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Screening of microorganisms from pineapple waste for fructooligosaccharides production

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Abstract. Fructooligosaccharides (FOS) are one of the well-known low caloric value sweeteners with prebiotic properties that promote positive effects on consumer's health. They are synthetically produced by transfructosylation of sucrose via microbial enzymes which are β -fructofuranosidases (FFase) (EC 3.2.1.26) and fructosyltransferase (FTase) (EC 2.4.1.9). Despite the large number of microbial FTases that are produced, the yield of FOS is low and has poor stability, thus, only a few of them have the potential for industrial application. Research for a new source of microbial enzyme for FOS production becomes necessary due to the high demand for FOS in the pharmaceutical and food industry. Fruit waste such as pineapple waste can be an alternative source of microbial enzyme for FOS production beside can be recycled as FOS substrate. This will reduce the dumping and open burning of these waste which eventually will lead to environmental pollution. This paper presents an experimental study of microbial screening from pineapple waste that can catalyze FOS production. Three different parts of pineapple waste were used in this study which are peels, pulps, and leaves. From screening, all the five isolated bacteria which belong to gram-positive groups did possess both hydrolytic and fructosyltransferase activity with bacteria isolated from leaves showed the highest fructosyltransferase activity which is 0.91 U/ml. Bacterial identification using sequencing of 16S rRNA showed that the isolated bacteria is from the genus *Bacillus sp.*

1. Introduction

Recently, enzyme produced by microorganisms had been used for production of essential foods, such as bread, cheese, alcoholic drink and others. Beside that it has also been used for the production of prebiotic oligosaccharides. Prebiotics are non-digestible food ingredient that beneficially affect the host by promoting the growth and activity of beneficial microorganisms in the colon [1]. Among them, fructooligosaccharides (FOS) had created a great demand globally due to its benefits in disease prevention, growth stimulation of beneficial bifidobacteria in the digestive tracts by inhibiting growth of pathogens, non-carcinogenicity property and many more [2]. FOS also can be used as an alternative sweetener as it is water soluble and one third as sweet as sucrose [3].

FOS are short chain oligomers composed of monosaccharides units which containing kestose (GF2), nystose (GF3) and 1- β -fructofuranosyl nystose (GF4) [4]. Linked by β (2 \rightarrow 1) bonds, fructosyl units (F) with a range of 2 to 60 are often terminated in a glucose (G) unit [3]. FOS can be found in vegetables such as bananas, rye, onion, garlic, asparagus, wheat and tomatoes [5]. The commercial



production of FOS is mainly relying on enzymatic transformation of sucrose by microbial fructosyltransferase (FTase, EC 2.4.1.9) and β -fructofuranosidase (FFase, EC 3.2.1.26) [6]. FTase (EC 2.4.1.9) possess only transfructosylating activity, cleave the -1,2 linkage of sucrose and transfer fructosyl group to an acceptor molecule leading to formation of FOS and release of glucose [7]. While FFase (EC 3.2.1.26) catalyze both hydrolytic and transfructosylating reactions, however, latter is evidenced only with higher sucrose concentrations [8]. Enzymatic reaction by FFase (EC 3.2.1.26) releases a glucose molecule from the sucrose by cleaving the β -1, 2 linkage and transferring the fructosyl group to sucrose and fructooligosaccharides. Thus, fructooligosaccharides are formed containing 1-ketose (GF2), nystose (GF3) and 1- β -fructofuranosyl nystose (GF4) having fructosyl units (F) linked at β (2 \rightarrow 1) position of the sucrose molecule (GF) [9].

Both enzymes (FFase, EC 3.2.1.26) and FTase (EC 2.4.1.9) can be obtained from plants, bacteria or fungi [10]. Synthesis of FOS normally carried out under two-stage processes in which enzyme is produced in the first step and FOS is yielded by using bio-transformation process under controlled conditions. Several microorganism have been reported as potent producers of these FOS producing enzymes, between them fungi are the most reported strains: *Apergillus japonicus* [11], *Aspergillus niger* [12], *Aspergillus oryzae* [13], *Bacillus subtilis* [14], *Bacillus macerans* [11] are the example of bacterial strain that is isolated for FOS production. However, the production of FOS by using enzymes originated from plants is quite low and the mass production of enzymes are affected by seasonal conditions [15].

Exploration and identification of new microorganisms that produce FOS-producing enzymes are still ongoing. To suit industrial conditions, an enzyme with higher activity and stability are preferred. An enzyme with high transfructosylating activity and the product with more oligosaccharides and less of monomeric sugars will be a plus point [16]. Until now, *Aureobasidium*, *Aspergillus* and *Penicillium* are the most studied microorganism for FOS-producing enzymes [3]. Furthermore, until now, there is only limited bacterial strains that had been reported to produce FOS-producing enzymes compared to the fungal strains which has been widely reported and well-studied. Hence, the main objective of this study was to find newstrains having high transfructosylating activity for biotransformation of sucrose to FOS production from pineapple wastes.

On farm waste for fruit plantation contributes significantly to total generation of waste. The increasing production of pineapple processed product due to the higher demand from consumer results in massive waste generation [17]. This is because certain components of pineapple are not suitable for human consumption, therefore, the producers select and eliminate those parts that cannot be consumed. The disposal of fruit processing waste requires a huge capital investment [18]. This results in the dumping and open burning of these fruit processing waste in open field. This will lead to environment pollution. Pineapple wastes can be classified into pineapple on farm wastes (POFW) and pineapple processing waste (PPW). Leaves, stem and roots left in the field after the pineapples are harvested are considered as POWF while PPW is the waste left after the pineapple are processed to one of the commercial products [18]. The pineapple waste composed of carbohydrate with PPW mainly composed of simple sugars which are glucose, fructose and sucrose [19]. Hence, besides being a source for microbial with transfructosylating activity, it has a potential to recycle as FOS substrate too.

2. Materials and methods

2.1 Materials

All the reagents Protease Peptone, Beef extract, Yeast extract, D(+) Glucose, Sodium Acetate, Ammonium Citrate, Dipotassium phosphate, Manganese Sulphate, Magnesium Sulphate, Agar, L-cysteine, Sodium Propionate, and Dipotassium hydrogen phosphate were purchased from Sigma Aldrich.

2.2. Sample collections

Different parts of the pineapple such as leaves, pulp, and crown were chopped and blend into fine particles. 1 g of each pineapple waste samples was taken and diluted with 99 mL of distilled water. It was then followed by a series of 10-fold dilution from 10^{-1} to 10^{-10} .

2.3 Bacteria isolation

For isolation of bacteria, 0.1 mL of each pineapple waste samples from the dilution of 10^{-6} to 10^{-10} were spread on nutrient agar and incubated at 30°C for 1 - 2 days. A streaking method was applied then to isolate the bacteria. Several discrete colonies from each from pineapple waste samples have been choose and then streaked into new nutrient agar and followed by incubation at 30°C for 1 - 2 days. The streaking method has been repeated for three times to get a pure strain of bacteria.

2.4 Gram staining of bacteria

A small loop of bacteria was transferred to the surface of the microscopic slide by using an inoculum loop. There are four types of solutions used in this staining technique which are Gram's crystal violet solution, Gram's iodine solution, Gram's decolorizer solution, and Gram's safranin solution. The microscopic slide was observed under a microscope to identify the gram staining group of each isolated bacteria.

2.5 Crude enzyme preparation

The selected bacteria from gram staining results were cultivated at 37°C overnight in a 250 ml flask containing 100 ml of nutrient broth. The removal of the cells was done by using centrifugation at $10,000 \times g$ for 10 minutes. The supernatant which contained extracellular FOS-producing enzymes were further used for enzymatic assays.

2.6 Screening of microbial with sucrose degrading activity

The supernatant which contained the extracellular FOS-producing enzymes were transferred into a well in the agar plate containing a substrate (sucrose) that consist of a well. They are incubated at 30°C for 1 - 2 days. Staining the agar plates. The agar plate was kept in the dark for 20 minutes and washed with 0.1M of acetate buffer. The appearance of the red zone around the well was confirmed for hydrolytic activity [20].

2.7 Enzymatic assay

2.7.1 Assay of fructosyltransferase activity

The filtrate was taken as a crude enzyme with a 50% sucrose solution as a substrate at pH 5.50 (0.1 M sodium acetate buffer). The mixture was incubated for 2 hours at 50°C . The presence of reducing sugars was estimated by Dinitro-salicylic (DNS) acid reagent. The mixture was transferred into a test tube and kept in a water bath at 100°C for 10 minutes to terminate the enzymatic reaction [20]. The absorbance value for each sample are determined by spectrophotometer (UV-VIS). One unit of sucrose hydrolytic activity was considered as the amount of enzyme required to produce $1 \mu\text{mol}$ of glucose.

2.7.2 Assay of hydrolytic activity

The filtrate was taken as a crude enzyme with a 5% sucrose solution as a substrate at pH 5.50 (0.1 M sodium acetate buffer). The mixture was incubated for 2 hours at 50°C . The presence of reducing sugars was estimated by Dinitro-salicylic (DNS) acid reagent. The mixture was transferred into a test tube and kept in a water bath at 100°C for 10 minutes to terminate the enzymatic reaction [20]. The absorbance value for each sample is determined by spectrophotometer (UV-VIS). One unit of sucrose hydrolytic activity was considered as the amount of enzyme required to produce $1 \mu\text{mol}$ of glucose.

2.8 Identification of the selected isolates using automated microbial identification

The several selected isolated bacteria that able to produce the FOS-producing enzymes were identified for their genus and species by using 16S rRNA sequencing. The phylogenetic tree was then constructed based on the BLAST results of the resulted 16S rRNA sequence.

3. Results and discussion

3.1 Bacteria isolation.

Different parts of the pineapple wastes which are leaves, pulps, and peels have been used for isolation of bacteria. Serial dilution has been carried out and followed by overnight incubation at 30°C. Several discrete colonies were selected to be used for further analysis. Table 1 below shows the selected dilution from different parts of the pineapple waste samples. Three different colours of the colonies were observed which are white, yellow, and pink. All these samples were proceeded for the Gram's staining analysis to identify the Gram's group and the shape of the isolated bacteria.

Table 1. list of selected isolated bacteria samples

Parts	Dilution	Color of colony
Leaves	10 ⁻³	White
	10 ⁻⁵	Yellow
	10 ⁻⁵	White
	10 ⁻⁷	Yellow
	10 ⁻⁷	White
	10 ⁻⁸	Yellow
	10 ⁻⁸	Pink
Peels	10 ⁻¹	White
	10 ⁻³	White
	10 ⁻³	Yellow
Pulps	10 ⁻⁶	White
	10 ⁻¹⁰	White

3.2 Gram staining characterization

Gram staining results for all the selected isolated bacteria is shown in Table 2. From the nine selected isolated bacteria, only two of the isolated bacteria belongs to gram-negative group with a rod shape. While the remaining of six selected isolated bacteria belongs to gram-positive group with a rod shape. It was also observed that one of the isolated bacteria which is from the leaves samples did have a cocci shape with a gram-positive group. An example of both rod and cocci shapes of the selected isolated bacteria is shown in Figure 1(a)-(c).

The difference between gram-positive bacteria and gram-negative bacteria is mainly the thickness of the cell wall. Gram-positive bacteria usually have a thicker cell wall which is around 20 – 25 nm, while gram-negative bacteria are generally thinner which is around 11 – 15 nm. The rod-shaped bacteria are usually from the genus *Bacillus*. *Streptococcus pneumonia*, *Streptococcus pyogenes*, while *Staphylococcus aureus* is some of the examples of cocci-shaped bacteria [21].

Study by Liu [21] stated that *Bacillus paranthracis*, *Bacillus pacificus*, *Bacillus tropicus*, *Bacillus albus*, *Bacillus mobilis*, *Bacillus luti*, *Bacillus proteolyticus*, *Bacillus nitratireducens*, and *Bacillus paramycoides* are normally Gram-stain positive, rod-shaped bacteria. The colonies are off white, white or milky white in colour. There are other gram-positive bacteria including *L. monocytogenes*, *S. pneumoniae*, *C. perfringens* and *S. mutans* [22].

Table 2. Gram staining results for the nine selected isolated bacteria from different parts of pineapple waste

Parts	Dilution	Colour of the colony	Shape	Gram positive / Gram negative
Leaves	10 ⁻³	White	Rod	Negative
	10 ⁻⁵	Yellow	Rod	Positive
	10 ⁻⁷	Yellow	Rod	Positive
	10 ⁻⁷	White	Rod	Positive
	10 ⁻⁸	Pink	Spherical	Positive
Peels	10 ⁻¹	White	Rod	Negative
	10 ⁻³	White	Rod	Positive
Pulps	10 ⁻⁶	White	Rod	Positive
	10 ⁻¹⁰	White	Rod	Positive

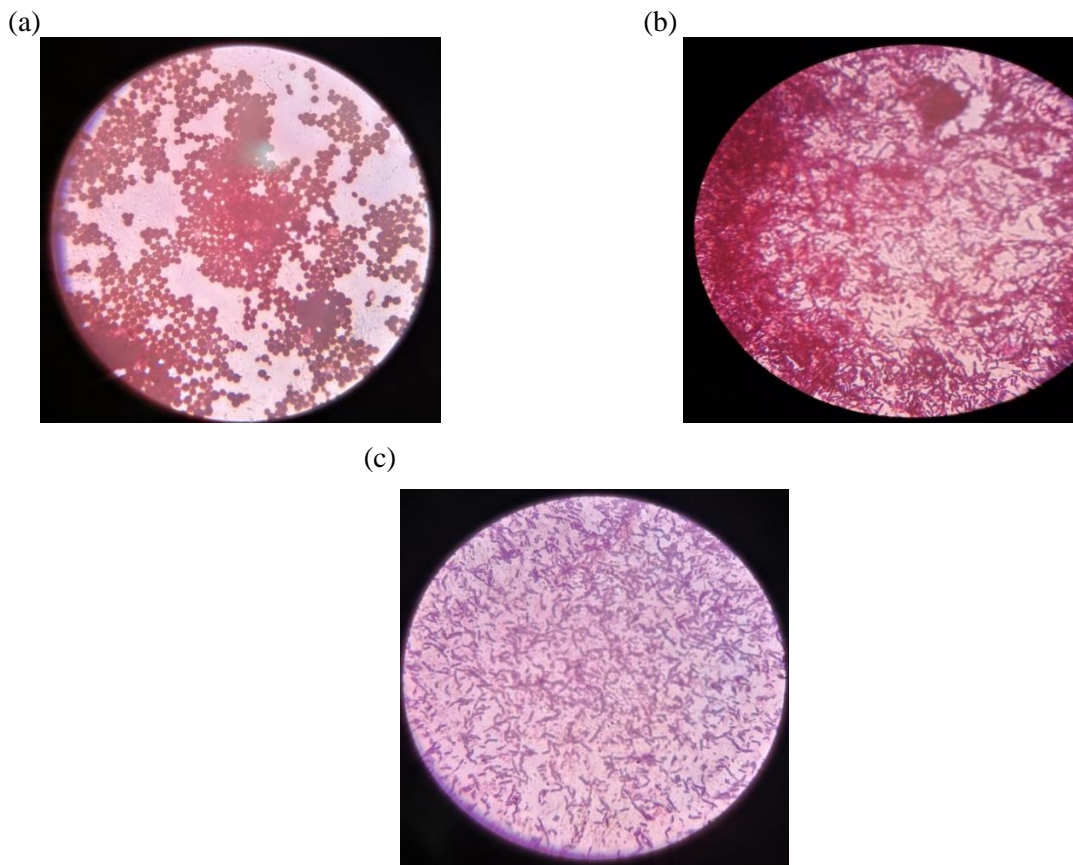


Figure 1. Gram staining results (a) gram positive cocci shape bacteria of leaf sample (10⁻⁸), (b) gram negative rod shaped bacteria of peel sample (10⁻¹), (c) gram positive rod shaped bacteria of leaf sample (10⁻⁵)

Table 3 shows the final list of the elected isolated bacteria for further enzymatic assay and sequencing analysis. These samples are selected based on the different colours of colony and parts of pineapple wastes.

Table 3. The final list of selected isolated bacteria samples

Parts	Dilution	Color of colony	Gram positive / Gram negative
Leaves	10^{-5}	Yellow	Positive
	10^{-7}	White	Positive
	10^{-8}	Pink	Positive
Peels	10^{-1}	White	Negative
Pulps	10^{-6}	White	Positive

3.3 Screening of microbial with sucrose degrading activity

The final selected isolated bacteria samples (as listed in Table 3) were then used for screening purposes. This screening method was used to screen the bacteria with sucrose degrading activity. All the samples were sprayed with triphenyltetrazolium chloride (TTC) reagent to determine the appearance of zone of hydrolysis. Triphenyltetrazolium salts are originally colourless and become coloured after being reduced to formazans. It has been widely used in selective agar medium where the colonies colour will change from white to marron as soon as the formazan precipitated within the colony [23].

From the results, it showed that all the selected isolated bacteria exhibited the appearance of the red zone after sprayed with triphenyltetrazolium chloride (TTC). The red zone represents the zone of sucrose hydrolysis by the bacteria samples. This indicates that the isolated bacteria have the FOS-producing enzymes that could hydrolyze the sucrose media that contain in the agar plate into glucose and fructose. Hence this result showed that the isolated bacteria can be a potential source to produce β -fructofuranosidases (FFases) enzymes.

A study by [20] also used triphenyltetrazolium chloride (TTC) reagent in a preliminary screening of β -fructofuranosidases producers from yeasts, mold and bacteria. In this study, the highest range of zone of hydrolysis was found to be of filamentous fungi among the three classes of microbes.

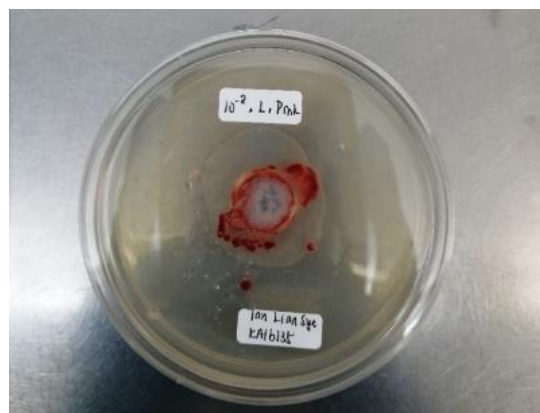


Figure 2. Zone of hydrolysis by one of the isolated bacteria sample

3.4 Enzymatic assay of fructosyltransferase and hydrolytic activity

The fructosyltransferase and hydrolytic activity for each of the isolated bacteria are tabulated in Table 4. In overall, the fructosyltransferase activity is generally higher than hydrolytic activity. Isolated bacteria from the leaves sample (10^{-5}) shows the highest fructosyltransferase activity which is 0.9089 U/ml while isolated bacteria from the pulps sample (10^{-6}) show the lowest activity which is 0.7326 U/ml. While isolated bacteria from the leaves sample with dilution 10^{-7} only showing 0.5325 U/ml of hydrolytic activity. It was also observed that isolated bacteria from the peel samples (10^{-1}) showed the highest Ftase/hydrolytic ratio with 1.5. The ratio of Ftase/hydrolytic determines the efficacy of transferase activity for the synthesis of FOS [20]. Hence the higher the ratio, the higher the efficacy of transferase activity. A study by [20] showed the highest ratio of Ftase/Inv with 1.6 of Based on [7], the Ftase production has been reported to be in the range of 0.053 IU to 660 IU/ml.

Table 4. total hydrolytic and fructosyltransferase activities in different samples

Sample		Hydrolytic activity (U/ml)	Fructosyltransferase activity (U/ml)	Ftase/Hydrolytic ratio
Leaves	10^{-5}	0.7400	0.9089	1.3
	10^{-7}	0.5325	0.7493	1.4
	10^{-8}	0.5574	0.7999	1.4
Peel	10^{-1}	0.5637	0.8573	1.5
Pulp	10^{-6}	0.6443	0.7326	1.1

β -fructofuranosidases (FFase) which possessing both transfructosylating and hydrolytic activity are normally used enzyme in producing FOS. It releases a glucose molecule from the sucrose by cleaving the β -1, 2 linkages and transferring the fructosyl group to sucrose and fructooligosaccharides. Thus, fructooligosaccharides are formed containing 1-ketose (GF2), nystose (GF3) and 1- β -fructofuranosyl nystose (GF4) having fructosyl units (F) linked at β (2 \rightarrow 1) position of the sucrose molecule (GF) [15]. Some microorganisms that produce this kind of enzymes are *Bacillus* sp., *Aspergillus* sp., and *Pseudomonas* sp.

3.5 Identification of the selected isolated bacteria using 16S rRNA sequencing

16S rRNA sequence was carried out and subjected to BLAST to identify the library sequence that resembles the 16S rRNA of the selected isolated bacteria. The results showed the strain isolated in the leaf sample belonged to the genus *Bacillus*. The closest relative for the selected isolated bacteria is *Bacillus paramycooides* strain MCCC 1A04098. Figure 3 below shows the phylogenetic tree generated based on neighbour-joining method in order to show the relationship among the closest relatives of 16S rRNA of selected isolated bacteria with the 16S rRNA of bacillus genus.

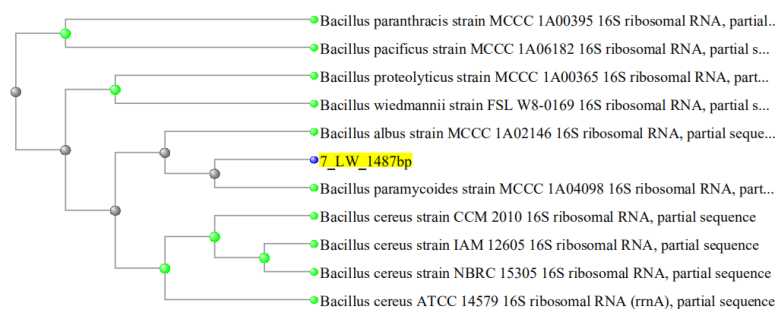


Figure 3. phylogenetic tree based on neighbour-joining method showing relationship among the 16s rRNA sequence of the sample and other close homologous.

There are several studies that showed *Bacillus sp* exhibit an enzyme that catalysed the FOS production. Study by [25] reported that 97% of levan-type FOS were obtained when 1.0 U/mL of LevB1 reacted with 100 g/L of levan produced by the levansucrase from *Bacillus subtilis*. While [26] reported that they successfully identified and characterized two fructosyltransferases from *Bacillus agaradhaerens* WDG185.

4. Conclusion

The study aimed to isolate the microbial strain from pineapple wastes which catalyze the production of FOS by fructofuranosidase (FFases). The screening of isolated bacterial strain resulted for the genus *Bacillus* with the highest fructosyltransferase activity at 0.91 U/ml and the closest relative is *Bacillus paramycooides strain MCCC IA04098*. This study revealed a higher Ftase: hydrolytic ratio for the isolates which can be further enhanced upon optimization experiments. Maximization of FOS synthesis could also be done for further experiments.

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