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Research article

Optimization of Hydrolysis for Reduced Sugar Determination from Avocado Seed Wastes

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Abstract

Several measurement methods currently employed in the determination of total reduced sugar content. In this study, to determine the total reduced sugar content, optimum conditions for acid hydrolysis, hydrolysis time, and hydrolysis temperature were investigated. The result showed that the maximum sugar content of 60.488±1.328 and 53.309±0.701%(w/w) was achieved using phenol-sulfuric acid and Fehling method, respectively, from hydrolysis of 10 % biomass concentration at 2 % acid concentration, 30^{o}C hydrolysis temperature, and 24 hrs of hydrolysis time, and a minimum yields of 31.658±0.376 and 27.903±0.664 %w/w sugar content at 24 hrs hydrolysis time, 25^{o}C hydrolysis temperature, and 0% acid concentration using phenol-sulfuric acid and Fehling method, respectively.

Keywords: Acid Hydrolysis, Avocado seed wastes, Fehling's method, Phenol-Sulphuric acid method, Reduced Sugar

Introduction

Several physiological studies of growth and preserve allocation in plants need the separate measurements of sugars and starch in tissues. In general, sugars that are soluble in water extracted from the tissue sample and starch content

is determined in the leftovers. Plant extracts include various mixtures of sugars. The presences of glucose, fructose, galactose, sucrose, maltose, melibiose, raffinose and stachyose have been reported from tissues of beech, aspen, poplar and birch. Though these sugars can be individually determined by high-performance liquid chromatography (HPLC) and gas chromatography (GC), the process is costly especially when only the total amount of glucose equivalents is of interest (which is the case of the this study) as cited by Chow and Landhäusser (2003).

Determination of carbohydrate concentration in aqueous solutions is very vital component of numerous areas of environmental research as well as industrial uses in the pharmaceutical, petroleum, and food industries. The broad variety of carbohydrates involved in these areas has put pressure to the development of several analytical techniques for determining carbohydrate concentrations including, capillary electrophoresis infrared (IR) spectroscopy, light scattering detection and Nuclear Magnetic Resonance (NMR) spectroscopy as referenced by Albalasmeh et al, (2013).

The different methods for the determination of carbohydrates in general, need considerable financial investment, advanced analytical skills, and time. One of the most all-around, relatively simple and low-cost for sugar concentration estimation is the calorimetric method. The principle of this method is that carbohydrates, when dehydrated by reaction with concentrated sulfuric acid, generate furfural derivatives. Extra reaction between furfural derivatives and phenol creates detectible color in the visible range of the electromagnetic spectrum. Reagents commonly used for color development include phenol (C_6H_5OH) (Dubois et al, 1956), alkaline ferricyanide ($2K_4Fe(CN)_6$) (Englis & Becker, 1943), and anthrone ($C_{14}H_{10}O$) (Dreywood, 1946).

Among the colorimetric methods for carbohydrate analysis, the Phenol–Sulfuric Acid method is the easiest, most common and reliable method and has been extensively used in a broad range of fields (DuBois et al., 1956). The Phenol–Sulfuric Acid method depends on dehydration of hydrolyzed saccharides to furfural derivatives during reaction with concentrated sulfuric acid (Chow and Landhäusser, 2003; Asghari & Yoshida, 2006; Dubois et al., 1956; Albalasmeh et al, 2013). Besides, the carbohydrate concentration can be determined using Fehling's method (Browne and Zerban, 1941).

Acids can be used as catalysts for hydrolysis of sugars from sugar containing feedstocks such as sugarcane bagasse. Because these acids can break down heterocyclic ether bonds between sugar monomers in the polymeric chains, which are produced by hemicellulose and cellulose (Aguilar et al., 2002; Laopaiboon et al, 2010). H_2SO_4 (Sun and Cheng, 2002; Kumar et al., 2009) is the potential acid which can be used to hydrolyze avocado seed wastes.

Kahn (1987) stated that avocado seeds have around 30% starch content, and hence it serves as a potential starch source. He also mentioned that the microscopic evaluation of this starch showed the presence of characteristics similar to those of corn. These seeds have some anti-nutritional properties like hydrocyanic acid, cyanogenic glycosides, condensed polyphenols and some tannins, which could act negatively on their possible use. However,

the great majority of those substances are thermolabile; as a result, an appropriate heat treatment (cooking) would demolish them (Kahn, 1987**)**.

This study is series, and aims to determine the optimum hydrolysis of avocado seed wastes to obtain the hydrolysate containing high reduced sugar through Phenol-sulfuric acid and Fehling method, and conserved for a continues study on bioethanol production from the current hydrolysates.

Experimental Part

Materials and Chemicals

Drying oven (GALLENKAMP), electrical grinder (ZAIBA super blender), Electrical Balance (ae ADAM, PW 124), UV-Vis spectrometer (NV203 spectrophotometer), digital pH meter (pH meter 3310, JENWAY), methylene blue indicator, Fehling A (prepared by dissolving 34.6 g of copper (II) sulfate pentahydrate in 500 mL), Fehling B (prepared by dissolving 125 g of potassium hydroxide and 173 g of potassium sodium tartrate tetrahydrate in 500 mL of distilled water), sulphuric acid, D (+) – glucose (PANREAC, MONTPLET & ESTEBAN SA, Barcelona. Madrid), and calcium hydroxide are the major materials and chemicals used in this study.

Sample Collection

Avocado (*Persea Americana)* seeds were collected from the juice houses found in Bahir Dar city, Ethiopia. The seeds were first washed with tap water in order to remove the dirty particles from the cover, and cracked to obtain the kernels. Then, the kernels were cut using chopper to reduce the size of the sample and allowed to dry through direct sunlight for few days. The dried samples were ground with grinder machine to reduce the particle size to 2 mm.

Hydrolysis

The methods used for bioethanol production includes hydrolysis, fermentation and distillation process. Different quantities of the substrates were weighed and placed in triplicate conical flasks. 10 g of avocado seed powder was hydrolyzed with different concentrations of sulphuric acid (0- 4) % at different temperatures (25-40)^{o}C and different hydrolysis time (12, 24, 36, 48, 72, and 96) hrs for optimization. The mixture was added in glass bottles and sealed to prevent contamination and vaporization of acid due to heat. After hydrolysis the liquid fraction of the hydrolysate samples were filtered, collected, and their sugar content was determined as follows.

Analytical Methods

The sugar content was determined through phenol-sulfuric acid method (Albalasmeh et al, 2013; Coates and Meyers, 2000) by taking anhydrous D (+) – Glucose as standard (PANREAC, MONTPLET & ESTEBAN SA,

Barcelona. Madrid), and Fehling method (Muche and Sahu, 2014; Periyasamy et al, 2009; Sahu, 2014) as described below.

Fehling method

50 mL of hydrolyzed filtered sample solution was neutralized with 4 M NaOH and 2.5 M HCl and the solution was made up to a volume of 300 mL and taken into the burette. Then, 5 mL of Fehling A (prepared by dissolving 34.6 g of copper (II) sulfate pentahydrate in 500 mL) and 5 mL of Fehling B solution's (prepared by dissolving 125 g of potassium hydroxide and 173 g of potassium sodium tartrate tetrahydrate in 500 mL of distilled water) were taken and mixed with 90 mL of distilled water in 250 mL Erlenmeyer flask and methylene blue indicator was added. The solution in the flask was titrated with solution in the burette in boiling conditions until disappearance of blue color and the volume at which brick red color observed were recorded (Figure 1). For each sample the sugar content was calculated by using Eqn. (1) (Muche and Sahu, 2014; Periyasamy et al, 2009; Sahu, 2014).

$$\text{Sugar content (\%)} = \frac{300mL.f}{V} \text{ x } 100 \qquad (1)$$

Where: f = 0.051 (Fehling factor), V is volume used in the titration (titrate value) (mL) and mL is milliliter

Phenol-sulfuric acid method

The total sugar concentration was determined by using UV-visible spectrophotometer (NV203 Spectrophotometer) at 540 nm wavelength of glucose absorbance and the quantification was made from calibration curve using glucose as standard (Figure 2) and calculation was performed by equation of the linear regression obtained from calibration curve. The standard procedure of this method is as follows. A 2 mL aliquot of a sample solution was mixed with 0.4 mL of 5% aqueous solution of phenol in a test tube. Subsequently, 2 mL of concentrated sulfuric acid was added rapidly to the mixture. The test tubes were allowed to keep for 10 min at room temperature, and placed in a water bath for 20 min for color development. Then, light absorption at 540 nm was recorded on a spectrophotometer. Blank solutions were prepared in the same way as above, except that the 2 mL aliquot of a sample solution was replaced by distilled water (Albalasmeh et al, 2013).

Standard and Reagent Solution Preparation

Stock glucose solution was made by dissolving 3.6 g of glucose in 100 mL of distilled water. Various dilutions of the stock glucose solutions were made separately by pipetting a known volume of the stock solution (1, 2, 3, 4 and 5 mL) into a 100 mL volumetric flask and filling the volume with distilled water up to the mark. The concentrations made for this study were: 0.036, 0.072, 0.108, 0.144 and 0.181 g/mL.

To determine the calibration curve for standard glucose, 2 mL of each of the standard solutions were pipetted out and taken into a separate test tube. Then 0.4 mL of 5% aqueous solution of phenol reagent and 2 mL of 96% sulfuric acid were added. The mixture was kept for 10 min at room temperature, and placed in a water bath for 20 min. Then

the absorbance was read at 540 nm using UV-visible spectrophotometer (NV203 Spectrophotometer). Blank solutions were prepared in the same way as above, except that the 2 mL of the standard solution was replaced by distilled water. Then, the amount of total reduced sugar content present in the sample solution was calculated using the standard graph and expressed as gram glucose equivalents (GE) per 10 g of sample (Albalasmeh et al, 2013; Miliauskas et al, 2004).

Data Analysis

An OriginPro8 software and Microsoft excel 2007 were used to determine the effect of operating variables of the hydrolysis for total sugar content determination from avocado seed wastes.

Results and Discussion

In this study, the total reduced sugar content through hydrolysis process was investigated. The powdered avocado seed through hydrolysis at different acid concentration, hydrolysis time, and hydrolysis temperature on the amount of sugar produced was investigated, and the results are shown below.

Effect of Different Parameters for Hydrolysis

Total sugar content of avocado seed sample was determined by using phenol-sulfuric acid and Fehling's method.

From Fehling method, when the solution in the flask was titrated with the sample solution in the burette under boiling conditions, the blue color was disappeared and brick red color was formed. Because, the reduced monosaccharide's were oxidized by the copper ion in solution to form a carboxylic acid and a reddish precipitate of copper (I) oxide appears within a short period of time (Figure 1).

Figure 1: Experimental observation of Fehling method for total sugar determination (a) before titration, (b) at end point (brick red color), and formation of red precipitate due to Cu^{+1} formation

From phenol-sulfuric acid method, the carbohydrates in the sample, when dehydrated by reaction with concentrated sulfuric acid, they produce furfural derivatives. Further reaction between furfural derivatives and phenol develops detectible color (Figure 3). The glucose equivalent (GE) was calculated from the calibration curve of glucose

standards. The concentrations of unknown sugar samples were determined from a standard curve of glucose (y = 0.05797x + 0.00807; R^2 = 0.996) (Figure 2).

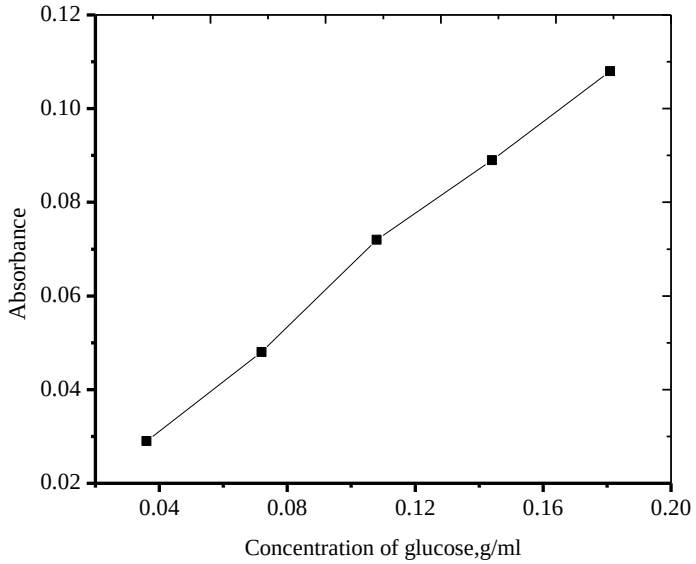

Figure 2: Calibration curve of glucose standard for determination of total sugar content

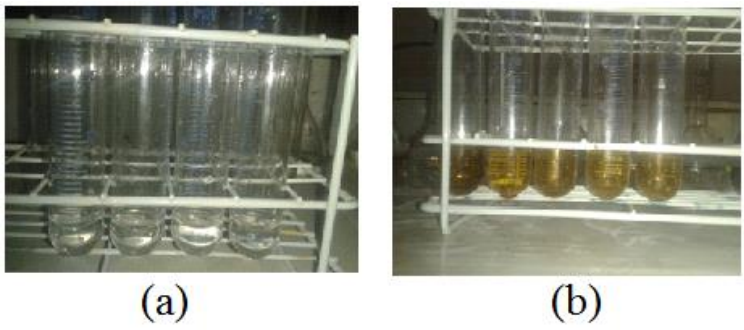

Figure 3: Experimental observation of glucose standard (a) before addition of sulphuric acid nad phenol (b) after addition of sulphuric acid and phenol for total sugar determination

Effect of Hydrolysis time

The effect of hydrolysis time (12, 24, 36, 48, 72 and 96 hrs) on total reduced sugar yield was investigated under the constant conditions of temperature (25 ^{o}C) and acid concentration (2 % sulfuric) (Table 1).

Table 1: Amount of sugar content (% w/w) using the two methods at different hydrolysis time of 10% biomass concentration, 2 % H_2SO_4 and room temperature (25^oC).

Time (hours)	Amount of sugar content (% w/w)	
	Phenol-sulfuric acid method	Fehling method
12	39.403±0.664	38.754±0.427
24	54.003±1.757	48.61±1.506
36	39.787±0.664	37.036±1.051
48	36.337±0.664	35.863±0.489
72	33.837±0.625	33.025±0.409
96	31.353±0.664	33.025±0.409

As shown in Table 1**,** hydrolysis time significantly increased the reduced sugar concentration from 12 hrs to 24 hrs and then started to turn down after 24 hrs hydrolysis. Table 1 showed that at 12, 24, 36, 48, 72 and 96 hrs hydrolysis of avocado seed powder, 39.403±0.664 and 38.754±0.427, 54.003±1.757 and 48.61±1.506, 39.787±0.664 and 37.036±1.051, 36.337±0.664 and 35.863±0.489, 33.837±0.625 and 33.025±0.409, 31.353±0.664 and 33.025±0.409% of sugar content were obtained by phenol-sulfuric acid and Fehling method, respectively. The highest reduced sugar content, 54.003±1.757 and 48.61±1.506% was achieved at 24 hrs hydrolysis time for both phenol-sulfuric acid and Fehling methods, respectively. However, as hydrolysis time goes beyond 24 hrs it resulted in decreasing reduced sugar content. This may be due to the longer residence time, which makes the sugars degraded to form inhibitors (furfural and HMF) (Nutawan et al, 2010).

Effect of Acid Concentration on Hydrolysis

The effect of acid concentration on the amount of total reduced sugar from Avocado seeds was studied by using different sulfuric acid concentrations (0, 1, 2, 3, and 4) % v/v. From Table 2, the greatest reduced sugar content of 54.003±1.757 and 48.61±1.506% by phenol-sulfuric acid and Fehling method, respectively, was produced using 2 % acid concentration and 25^oC of avocado seed samples followed by 38.253±1.757 and 36.471±1.541 % by phenol-sulfuric acid and Fehling method at 3% sulfuric acid, respectively. However, minimum reduced sugar content of 31.658±0.376 and 27.903±0.664 by phenol-sulfuric acid and Fehling method, respectively, was achieved at 0 % acid concentration and 25^oC.

Table 2: Amount of sugar content (% w/w) using the two methods at different hydrolysis of acid concentration of 10% biomass concentration, room temperature and 24 hrs hydrolysis time.

Acid Concentration (%)	Amount of sugar content (% w/w)	
	Phenol-sulfuric acid method	Fehling method
0	31.658±0.376	27.903±0.664
1	38.253±1.328	34.042±0.466
2	54.003±1.757	48.61±1.506
3	38.253±1.757	36.471±1.541
4	37.487±0.664	32.101±0.391

The decrement of reduced sugar content with increasing of acid concentration from 2-4 % may be due to decomposition of the sugars and the formation of some inhibitor such as Furfural and 5-Methylhydroxy furfural. These substances are toxic substances for yeast and can inhibit the yeast growth (Nutawan et al, 2010).

When the acid concentration is higher than 6%, the lower glucose concentration and an increase in inhibitor was observed. When acid concentration was raised to 10 %, the concentration of HMF and furfural became higher (Fadel, 2000). In this study the maximum reduced sugar from powdered avocado seeds was achieved at 2 % sulfuric acid, which is an optimum condition in acid concentration.

Effect of temperature on hydrolysis

Temperature is one of the principal factors on determination of reduced sugar, because temperature exerts an insightful effect on conversion of cellulose or hemicellulose to simple sugars. To optimize the temperature for sugar production, the hydrolysis media were kept at 25, 30, 35, and 40^{o}C (Table 3).

Table 3: Amount of sugar content (% w/w) using the two methods at different hydrolysis temperature of 10% biomass concentration, 2 % H_2SO_4 and 24 hrs hydrolysis time.

Temperature (^{o}C)	Amount of sugar content (% w/w)	
	Phenol-sulfuric acid method	Fehling method
25	54.003±1.757	48.61±1.506
30	60.488±1.328	53.309±0.701
32	49.370±1.150	41.754±1.292
35	43.237±1.757	36.146±0.489
40	38.253±1.328	34.258±0.446

As the temperature increases from 25^{o}C to 30^{o}C the reduced sugar also increased. The maximum reduced sugar was achieved at 30^{o}C with 60.488±1.328 and 53.309±0.701 % by phenol-sulfuric acid and Fehling method, respectively. However, when the temperature lowers (25^{o}C), a metabolic function was suppressed and the rate of conversion of substrate into reduced sugar slows down. On the other hand, when the temperature further increases beyond 30 ^{o}C the sugar content was decreased significantly. This is due to degradation of sugar in to unwanted materials like 5-HMS and Furfural that are toxic for S. cereviciae in fermentation (Nutawan et al, 2010; Sahu, 2014; Tsigie et al, 2013). Besides, the minimum sugar yield was obtained at 40^{o}C with 38.253±1.328 and 34.258±0.446 % by phenol-sulfuric acid and Fehling method, respectively. This is due to the aforementioned problems. Generally, powdered avocado seeds has a maximum reduced sugar of 60.488±1.328 which is in a good agreement with previously reported works such as Olive-tree biomass (Kumar et al, 2009), Sweet potato (Kumar et al, 2014), etc having 83%, and 78.19% total reducing sugar, respectively.

Conclusion

Determination of total reduced sugar from avocado seed wastes and optimization of different factors in the hydrolysis were studied. The optimum conditions in producing reduced sugars from powdered avocado seed wastes were 2 % hydrolysis acid concentration, 24 hrs hydrolysis time, and 30^{o}C hydrolysis temperature. Comparable results have been achieved using both phenol-sulfuric acid and Fehling method.

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