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Isolation of *Gluconobacter oxydans* PIN7 as a Potential Producer of Cellulose from Fruit Wastes

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ABSTRACT

Cellulose is an essential polymer that is commonly produced from plants and bacteria in a number of industrial processes, such as the biomedical and food industries. While cellulose production from plants is costly, bacterial cellulose (BC) is recognised as one of the best types of cellulose produced. The aim of this study was therefore to generate BC from different types of fruit waste through isolation, identification and characterization procedures. A total of 50 fruit waste samples (watermelon, pineapple, mango, apple and grape) were collected in Serdang, Selangor, Malaysia, where 10 isolates from these samples were capable of producing BC. Ten strains of all these fruits were analysed for morphological and biochemical testing. The findings showed that all isolates matched the characteristics of the BC producers. The BC production of each isolate was then evaluated using a batch culture technique cultivated in the Hestrin-Schramm medium. Of the 10 strains, only pineapple isolates showed high BC production (3.34 g/l ± 0.06). The BC produced by the PIN7 isolate was identified using 16S rRNA analysis and indicated that it was closely linked to *Gluconobacter oxydans* with a sequence identity of 98%. The strain has been identified as *G. oxydans* PIN7. The potential for PIN7 isolation was therefore tested and demonstrated to have a high BC production capability.

Key words *: Bacterial cellulose, Fruit waste, Isolation, Gluconobacter oxydans*

Introduction

Cellulose is produced from several sources, such as plants and bacteria. However, due to several intrinsic properties, bacterial cellulose (BC) is one of the best types of cellulose to be produced than plant cellulose. These properties include being mechanically robust, possess high quality of nanoscale network structure, crystallinity and water-content capacity, as well as having excellent biocompatibility that could be broken down naturally into harmless compounds (Zhai *et al.*, 2020). BC is also free from any impurities such as pectin, hemicellulose, and lignin (Cheng *et al.*, 2009). These essential qualities have permitted BC to be atan advantage over other substances as an alternative resource for several industries, such as the biomedical and the food industries. For example, BC has medicinal components that are

useful in the dressing for wounds, in blood vessels and in supporting the construction of cartilage and bone within the field of tissue engineering (Blanco Parte *et al.*, 2020; Wang *et al.*, 2019). On the other hand, the food industry would apply BC to the manufacturing of specific products such asnata de coco, Kampuchea or Manchurian tea (Nugroho and Aji, 2015). Other industries that benefit from use of BC include Bible paper production, currency notes, high-performance speaker diaphragms, flexible display screens, electronic paper displays of cosmetic pads, anti-ageing agents, and paint enhancers (Iguchi *et al.*, 2000; Nakagaito *et al.*, 2010; Shah and Brown, 2005).

Various bacterial genera have been reported to produce cellulose, such as *Azotobacter, Rhizobium, Agrobacterium, Aerobacter, Komagataeibacter, Sarcina, Gluconacetobacter, Achromobacter, Pseudomonas* and *Alcaligenes* (Andersson *et al*., 2003; Brown 2004). Among these bacteria, the most common species for BC production are *Acetobacter. xylinus, A. nataicola* and *A. rhaeticus,* which strains can produce high amounts of cellulose (Ross *et al.*, 1991; Ruka *et al.*, 2012). These bacteria are usually present in most edible plants such as vegetables, flowers, sugar cane, nuts and decomposing fruits (Park *et al.*, 2003; Valla and Kjosbakken, 1981).

Regular cultivation techniques that use Hestrin and Schramm (HS) as the standard medium (Hestrin and Schramm, 1954) are used to cultivate the production of BC from the strains of these bacteria. The growth of BC can be observed through the formation of white pellicles on the surface of the fluid medium, but this technique requires a significantly longer time and a larger area for fermentation. Also, non-BC secreting mutants are generated (Valla and Kjosbakken, 1981) during the shaking procedure. Thus, an improved condition of culture that allows static fermentation process and isolation of the most efficient BC-producing strains is highly desired.

Malaysia is known to have a high humidity condition and temperature throughout the year, with a bountiful gathering of local and imported fruits that are rich in substrates for BC-producing microorganisms. The aim of this study was to obtain a new isolate from rotten fruit because it contains abundant sugars, such as glucose and fructose, which could be bio-converted into useful products capable of producing cellulose.

Materials and Methods

Sample collection and storage

Ten types of fruit waste samples from watermelon, pineapple, mango, grapes and apples were collected from various stalls on the local market in Serdang, Selangor, Malaysia. These samples were collected in sterile containers and transported immediately to the microbiology laboratory of the School of Biosciences and Biotechnology at the Faculty of Science and Technology, Kebangsaan University of Malaysia. Samples were stored in the refrigerator at 4°C for a maximum of one week prior to analysis.

Culture medium

In the initial procedure of analysis, 10g of each fruit waste sample was transferred into a 250mL flask that was filled with 90 ml HS medium. The flasks were left for an incubation period of approximately seven days at 30 °C under static conditions. After the incubation period, pellicle-forming flasks were selected, which diluted the cultured broth of these flasks with 0.85% DW (w/v). Then 0.1 ml of diluted broth was spread over the HS agar medium and incubated at 30 °C for three days until visible colonies appeared on the agar surface. Pure cultures were tested for microscopic characterization by Gram staining. Only rod-shaped Gram-negative cells showing the ability to produce cellulose have been selected for further study. Colonies which had a clearance zone were collected and transferred to a small tube of HS medium (5 ml) followed by an incubation period of 3 to 7 days at 30 °C. The white pellicle that formed on the surface of the medium was collected forpurified colonies, which were then mixed with 0.1N NaOH and placed into a boiling water bath at 121 °C for 15 minutes to ensure the mixture was free from bacterial contaminants. The treated cellulose was then dried at 80 °C for 48 hours in a hot air oven. After purification, the isolate that producedthe highest yield was selected, and the distinctive strain was coded accordinglyto the 16S rRNA sequence.

A total of ten isolates showing gram-negative characteristics with a spore-forming capability were tested and yielded positive results for BC production as indicated in FESEM analysis. Among the ten BC-producing culture that were isolated, the one that produced BC at a maximum rate was the initially isolated culture of pineapple fruit waste.

Identification of *a Gluconobacter strain*

The technique that had been previously described was used to identify the morphological, biochemical and physiological properties of the selected isolate (Asai *et al*., 1964; Sokollek *et al*., 1998; Valla and Kjosbakken, 1981). A DNA extraction kit (Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA) was used to extract the DNA for *Gluconobacter oxydans* strain PIN7. The procedure adhered to the protocol stipulated by the manufacturer, whereby the total reaction volume of 25ul containing gDNA was purified using in-house extraction method. Then, the amplification of 16S rRNA was achieved using universal primers 27F and 1492R. Standard practice was used to refine the PCR before being sequenced directly with primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' CCGTCAATTCMTTTRAGTTT 3') using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The amplified DNA fragments of the PCR were further purified using a commercial kit of Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified DNA fragments were then sent to 1st Base, Malaysia, for sequencing using a Biosystems Genetic Analyzer (BigDye® Terminator v3.1 cycle sequencing kit chemistry).

Results and Discussion

Isolation and screening for bacterial cellulose producing bacteria

From 50 fruit waste samples tested for BC-producing bacteria, only 10 samples showed results of BC production BC. After 48 hours of growthon HS agar plates, the colonies of these ten strains were found to bedense, pale yellow, viscous, smooth, convex and circular or irregular in shape. These isolates were Gram-negative, with similar cell morphology (short rod; rod-shaped) and occurred as single colonies. The morphological results obtained in this study were consistent with previous studies on *Gluconobacter sp.* (Lisdiyanti *et al*., 2006; Suwanposri *et al*., 2013; Valla and Kjosbakken, 1981; Yamada *et al*., 1999).

Besides, the results from the isolates were also showed negative for oxidase but positive for catalase production. These preliminary tests revealed that the isolates had the same properties to BC-producing *Gluconobacter* spp (Lavasani *et al*.,.

After incubation of all isolates in HS media for 7 days, the results showed observable BC production at different rates as shown in Fig. 1. The highest BC production was in isolate PIN7, which was taken from the pineapple fruit. These results were supported a study by Suwanposri *et al*., 2013, which isolated *Glucon acetobacter* strains from tropical fruits in Thailand and detected the isolates from pineapple fruits produced high BC compared to isolates obtained from other sources. The high production of BC (3.34 g/l) in isolate PIN7 could be justified bythe argument that a high amount of sugar, specically fructose, which was a better source of carbon than glucose, increased BC production (Santos *et al*., 2013). The study had reported a maximum output of 3.0 g/l of BC production uponusing *Gluconacetobacter sucrofermentans* CECT 7291 after optimization of carbon and nitrogen sources (Santos *et al.,* 2013). Therefore, the results of this present study further indicated that pineapple contained a high percentage of sugar, in particular fructose.

Results from the 10 cellulose-producing that were isolated in this study also showeda relationship be-

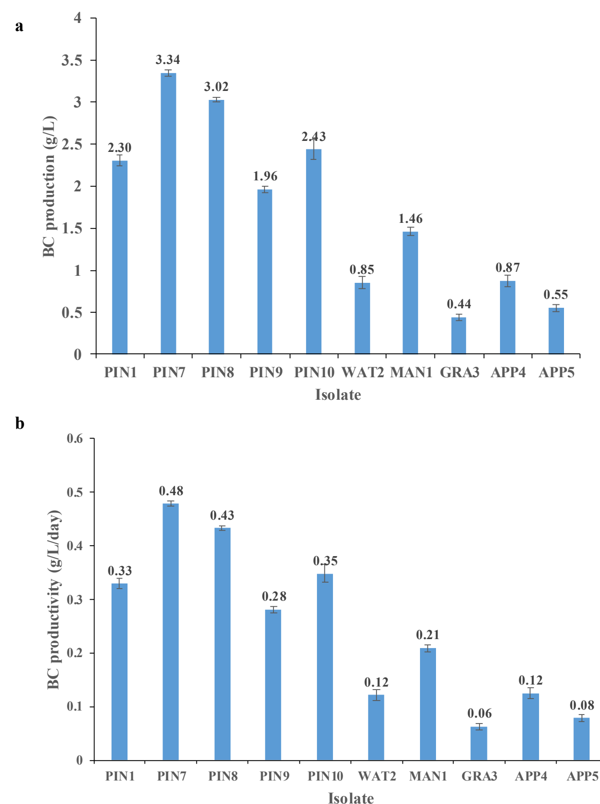

Fig. 1. The isolates producing cellulose (a) BC production (b) productivity

tween the number of isolates and the types of fruit wastes. The highest number of isolates from pineapple (50%) was obtained,while the lowest quantity of isolates from grapes was 10%. Apple had 20% of isolates, and both watermelon and mango had 10% of isolates each. These findings suggested that the unique properties in pineapple wastes, which was abundant in carbon and other nutrients, increased the fermentation process (Kurosumi *et al*., 2009).

One-way ANOVA was usedto compare the productivity of BC between the ten isolates. Before analysis, the Shapiro-Wilk test of normality was carried out to ensure all the variables were distributed normally. Besides, the homogeneity of variances test for BC ($F = 1.415$, $p > 0.001$) and productivity ($F = 1.452$, $p > 0.001$) indicated that the variables were homogenous. The results for one-way ANOVA, which revealed significant differences of BC ($F = 301.238$, $p < 0.001$) and productivity ($F = 299.408$, $p < 0.00$)among the ten isolates. The Duncan post hoc test was applied to further compare all ten isolates for BC production and productivity (Table 1).

From the ten different samples of fruit wastes from pineapple, only PIN7 produced the highest level of BC and productivity, which could be due to the different conditions when the samples were collected. These conditions included the specific origin of the sample, the stage of fermentation of the wasteand the conditions of the waste. As a result, the PIN7 isolate was selected for further examination.

Table 1. Post hoc test using the Duncan test.

	BC Men & SE	Productivity Men & SE
PIN1	2.3033 ± 0.1124^{c}	0.01582 ± 0.01582^{c}
PIN7	3.3433 ± 0.0611^{a}	0.00874 ± 0.00874^{a}
PIN8	3.0233 ± 0.04933^{b}	0.00723 ± 0.00723^{b}
PIN9	1.9633 ± 0.06429^{d}	0.00907 ± 0.00907^{d}
PIN10	2.4333 ± 0.19858^{c}	0.02875 ± 0.02875^{c}
WAT2	0.85 ± 0.1253^{f}	0.01804 ± 0.01804^{f}
MAN1	1.46 ± 0.08^{e}	0.0115 ± 0.0115^{e}
GRA3	0.4367 ± 0.06658^{g}	0.00917 ± 0.00917^{g}
APP4	0.8733 ± 0.11504^{f}	0.0165 ± 0.0165^{f}
APP5	0.55 ± 0.07937^{g}	0.01115 ± 0.01115^{g}

*Means with a different letter are significantly different at 0.01 level

Identification of isolate PIN7 by 16S rRNA amplification

Isolate PIN7 was identified and characterised using the 16S rRNA sequence. The Agarose electrophoresis was performed to estimate the amplified PCR fragments, whereby a ladder marker was administered and compared with the dsDNA sample. The findings revealed that the dsDNA sample of PIN7 was aligned to the positive control and DNA ladder, which had the nucleic acid size of about 1500 bp. The analysis of 16S rRNA gene sequence on isolate PIN7 had yielded the isolate to belong to the genus *Gluconobacter.*

Using the Mega 7 software, trimmed sequences were subjected to BLAST against the GenBank (NCBI) database, which indicated that the PIN7 strain was closest to *Gluconobacter oxydans* strain DSM 3503 with a 98% homology (Figure 2). The evolutionary history inferred using the Neighbour-Joining method exhibited the optimal tree with the sum of branch length = 74.57164556. The tree, with the branch lengths, were drawn to scale within the same units of the evolutionary distances to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and were measured according to the number of units of base substitutions per site. The differences in the composition bias among the sequences were considered in the evolutionary comparisons (Felsenstein, 1985). The proportion of sites where at least one unambiguous base was present in at least one sequence for each

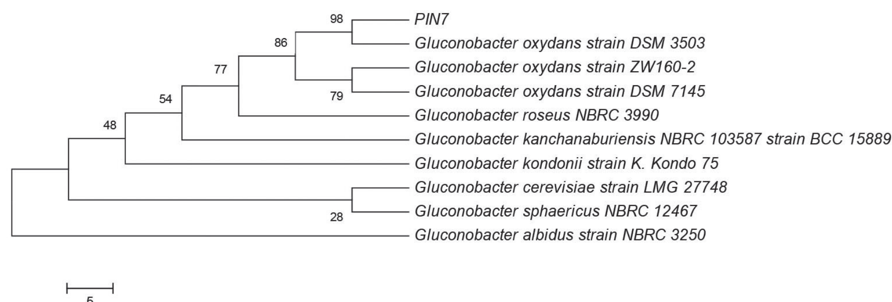

Fig. 2. The Phylogenetic tree of *Gluconobacter oxydans* PIN7 in MEGA 7 software

Table 2. BLAST results for the top 9 highest match against PIN7

Organisms	Identification percentage (%)	Accession no.	Source
*Gluconobacter oxydans*strain DSM 3503	99.93	NR_026118	NCBI
Gluconobacter oxydans strain ZW160-2	99.68	NR_112534	NCBI
*Gluconobacter oxydans*strain DSM 7145	99.93	NR_118196	NCBI
Gluconobacter roseus NBRC 3990	98.93	NR_041049	NCBI
Gluconobacter cerevisiae strain LMG 27748	98.22	NR_117735	NCBI
*Gluconobacter albidus*strain NBRC 3250	98.22	NR_041047	NCBI
Gluconobacter kanchanaburiensis NBRC 103587 strain BCC 15889	98.15	NR_112822	NCBI
Gluconobacter sphaericus NBRC 12467	98.15	NR_041050	NCBI
Gluconobacter kondonii strain K. Kondo 75	98.15	NR_104680	NCBI

descendent clade was shown next to each internal node of the tree.

The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1355 positions in the final dataset. It also seems that the highest match (> 90% homology) for PIN7 against the NCBI database was among the *Gluconobacter* genus with species such as *G. oxydans*, *G. kanchanaburiensis*, and *G. sphaericus* (Table 2). Sequences for strain PIN7 have been deposited in the GenBank database under the accession number MN524152. Based on the above analysis, the BC-producing strain PIN7 was designated as *Gluconobacter oxydans* PIN7.

Conclusion

The producers of bacterial cellulose (BC) from fruit waste were successfully isolatedin this study. The isolate *G. oxydans* PIN7, obtained from pineapple, had the highest production of BC due to an increase in fermentation due to the abundance of carbon and nutrients. The 16S rRNA sequence has shown that the isolate PIN7 belongs to *G. oxydans* with a sequence identity of 98%. PIN7 also produced high concentrations of BC, indicating that the strain was a good BC producer. The findings of this study may potentially be applied in number of industries involving the use of BC, such as manufacture of paper and packaging products, construction, automotive, furniture, electronics, pharmaceuticals, cosmetics, and biomedical products.

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