total Sugars	0.477	2297	0.23	0.23 ± 0.000	1.64
			0.471	2265	0.23		
		Reducing Sugars	0.360	1388	0.14	0.14 ± 0,000	
			0.362	1387	0.14		
2	Sulfuric acid solvent	Total Sugars	0.674	3287	0.33	0.32 ± 0.007	2.13
			0.673	3177	0.32		
		Reducing Sugars	0.383	1471	0.15	0.15 ± 0.000	
			0.383	1483	0.15		

Values of concentration are expressed as mean ± standard deviation (SD) (n =2)

Table 4: *In vitro* oligosaccharide testing

No	Sample	Parameter (CFU/mL)	Result (CFU/mL)	Average
1	Cashew, citric acid	Total Plate Number	$1.6x10^6$	$1.4x10^6$
	solvent		$1.2x10^6$	
2	Cashew nut sulfuric	Total Plate Number	$1.2x10^6$	$1.5x10^6$
	acid solvent		$1.7x10^6$	

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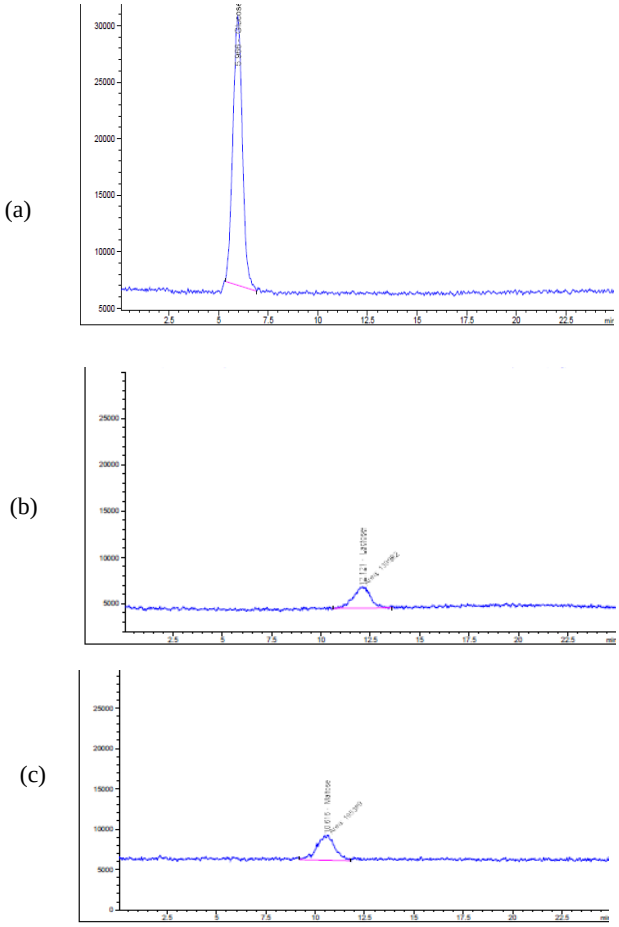

Figure 4: Chromatogram Results Standard Standard Solution (a) Glucose; (b) Lactose; (c) Maltose

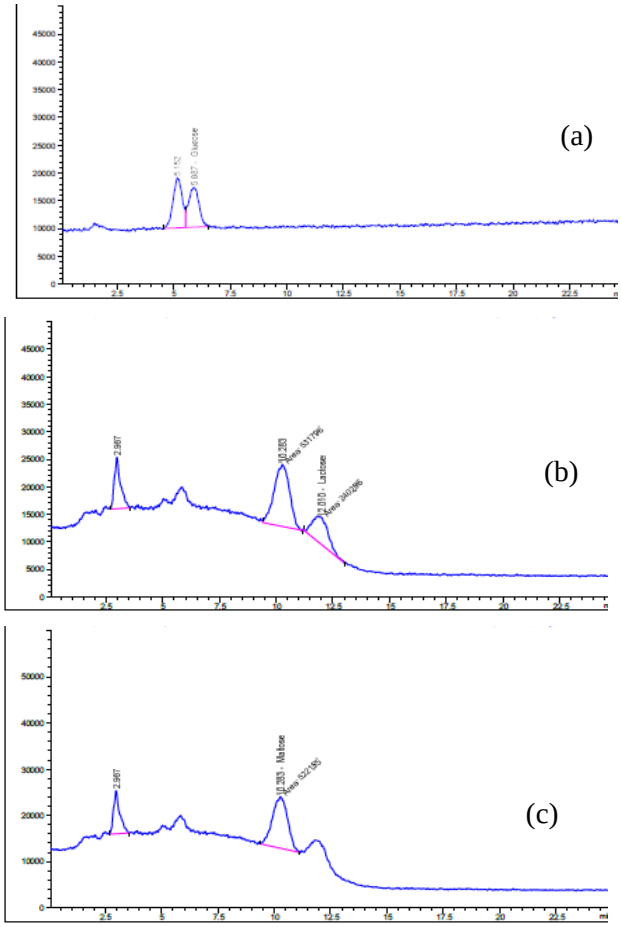

Figure 5: Results of Sample Chromatogram with Citric Acid Solvent (a) Glucose 100x Magnification; (b) Lactose 10x; (c) Maltose 10x

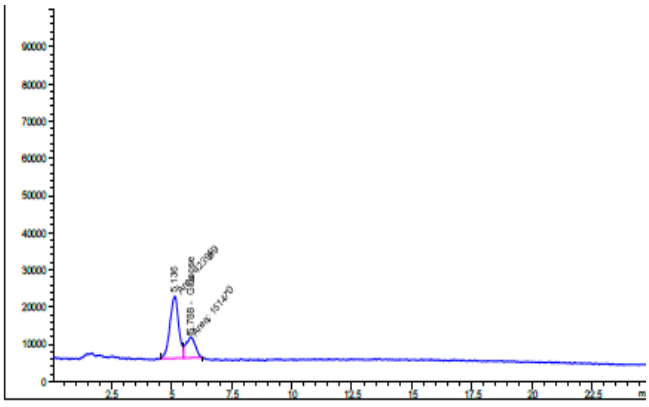

Figure 6: Results of Sample Chromatogram with Sulphuric Acid Solvent

Conclusion

The hydrolysis of cashew pseudo fruit waste using citric and sulfuric acid can convert polysaccharides into oligosaccharides. The best hydrolysis is obtained by using sulfuric acid solvent with the resulting oligosaccharides in the form of lactose (848.4 mg/L) and maltose (1242.60 mg/L) the amount of LAB as $1.5x10^6$ CFU/mL. Therefore, the cashew pseudo fruit has the potential to be used as a source of prebiotics that can support the growth of probiotic bacteria so that it can increase its economic value.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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