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Research Article

Antibacterial Properties of Purified Sago Frond Sugar Against Food-Borne Associated Disease Bacteria

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ABSTRACT

Sago palm is recognised as key to sustainable food security due to its advantages resilient against extreme conditions such as wildfire and flood associated with adaptability to climate change. Sago palm is also known to remain solid after being attacked by pests and infected by the disease. Unfortunately, for the last ten years, the Sago palm industry experiences a significant decrease in plantation area and productivity. The long maturation period is identified to be the major factor that is responsible towards the respected issue. Thus, alternative commodities from the growing sago palm must be explored to offer a better perspective on the sago industry. Sago frond (SF) was utilised into Sago Frond Sugar (SFS) via enzymatic hydrolysis using cellulase enzyme containing cellobiose and glucose as main sugar at 9-10 g/L and 5-6 g/L concentration respectively. SFS was purified (PSFS) using Powdered Activated Charcoal (PAC) to remove the impurities. Antibacterial analysis shows that PSFS able to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi* at 23.5 mm, 22.5mm and 13.25 mm clearing zone respectively. However, the growth of Listeria monocytogenes seems unaffected by the presence of PSFS. Promoting the versatility of sago frond as raw material to synthesise high-value products such as SFS will extend the potential of the sago palm to be recognised as an important crop to ensure global food security and safety.

Key words: Cellobiose, enzymatic hydrolysis, food-borne disease, powdered activated charcoal

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INTRODUCTION

About 2.2 million people were estimated to be killed by diarrhoea in 1998. Most of them are kids under 5 years old. Approximately 4 billion cases of diarrhoea worldwide are reported every year with developing and poor countries being the most affected. Diarrhoea is commonly caused by the consumption of food that has been contaminated by food-borne associated viruses, bacteria and parasitic. *Escherichia coli* (*E. coli*) and *Salmonella* sp. are the most common and widely distributed food borne diseases associated bacteria that can cause diarrhoea. *E. coli* usually can be found in meat, vegetables and fruits while *Salmonella sp.* usually can be found in meat and vegetable that have been contaminated with animal manure. Both bacteria can cause infection due to improper management of food handling, storage and cooking style especially undercooked meat (WHO, 2001).

Antimicrobial agents are widely used as antibiotics in humans and animals. Antibiotics have been used in animals not only as an antimicrobial agent but also as a growth promoter and improvement in growth performance. The use of such antimicrobial agents has emerged as livestock systems intensified around the world to encounter a growing demand for meat, milk and eggs. Antibiotics are used normally in animal feed at a rate of 2 to 50 g/ton. The benefit of antibiotic use in animal feed is increasing the digestion efficiency and growth rate, treating clinically sick animals and preventing the infection of diseases (University of Delaware, 2016).

To increase the effectiveness of the antimicrobial agents and reduce production costs, artificial methods in the production of antimicrobial agents are introduced. Production of antimicrobial agents would be produced from non-natural compounds that are chemically foreign to the environment such as xenobiotics. Advances in synthetic chemistry allow the production of antimicrobial agents faster and cheaper. Rather than being limited to “one vessel, one reaction” as in most serial synthetic chemistry, combinatorial chemistry is characterized by exponential increases in the number of compounds

that can be synthesized in each reiterative cycle of reactions. Advances in assay automation, robotics, imaging, and miniaturization are transforming how active molecules are discovered. Assimilation of advances in high-throughput screening with combinatorial chemistry offers opportunities for formulating new patterns for antibiotic development during the next decade. Huge multimillion-member libraries of compounds can be generated without the costs of traditional synthetic chemistry (Cassell & Mekalanos, 2001).

The concentrated sugar solution is usually used to preserve food products by creating osmotic pressure to prevent the manifestation of microorganisms. However, excessive sugar consumption can affect the nutritional benefit of the food and is commonly associated with obesity, diabetes and heart disease. Thus, non-fermentable sugar was proposed as an alternative to preserving food considering the similar capability to manipulate osmotic pressure as fermentable sugar (Kunz *et al*., 2012).

Cellobiose is a disaccharide that is categorised as non-fermentable sugar due to the strong β(1,4)–glycosidic bond between two molecules of glucose. The β-glucosidase enzyme is required to be hydrolysed into glucose. Due to the lack of β-glucosidase enzyme in the human digestive system, cellobiose considers a non-caloric sugar since it does not act as a carbon source for human cells. However, cellobiose exhibits prebiotic properties to human gut microflora such as lactic acid bacteria by their capability to metabolise cellobiose as a carbon source and without competition from human cells. The application of cellobiose as a carbon source significantly promotes *Lactococcus lactis* IO-1 growth performance and improves the viability of the cell (Ahmad *et al*., 2020). Cellobiose can be produced via enzymatic hydrolysis of the cellulosic component. The cellulase enzyme produced by *Trichoderma reesei* was identified to exhibit superior production of cellobiose due to a high concentration of endoglucanase and a minimum amount of β-glucosidase (Adeni *et al*., 2018).

Production of non-invasive antibacterial compounds is crucial to avoid the potential side effect of the synthetic antibiotic on human health and prevent pathogens from evolving into multidrug-resistant superbugs. Extraction of the active compound with antibacterial properties from medicinal plant material exhibits a magnificent protective effect against pathogenic microorganisms. Implementation of advanced biotechnological methods such as enzyme technology allows researchers to utilise biomass as a high-value product. According to Ren *et al*. (2012), the enzymatic hydrolysis technique was used to produce antibacterial agents from the bones of channel catfish. The enzymatic hydrolysis method produces fewer undesired by-products compared to chemical synthesis (Wyman *et al*., 2005).

The foundation of the research is based on a preliminary study on the inhibitory effect of Brown Sago Sugar (sugar that produces from enzymatic hydrolysis of sago starch) exhibits bacteriostatic activity towards pathogenic bacteria at 40% (w/v) concentration. Brown sago sugar manages to inhibit further growth of *E. coli, S. typhi, S. aureus and L. monocytogens* simultaneously (Sikem, 2017). Hence, a study on the antibacterial properties of sago frond sugar against food-borne disease bacteria was conducted to observe the potential of the respected sugar as a non-caloric bacteriostatic compound.

Therefore, the production of antibacterial compounds from agricultural biomass such as sago fronds associated to the enzymatic hydrolysis technique can produce effective food grade antibiotics as well as preservatives ingredient.

MATERIALS AND METHODS

Materials

Sago Frond (SF)

Sago frond leaves were removed and the fronds were cut into shorter sticks about 1-metre length. The SFs were deskinned to obtain the soft white pith which was further cut into smaller pieces about 2 cm^3 cubes to enhance the drying of the pith and later ease blending. The small cubes were dried at 105 °C for 24-30 h. Dried SF cubes were ground to produce Sago Frond Powder (SFP).

Sago Frond Sugar (SFS)

Sago Frond Sugar was produced through the enzymatic hydrolysis of sago frond. 6% (w/v) of SFP was used as substrate. About 10% (v/w) of the enzyme Celluclast (Novozyme, Denmark) was used to produce SFS from the frond. The hydrolysis was conducted at 45 °C for 48 h.

Powdered Activated Charcoal (PAC)

PAC used for the purification process was supplied by PhytoTechnology Laboratories.

Indicator strains

In this experiment, the antibacterial activity of SFS was evaluated using four types of indicator strains obtained from ATCC as below;

Escherichia coli
Salmonella typhi
Listeria monocytogenes
Staphylococcus aureus

Method

Purification of Sago Frond Sugar

SFS obtained from the enzymatic hydrolysis was purified using Powdered Activated Charcoal (PAC). Exactly 5 g of PAC was added into 100 mL SFS solution. The mixture was stirred for 5 minutes and then centrifuged at 10,000 rpm for 10 min at 4 °C. Purified SFS were concentrated to 50% of the initial volume.

Sugar analysis

The components of sugar in SFS were determined by using High-Performance Liquid Chromatography HPLC (Shimadzu, Japan) from the enzymatic hydrolysis of sago frond and determined the level of purity of cellobiose produced. Refractive Index (RID-10A) detector with a Biorad Fermentation Monitoring column and 0.005 M H_2SO_4 as mobile phase at 0.8 mL/min flow rate for glucose, ethanol and lactic acid determination and 1.0 mL/min for cellobiose determination at 60 °C column temperature. Retention times of cellobiose, glucose, lactic acid and ethanol were 3.1 min, 3.8 min, 5.5 min and 8.4 min respectively.

Disk diffusion method

In this research, a modified (Leclercq *et al*., 2013) disk diffusion method was used. In the disk diffusion method, disks were made up of Whatman filter paper and immersed in the concentrated Purified Sago Frond Sugar (PSFS) (20 g/L of cellobiose). The sterile cotton swab was dipped into the stock culture of the indicator strains. The inoculum was spread evenly over the whole surface of the Muller Hinton Agar (MHA) by swabbing in 2 directions. Then, sterile disks were immersed in the stock solution of cellobiose and laid on top of the agar. Inoculated agar plates were incubated for 24 h at 37 °C.

RESULTS AND DISCUSSION

Purification of sago frond sugar

Sago frond sugar was obtained from enzymatic hydrolysis of sago rachis by using the Celluclast enzyme. Purification was conducted to remove impurities from the sago frond sugar that may interfere with the efficiency of the sugar component in SFS to inhibit the growth of the selected pathogenic microorganism indicators. Table 1 shows the effect of purification on SFS.

Table 1. Effect of SFS purification

Sample	Unpurified SFS	Purified SFS
Colour		
Sugar Content (g/L)	16.65±0.18	14.73±0.12
Phenolic (g/L)	1.86±0.018	0.034±0.001
Flavonoid (g/L)	0.423±0.0031	0.018±0.001

Statistical analysis was determined using One ANOVA and Tukey tests. Each value is the mean ± SE of 3 replicates. Means with the same letter are not significant at $p<0.05$.

Based on the result, concentrated SFS have reduced the efficiency of the purification process. The concentrated SFS by evaporating 50% from the initial volume still have light brown colour after purification. Hence, concentration is only conducted after the purification process. The colour indicated the residual targeted compound that cannot be absorbed by the PAC due to the increase of the compound concentration in the sample. This was supported by (Tchobanoglous *et al*., 1991) concentration of the compound influenced the performance of the activated carbon. The higher concentration of the compound, the higher the consumption of the carbon. Hence, more PAC is required to purify high concentration of SFS.

Based on the table above, SFS contain a higher amount (51.67%) of phenolic compound compared to purified SFS (0.94%) from the amount of lignin. SFS and PSFS contain 0.423 g/L and 0.034 g/L, respectively. This shows that purification of SFS using powdered activated charcoal (PAC) reduced the amount of phenolic compound. About 98.17% phenolic compound was washed out due to the purification of SFS.

According to Dąbrowski *et al*., (2004) activated carbon is widely used for the absorption of phenolic compounds in the water treatment process. The ability of carbon to absorb oxygen greater than the absorption of other species. Absorption of oxygen by the surface of activated carbon lead to the formation of a carbon-oxygen functional group such as the carbonyl group. The presence of the carbonyl group at the surface of activated carbon promotes the adsorption of phenols by involving the formation of an electron donor–acceptor complex of the aromatic ring carbonyl group at the surface of the activated charcoal. Thus, enhance the absorption of the phenolic compound during the purification of the SFS. Therefore, most of the phenolic compounds absorbed by activated charcoal are stranded at the bottom of the centrifuge bottle after the centrifugation during the purification process.

The result shows that the flavonoid compound of Purified SFS slightly decreases from SFS due to the purification process. The recovery of flavonoid compound of SFS from lignin is 51.67% and the recovery of residual flavonoid compound in purified SFS is 0.97% from the total flavonoid content of SFS after purification. Thus, flavonoid compounds were absorbed during the purification process of the sago frond sugar. 95.74% of the

flavonoid compound was washed out due to the purification of SFS.

According to Vihakas and Salminen (2014), the flavonoid compound was differentiated into several subclasses according to the chemical differences in their ring C. The subclass has a double bond between C2-C3, a hydroxyl group at C3 or a carbonyl group at C4. Therefore, the formation of the carbonyl group allowed the flavonoid to be absorbed by active carbon which is known as the neo-flavonoid subclass of flavonoid compound.

However, the subclass of flavonoid compounds that have a double bond between C2-C3 at C3 has a hydroxyl group which is known as iso-flavonoid. According to Dąbrowski *et al*. (2004), the hydroxyl and carboxyl groups inhibited the adsorption and increased the affinity of the activated carbon towards water. Therefore, the remaining flavonoid compound in the purified sago frond sugar might be the iso-flavonoid subclass.

The ability of the activated charcoal to absorb the phenolic and flavonoid compound will give the advantage to purify the phenolic and flavonoid compound from the sago frond sugar solution. The phenolic and flavonoid compound that has been absorbed by the activated charcoal can be recovered through the desorption process of used activated charcoal. Thus, the purification of sago frond sugar can lead to two different beneficial products which are purified cellobiose for prebiotic and antimicrobial purposes and the phenolic and flavonoid compound can be used for pharmaceutical purposes (Sikem, 2016).

Antibacterial activity of purified sago frond sugar

Experiments were conducted to test for antimicrobial activities of purified SFS (PSFS) at (20 g/L sugar concentration) on four different indicator strains. The purified SFS was concentrated to 50% from the initial to reach 40% (g/L) sugar concentration as suggested by (Sikem, 2017). Table 1 shows the antibacterial activity of purified SFS.

Table 2. Antibacterial activities of Purified Sago Frond Sugar (PSFS)

Indicator Strain	Inhibition Zone (mm)
Staphylococcus aureus	23.500^{a}±0.289
Salmonella typhi	13.250^{b}±0.854
Escherichia coli	22.500^{a}±0.645
Listeria monocytogenes	0.000^{c}±0.000

Statistical analysis was determined using One ANOVA and Tukey tests. Each value is the mean ± SE of 3 replicates. Means with the same letter are not significant at $p<0.05$

It is evident from these results that the two gram-negative bacteria were highly affected by the presence of purified Sago Frond Sugar (PSFS, at 20 g/L sugars initially, 41 g/L after concentration). The clearing or inhibiting zones were largest for *Staphylococcus aureus* and *Escherichia coli* at 23.5 mm and 22.5mm clearing zone, respectively. The clearing zone was slightly less on the gram-positive bacteria, represented by *Salmonella typhi* at 13.25 mm, while the growth of *L. monocytogenes* seems unaffected by the presence of PSFS.

The β-glucosidase enzyme was responsible to metabolise cellobiose into glucose. Without the respected enzyme, cellobiose is unable to be metabolised by the cell due to its complex structure and strong glycosidic bond between two glucose molecules. Before that, the strain must possess a cellobiose protein acceptor to allow cellobiose to be transported into the cell as a carbon source. The capability of the selected strain indicator to produce β-glucosidase enzyme and possessed cellobiose protein acceptor is shown in Table 3.

Table 3. β-glucosidase and PTS gene expression of the strain indicator

Indicator Strain	β-glucosidase	PTS
Staphylococcus aureus ATCC 25923	*bglA* Aryl-phospho-beta-D-glucosidase *bglA*	Non
Salmonella typhi ATCC 14028	*bglA* 6-phospho-beta-glucosidase *bglA*	Non
Escherichia coli ATCC *25922*	2 X *bglA* 6-phospho-beta-glucosidase *bglA*	1 X *celA* PTS system cellobiose-specific EIIB component
Listeria monocytogenes ATCC 19116	*bglA* 6-phospho-beta-glucosidase *bglA*	4 X *celA* PTS system cellobiose-specific EIIB component

(Source: ATCC, 2023)

Complete genome analysis on *S. aureus* and *S. typhi* show the absence of a gene encoding for the formation of a cellobiose protein acceptor in the form of a Phosphotransferase System (PTS). Without PTS, both strains are unable to transport cellobiose into the cell through the proper channel; meanwhile, E. coli only possessed one gene expression site over the whole genome is insufficient to translocate cellobiose efficiently into the cell. Both situations are plausible factors in which excessive concentration of cellobiose triggers glucose repression blocking the cell from consuming glucose and then leading to carbon starvation. A similar situation occurs with a

high concentration of cellobiose during the initial growth stage repressed sporulation and hence the viability of *C. cellulilyticum* as well as low cellobiose concentration that prohibit the induction of proteolytic activities crucial for the sporulation (Gehin *et al*., 1995). The related inhibition mechanism also exhibited by bacteriocin as produced by the lactic acid bacteria inhibits the nutrient uptake by bacteriocin-intolerant strains (Waite & Hutkins, 1998). Excessive concentration of cellobiose may create competitive inhibition with the glucose that prevents glucose uptake leading the cell to starve to death. On the other hand, *L. monocytogenes* possessed four PTS gene expression sites over the whole genome indicating the superiority of the strain to transport cellobiose into the cell system and metabolise it as a carbon source internally. This condition associated with the discovery of *bglA*-gene possessed by *L. monocytogenes* indicates the capability of the respected strain to produce β-glucosidase completely neutralised the inhibition effect by the cellobiose in the PSFS.

All three indicator strains inhibited by PSFS are categorised by food-borne associated disease bacteria. All of these strains can be found in the human digestive system. As such, there is a high possibility that PSFS can be used as an antibacterial agent to cure bacterial infections that can cause food poisoning.

CONCLUSION

In conclusion, purification of SFS by using PAC almost completely washed out phenolic and flavonoid from the stock solution to prevent the active compound from interfering with the evaluation of cellobiose antibacterial properties of PSFS against food-borne associated disease bacteria. Lack of phosphotransferase system in *S. aureus, S. typhi, and E. coli* prevent the strains from properly metabolising cellobiose. Meanwhile, *L. monocytogenes* possessed a significantly effective cellobiose protein acceptor coupled with the synthesis of β-glucosidase enzyme allowing the strain to absolutely neutralise the antibacterial effect of cellobiose from PSFS. Hence, high concentrations of cellobiose potentially elevate osmotic pressure and trigger carbon starvation leading the cells to starve to death. This study will initiate the possible application of non-fermentable sugar and non-caloric sugar as cellobiose as preservative agents in the production of food to offer healthier options to the consumer.

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