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Biochemical Characterization, 16S rRNA Sequence Analysis and Multiple Sequence Alignment of Bacteria Isolated from Fermented and Unfermented Coconut

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Author contributions

This research was carried out in collaboration between all authors. All authors designed and conducted the study. Author HWH managed literature search, performed biochemical, molecular and bioinformatics analysis and compiled the manuscript. Author AS supervised the research. All authors read and approved the final manuscript

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ABSTRACT

Coconut (*Cocos nucifera L.*) is a tropical and subtropical plant which has great versatility due to many benefits of its different parts. Appropriate methods such as biochemical, morphological, serological, physiological and molecular techniques are required to clearly differentiate beneficial from harmful bacteria that are found in coconut. The objectives of this research were to characterize bacterial diversity in coconut based on biochemical tests and molecular techniques, to study the evolutionary relationship among the bacteria isolates based on 16S rRNA BLAST

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analysis and Multiple Sequence Alignment. Eight bacteria isolates, 3 from fermented and 5 from non-fermented coconut were identified. Biochemical characterization, polymerase chain reaction amplification of 16S rRNA using universal primers (63f-1387r), BLAST analysis and multiple sequence analysis were performed. The result indicated that the bacteria were detected as *Klebsiella pneumonia, Enterobacter aggromerans, Pseudomonas, Ralstonia pickettii* and *Burkholderia.* The first two were carbohydrate fermenting while the last three were non-fermenting bacteria. All these species are related to human pathogenicity. This can be related to hygiene in food process and handling procedures. Therefore, to favour the selective growth of beneficial bacteria, appropriate environmental hygiene is required.

Keywords: 16S rRNA; Bacteria; Biochemical; BLAST; Multiple sequence analysis; PCR.

1. INTRODUCTION

Coconut (*Cocos nucifera* L.) has been envisaged as a valuable source of several commercial products for human life [1], as well as the most important and hugely grown palm tree worldwide [2]. Each sections of the plant is useful and, in many cases, human is dependent on it [3]. The leaf and trunk supply building material, and the root is utilized as medicine [4]. The fruit is the most demandable part for food and sale. The envelope called mesocarp is processed into rope, carpets, geotextiles and growing media. The hard brown shell (endocarp) can be processed into very high-quality activated charcoal. The inner part of the nut (endosperm) is divided into two edible parts: a white kernel and a clear liquid (coconut water). Moreover, Coconut oil is consumed in tropical countries for many centuries. Studies done on native diets high in coconut oil expenditure reveal that the population is usually in good health. Coconut oil carries a long shelf life and is capitalized in baking industries, processed foods, infant formulae, pharmaceuticals, cosmetics and as hair oil [5].

Coconut meat can be used either as fermented or unfermented form. The protein content, crude fat and crude fiber of fermented defatted coconut increased while the carbohydrate, moisture and ash content decreased as the fermentation period increased [6].

Most mercantile grade coconut oils are derived from copra, the dried kernel of coconut. It can be produced by: smoke drying, sun drying, kiln drying, or derivatives or a merger of these three. *Nevertheless, virgin coconut oil* has more health benefits as compared to coconut oil extracted from copra and it is very durable with a shelf life of several years [5,7].

Coconut milk raw material used for virgin oil production mainly consists of (w/w) 21.3% fat, 2.0% protein, 2.8% carbohydrate and 2.1% of sugar together with some vitamins and trace elements. Virgin Coconut Oil (VCO) consists of C_8, C_{10}, C_{12} and C_{14} fatty acid with lauryl acid has the most composition of 47-53% among all saturated fatty acid [8,9]. Around 45 to 50% of fatty acids of coconut oil form lauric acid. This acid is cognizant to kill viruses, bacteria, yeast, and fungi etc. that are enveloped in a phospholipid membrane. [5,10].

Some microorganisms however, have adapted with the environments circumstances and could actively break the compounds in coconut milk and emerged as either beneficial or harmful. Additionally, disintegration of the linkage of the protein, fat and carbohydrate could be conducted by the inoculation of effective microbes having proteolysis and amyloid enzymes on to the freshly extracted coconut milk to coagulate the protein and further centrifugation can separateprotein from fat, carbohydrate water. With this method, lauryl acid will not be decomposed [9,11].

Under the non-sanitary environmental conditions low quality Virgin Coconut Oil is produced. The wild organism may grow producing several compounds, which affects the quality of the oil. Therefore, prior and proper identification of microorganism is important to avoid microbial associated risks. Some bacteria can be identified by direct microscopic observation. However, definitive identification usually requires further tests. Immunological techniques, DNA probes and PCR-based methods are mainly used for recognizing the pathogenic and non-pathogenic bacteria present in the coconut [12]. Molecular biological methods using PCR amplification of the bacterial 16S ribosomal ribonucleic acid (rRNA) gene using universal bacterial primers and sequencing are commonly used for accurate identification and classification of bacterial isolates [13]. This research was aimed at isolation, biochemical and molecular

characterization of microorganisms in coconut which may have significant health concern.

2. METHODS AND MATERIALS

2.1 Materials and chemicals

The materials used in this research were: safety cabinet, laminar air flow, inoculation loops (spread and streak), erlenmeyer flask, bunsen burner, measuring cylinder, petri dish, test tubes, parafilm, plastic tape, autoclave, electric heater, electric balance, PCR thermocycler, PCR tubes, micropipettes, eppendrof tubes, electrophoresis equipments, GelDoc. The media and reagents used were: nutrient agar, Macconkey agar, Goat blood agar, distilled water, double distilled water, Genejet PCR kits, Geneaidg DNA isolation kit, TAE buffer (50X), agarose, Ethidium bromide (5mg/ml) [EtBr], ultrapure 6X grade gel loading dye.

2.2 Media preparation

As much as 52 gram of Macconkey agar was suspended in 1L of distilled water in erlenmeyer flask and mixed thoroughly; thenheatedfor 5 minutes to completely dissolve the powder. It was then autoclaved at 120ºC for 15 minutes, 1 atm [14].

28 gram of nutrient agar was suspended in 1 litter of distilled water in another erlenmeyer flask and mixed by boiling to dissolve completely. It was then sterilized in an autoclave at 120ºC for 15 minutes, 1 atm.

In the third erlenmeyer flask, 28g/l of nutrient agar was prepared, sterilized and goat blood agar was added to it when it cools to 40-50ºC. All the Medias were allowed to cool at 40-50ºC and transferred into different sterile petri-dishes for bacteria inoculation. The surfaces of the gels were dried for 2 days before inoculation.

2.3 Sample collection and bacteria Inoculation

Five samples from unfermented and three from fermented coconut were collected. The Unfermented were labeled as HR1, HR2, HR3, HR4 and HR5 whereas the fermented were labeled as with BP1, BP2, MC. All the petridish were labeled and spread technique was applied. Bacteria isolates were taken this previously enriched media using another loop and quadrant streaking technique was applied to isolate pure colonies on petridish containing nutrient agar, Macconkey agar and Goat blood agar. The petri dishes were incubated for 24-48 hours at 37ºC.

2.4 BIOCHEMICAL CHARACTERIZATION

A number of Biochemical tests were performed for the identification of bacterial isolates with the help of Oxoid manual [14]. Bacterial isolates were identified by their reaction to glucose, lactose, maltose, manitol, sucrose, Sulfide Indole Motility (SIM), oxidase and Simmon's citrate. To differentiate among *Enterobacteriaceae* group malonate broth, uria agar, Motility Indole Ornithine (MIO) and Lysine Iron agar (LIA) tests were conducted.

2.5 MOLECULAR CHARACTERIZATION

Molecular biological methods comprise a broad range of techniques that are based on the analysis and differentiation of microbial DNA. These methods test DNA directly from the microbial cells themselves and the nucleic acid can be cloned polymerase chain reaction (PCR), sequenced and analyzed.

2.5.1 DNA extraction

Bacterial genomic DNA was isolated based on Geneaidg DNA isolation kit. The detailed protocol is presented below in continuous steps:

2.5.1.1 Sample preparation

Up to 1 x 10^9 (1.5 ml) of bacterial cells were transferred from culture broth to a microcentrifuge tube. It was centrifuged at 14000xg for 1 minute and then the supernatant discarded. 200 µl of GT Buffer was added, vortexed for 5 seconds and incubated at room temperature for 5 minutes.

2.5.1.2 Lysis

200 µl of GB Buffer was added to the sample and mixed by shaking for 5 seconds. It was incubated at 60ºC for 10 minutes to ensure the sample lysate is clear. During incubation, the tube was inverted and shaken every 3 minutes for mixing. At this time, the required Elution Buffer (200 µl per sample) was pre-heated to 60ºC (for next step, DNA Elution).

2.5.1.3 DNA binding

200 µl of absolute ethanol was added to the sample lysate and mixed immediately by

votexing for 5 seconds. GD Column was placed in a 2 ml Collection Tube. All the mixture was transferred into the GD Column and then centrifuged at 14000xg for 2 minutes. The 2 ml Collection tube containing the supernatant was discarded. Then the GD Column was placed in a new 2 ml Collection Tube.

2.5.1.4 Washing

400 µl of W1 Buffer was added to the GD Column then centrifuged at 14000 x g for 30 seconds. The supernatant was discarded. The GD Column was placed back in the 2 ml Collection Tube. 600 µl of Wash Buffer two was added to the GD Column and centrifuged at 14000 x g for 30 seconds. Then supernatant was discarded. The GD Column was placed back in the 2 ml Collection Tube. It was centrifuged again for 3 minutes at 14000 x g to dry the column matrix.

2.5.1.5 Elution

The dried GD Column was transferred to a clean 1.5 ml microcentrifuge tube. 100 µl of the previously heated elution buffer was added. It was incubated at room temperature for at least 3 minutes to allow the Elution Buffer to be completely absorbed. It was centrifuged at 14000 x g for 30 seconds to elute the purified DNA. The DNA extraction result was kept at -21ºC for the next procedures used in the next days.

2.5.2 Biophotometer DNA (Measuring DNA quantity and quality)

50 µl of double distilled water (ddH_2O) was added into cuvette as a blank control. The Cuvette was then placed in the cuvette shaft of the biophotometer. The blank button was pressed and checked if the result is 0.000A. 5 µl of ddH_2O was pipetted from the cuvette and discarded. Then 5 µl of DNA sample from test tube with BP_2 label was added to the cuvette containing ddH_2O and mixed by micro-pipetting. The cuvette was placed in the cuvette shaft of the biophotometer. The sample button was pressed to display the result and the result was recorded. All the above procedures were repeated for the other samples.

2.5.3 16S rRNA PCR amplification

PCR technology is the simplest and currently the most widely used method to obtain 16S rRNA genes for detailed characterization of microbial communities by amplifying a single target molecule by millions-fold. PCR methods are highly sensitive and specific. It is the foundation upon which most genetic polymorphism-based techniques are based [13].

The key to PCR is the use of oligonucleotide primers designed to be complementary to the desired gene or genetic region. Polymerase Chain Reaction (PCR) amplification was done using a pair of universal primers (63f:5'-CAGGCCTAACACAT-GCAAGTC-3' and 1387r: 5'-GGGCGGAWGTGTACAAGGC-3') for amplification of 16S rRNA [15].

The extracted DNA products were used as DNA template for amplifying 16S rRNA gene. 25 µl PCR mix include: 1.25µl each (forward and reverse) primers, 2 µl DNA sample, 12.5 µl Kapa (which includes Tag polymerase, PCR buffer, MgCl2, dNTPs premixed together) and 8 µl of double distilled water (ddH_2O). The conditions of PCR were as follows: initial denaturation for 3 minutes at 95ºC, denaturation for 15 sec at 95^0C, annealing for 15 sec at 58ºC, extension for 5 sec at 72ºC, final Extension for 5 minutes at 72ºC, Post PCR at 4ºC, 35 cycles, cover temperature of 105ºC.

2.5.4 DNA electrophoresis and GelDoc

The PCR products were separated by 1% w/v gel agarose electrophoresis, a technique used to separate biological molecules such as DNA, RNA, Proteins, Enzymes, etc. based on their migration ability, size, charge and pH. Generally, horizontal electrophoresis is used for nucleic acids (DNA, RNA) while vertical electrophoresis is applied for proteins including Isozymes [16].

Tris-acetate-EDTA (TAE) buffer was provided as stock of 50X, therefore it should be diluted due to the reason that failure in diluting the buffer will result in slow migration of samples and causes excessive heating of gel. Dilution was done to get 1L of 1X TAE buffer based on the following formula:

$V_1C_1 = V_2C_2$
$V_1x50 = 1000mlx1$
V_1 =20 ml, hence 20 ml of 50X TAE was diluted in 980 ml distilled water.

1% w/v gel agarose was prepared by suspending 1 gram of agarose in 100ml of TAE (1X) buffer and dissolved by heating to boiling. It was then allowed to cool at 40-50^0C for almost 20 minutes. The two dams were placed in to the slots on each side of the gel plate and made tight. The

melted agarose was poured onto the gel plate electrophoresis box. Gel comb with eight teeth (well maker) was inserted in a gel. The comb was nearest to the cathode (negative) as DNA migrates towards anode (positive). About 250 ml of TAE (1X) buffer was poured.

5 µl of 1Kb DNA marker plus 2 µl loading dye was used as ladder in the first well. 3 µl of PCR products were loaded in each of the other wells. DNA electrophoresis was run for 50 minutes at 90V, 400mA using thermo-scientific electrophoresis machine. The electrophoresis result was visualized by staining with ethidium bromide for 15-20 minutes followed by washing (to detoxicate ethidium bromide) with distilled water for 10-15 minutes and illuminated with UV light Gel Doc.

2.5.5 DNA sequencing, BLAST analysis, Multiple sequences Alignment

Four carbohydrate non-fermenting bacterial 16S rRNA isolates were sent to 1st BASE laboratories, Singapore, for 16SrRNA sequencing. 16S rRNA molecule has major role of protein synthesis in microorganisms. The mechanism of protein synthesis doesn't vary significantly from one organism to another due to the reason that the nucleotide sequences of some portions of 16S ribosomal deoxyribonucleic acid (rDNA) are sealed which are responsible for conserved nature of 16S rRNA. Nucleotide sequencing of this molecule is used to identify organisms and rank biological phylogenetic nomenclature by comparing certain locations on the 16S rRNA molecule with database of previously identified micro-organisms whose 16S rRNA mark is already known [12].

The 16S rRNA sequences were analyzed using Basic Local Alignment Search Tool (BLAST: http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi) from National Center for Biotechnology Information (NCBI) to find local similarity between sequences. This program compares sequence database and calculates the statistical significance of matches. Multiple sequence alignment using CLUSTALW2 program from EMBL European Bioinformatics Institute (EMBL-EBI: http://www.ebi.ac.uk/Tools/msa/clustalw2/) was performed to obtain phylogenetic tree that would help species relationship and evolutionary trends.

3. RESULT AND DISCUSSION

3.1 Biochemical Test Result

Test tubes containing carbohydrate broths (glucose, lactose, maltose, manitol and sucrose) were inoculated with bacteria and incubated at 37°C for 24 h. After incubation, a positive result was noted as change of color from red to yellow (MC, BP1 and BP2) whereas no color was observed in negative results (HR1, HR2, HR3, HR4 and HR4) as shown in Fig. 1 and Table 1.

Phenole red broths of crabohydrates are used to determine the ability of organisms to ferement carbohydates. The change in color from red to yellow indicates an acidic pH change, which is a positive indicator for carbohydrae fermentation. For glucose fermenting bacteria gaseous byproducts are formed and a bubble was detected in inverted tubes.

For the citrate utilizing test, Simons citrate agar slants were inoculated and incubated at 37°C for 24 h. All bacteria isolates were positive as color change from green to blue was noted due to drop in pH. For urease test urea broth was used and change in color from yellow-orange to bright pink was considered as positive. A positive oxidase by *Pseudomonas*, *Ralstonia pickettii and Burkholderia* indicated as development of dark purple color. This test was used to assess bacteria isolates which produce the enzyme cytochrome c-oxidase, a component of electron transport chain of some specific organisms.

Sulfide-Indole-Motility (SIM) medium allows detecting three different traits (sulfide production, indole production from tryptophan and bacterial motility). The hydrogen sulfide (H_2S) production allows differentiation of family *Enterobacteriaceae* as positive organisms reduce sulfur into H_2S and black precipitate was formed due to the reaction of hydrogen sulfide with ferrous sulfate. Indole test is used to detect organism's ability to breakdown Tryptophan using tryptophanase into indole and pyruvic acid. Indole binds with Kovac's reagent p-dimethylaminobenzaldehyde in isoamyl alcohol. Positive Indole test result forms a pink red layer (complex) on the top of the broth due to the reaction between indole and the reagent.

Motility Indole Ornithine Medium (MIO Medium) was used as differential test medium to detect the ability of bacteria to produce indole from tryptophan, to decarboxylate the ornithine and

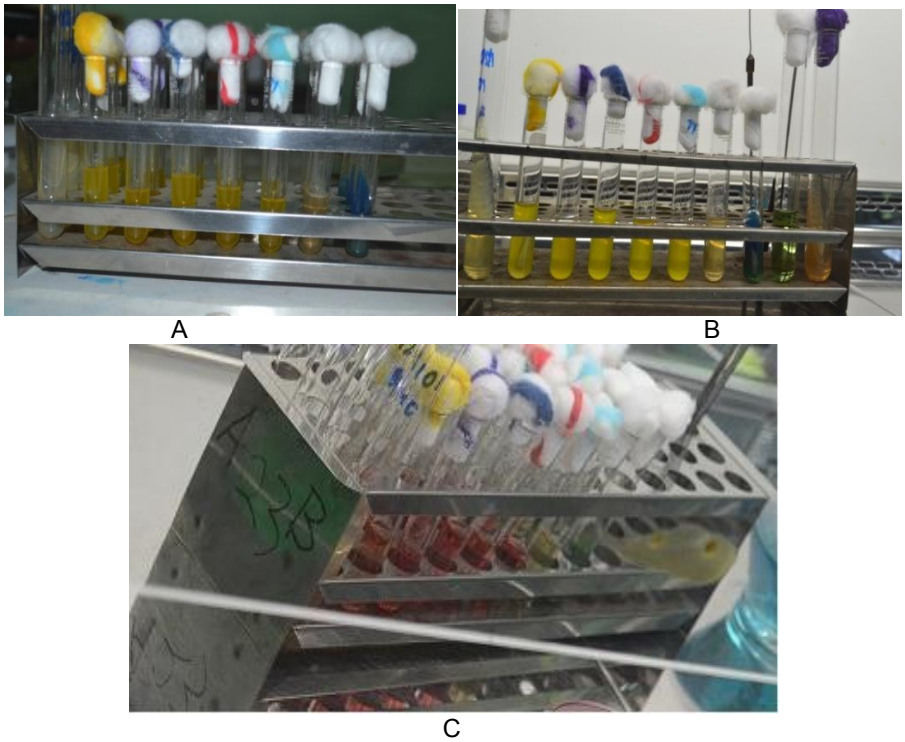
exibit motility by turbidity throughout of medium which enables the identification of *Enterobacteriaceae* on the basis of motility, indole production and ornithine decarboxylase activity. Motile organisms show either diffused growth or turbidity extending away from the inoculation line; while nonmotile organisms grow along the inoculation line. Organisms ferment dextrose to form acid which cause the bromo cresol purple which is the pH indicator to change from purple to yellow. Organisms which possess ornithine decarboxylase are able to decarboxylate ornithine to putrescine which increases the pH making it alkaline. The presence of casein enzymatic hydrolysate produces indole.

Lysin Iron Agar (LIA) test was used to differentiate gram negative bacteria to decarboxylate or deaminate lysine on the basis of lysine decarboxylase/deaminase and produce hydrogen sulfide (detected by ferric Ammonium Citrate indicator). L-Lysine helps to detect these two enzymes and sodium Thiosulfate acts as source of inorganic sulfur.

Based on Complete Biochemical test (tests used to further differentiate member of *Enterobacteriaceae* which include malonate, Uria, MIO and LIA), Sample BAP2 was detected as *Enterobacter agglomerans*, with results showing positive for carbohydrate fermentation, Citrate, Lysine Iron agar tests and H_2S production (black precipitate); but negative for malonate broth, urease and Ornithine tests.

On the other hand, MC1 and BP1 samples were detected as *Klebsiella Pneumonia*, with positive results for carbohydrate fermentation, citrate, malonate broth, uria agar, but negative for Sulfide Indole Motility (SIM) test, Motility Indole Ornithine (MIO) and H_2S production.

Samples HR1, HR2, and HR4were identified as *Ralstonia pickettii,* whereas sample HR3was identified as *Burkholderia* sp., (obtained from 16S rRNA sequence BLAST analysis) and Sample HR5 had negative results for carbohydrate and SIM test and but positive for citrate test; and was identified as *pseudomonas.*



A B C

Fig. 1. A) Biochemical test I (for genera identification of bacteria isolates form fermented coconut) B) Biochemical test II for species identification of bacteria isolates form fermented coconut C) Biochemical test I (for genera identification of bacteria isolates form unfermented coconut

Table 1. Biochemical test results of bacteria isolates from fermented and unfermented bacteria

Tests		Samples sources								Remark
		Fermented coconut			Unfermented coconut					+=yellow g=gas formation
		MC1	BP1	BP2	HR1	HR2	HR3	HR4	HR5	
Glucose		+g	+g	+g	-	-	-	-	-	
Lactose		+	+	+	-	-	-	-	-	
Maltose		+	+	+	-	-	-	-	-	
Manitol		+	+	+	-	-	-	-	-	
Sucrose		+	+	+	-	-	-	-	-	
SIM	Sulfide	-	-	+	-	-	-	-	-	
	Indole	-	-	+	-	-	-	-	-	
	Motility	-	-	+	-	-	-	-	-	
Simmon's citrate		+	+	+	+	+	+	+	+	+=blue
Oxidase test		NA		+	+	+	+	+	+	
Malonate broth		+	+	-						
Uria agar (urease test)		+	+	-	NA					
MIO	Motility	-	-	+						
	Indole	-	-	+						
	Ornithine	-	-	-						
LIA (Lysine Iron agar)	Slant	K	K	K						
	Butt	A	A	A						
	H_2S	-	-	+						

NA= Not applied

All these species are related to human pathogenicity. This can be related to hygiene in food processes and handling procedures. Therefore, to favour the selective growth of beneficial bacteria, appropriate environmental hygiene is required.

3.2 Molecular Characterization Result

3.2.1 DNA extraction and Biophotometer analysis

When checked by Eppendrof biophotometer the value of the bacterial DNA isolates at purity level of A260/280 ratio ranged from 1.88 to 2.0 (Table 2). DNA isolates are said to be pure and eligible to proceed to the molecular analysis if the A260/280 ratio values ranged from 1.8 to 2.0.

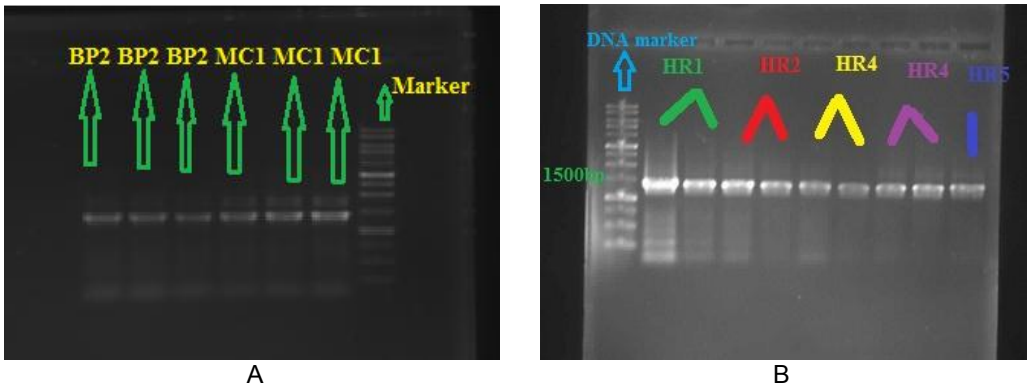
3.2.2 16S rRNA PCR amplification, DNA electrophoresis and GelDoc

PCR amplification of bacterial 16SrRNA gene using bacterial universal oligo-primers followed by sequencing of the PCR amplicons which help to determine bacterial species available in coconut. Depending on the data of nucleic acids obtained from PCR amplification of the bacterial 16S rRNA, the bacterial variants would be investigated. [12]. This produces a variety of community profiles in which complex banding patterns of DNA fragments on a gel, commonly referred to as a "fingerprint", represent the diversity of polymorphic genes of interest, which in turn reflects the diversity of microbial species in the community. [13].PCR amplified product sizes were determined by comparing the length of the DNA bands migrating with DNA markers of known size and concentration after running DNA electrophoresis. The results of 16S rRNA gene amplification with primers 63F and 1387r are found to be approximately 1,300 bp each as seen in a DNA marker size 1 Kb as shown in Fig. 2.

The denaturation of double stranded DNA at high temperature of 95°C separates the DNA into single stranded nucleotides. The two oligonucleotide primers (forward and reverse) anneal complementary regions of the denatured DNA at 58ºC. Finally a heat stable DNA polymerase created new stands of DNA for each template by using the complementary strand as a template to extend the primer.

Table 2. Yield of genomic DNA from 1 x 10^9 cells from fermented an unfermented coconut extracted by genomic DNA extraction kit (bacteria)

Sample source	Bacterial isolates	DNA concentration (µg/ml)	The ratio A260/A280
Fermented coconut	BP2 (1st replica)	3.1	1.91
	BP2 (2ndreplica)	3.6	1.88
	MC1 (1st replica)	6.5	1.95
	MC1 (2nd replica)	3.8	1.97
Unfermented coconut	HR1	7.3	1.99
	HR2	3.1	1.93
	HR3	8.2	1.96
	HR4	5.0	2.0
	HR5	7.6	1.98



A B

Fig. 2. DNA electrophoresis analysis of PCR products; the 16S rRNA products (amplified by using 63f and 1387r primers) were obtained from genomic bacteria DNA isolated from both fermented and unfermented coconut. The PCR products were separated by 1% w/v gel agarose electrophoresis

3.2.3 16S rDNA sequencing, BLAST analysis and Multiple Sequence Alignment

The sequences of four carbohydrate non-fermenting bacteria isolates were obtained from first BASE laboratories, Singapore. By using universal PCR primers complementary to conserved regions of the rRNA gene and subsequent cloning and sequencing of the PCR products, the 16S rRNA gene sequences are compared with other known 16S rRNA sequences to identify the bacteria species. The identity of some of the bacteria were determined by sequencing and BLAST analysis as shown in Table 3. 16S rRNA sequences enable the identification microorganisms because the fixed 16S rRNA contains species-specific variable regions that change according to different species [12].

Ralstonia pickettii is an infrequent invasive pathogen, but can cause infections, mainly of the respiratory tract, in immunocompromizd and cystic fibrosis patients [17]. *Ralstonia pickettii* and Ralstonia insidiosa were reported [18] as emerging waterborne bacteria pathogens and have survival capability in various water sources and grow in various water sources. They were found to be susceptible to antibiotics such as quinolones and sulfamethoxazole/trimethoprim. Apart from pathogenicity, *Ralstonia pickettii* possess a significant Biotechnological potential for bioremediation due to its ability to break down many toxic substance and xenobiotic pollutants such as toluene and trichloroethylene [19]. Some species of *Burkholderia* such as *Burkholderia pseudomallei* and *Burkholderia mallei* were associated to melioidosis and glanders [20,21].

The comparison of 16SrRNA sequence enables to construct phylogenetic trees which make it easier for studies of species diversity and molecular evolution as shown in Fig. 3. For recognition of closest species, the obtained sequences were match up with 16S rRNA gene sequence in GenBank databases using the National Center for Biotechnology Information [12]. The 16S rRNA was thus used for phylogenetic analysis as well as for the species-specific detection of bacteria. The partial 16S rRNA gene sequences are used in constructing phylogenetic trees based on CLUSTALW program to determine identity or nearest phylogenetic position.

Table 3. Identification of bacteria based on BLAST analysis of 16S rRNA sequence data

Isolate code	16S rDNA size (bp)	Similarity from BLAST	Accession	identity
HR1	1262	*Ralstonia pickettii* strain S-X17A 16S ribosomal RNA gene, partial sequence	KJ806364.1	97%
HR2	*1255*	*Ralstonia pickettii* strain VIT-SRM1 16S ribosomal RNA gene, partial sequence	KJ716446.1	98%
HR3	*1270*	*Burkholderia sp.* B38 16S ribosomal RNA gene, partial sequence	KF788057.1	97%
HR4	*1255*	*Ralstonia pickettii* strain S-X17A 16S ribosomal RNA gene, partial sequence	KJ806364.1	96%

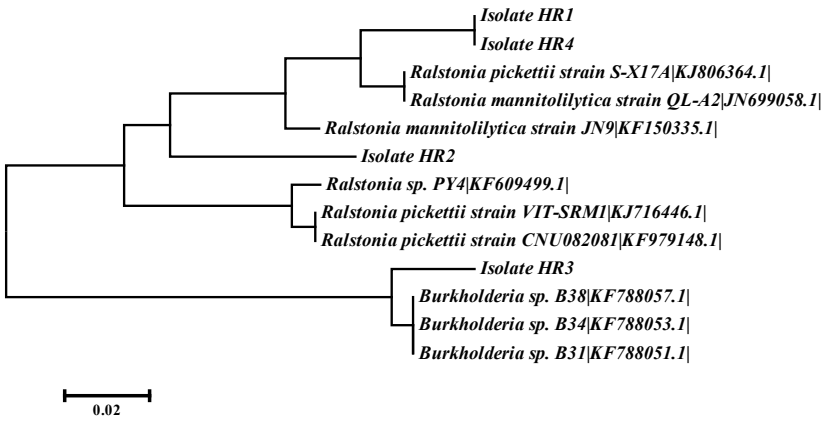


Fig. 3. Phylogenetic tree of 16S rRNA nucleotide sequence of bacteria from coconut; phylogenetic tree is based on 1300 bp (63f-1387r)

Based on phylogenetic tree analysis Isolate HR1 is 97% similar with | Ralstoniapickettii strain S-X17A, 97% similar with Isolate HR2, 96% similar with Isolate HR4 and 91% similar with Isolate HR3. Isolate HR2 is 98% similar with Ralstoniapickettii strain VIT-SRM1, 95% similar with Isolate HR4, and 91% similar with Isolate HR3. Isolate HR3 is 97% similar withBurkholderia sp. B38 but 90% similar with Isolate HR4. Isolate HR4 is 96% similar with Ralstoniapickettii strain S-X17A.

4. CONCLUSION

The combined application of biochemical and molecular techniques ensured to identify the bacteria species found in both fermented and unfermented coconut. Accordingly, *Klebsiella pneumonia* and *Enterobacter aggromelans* were found in fermented coconut whereas *Pseudomonas*, *Ralstonia pickettii* and *Burkholderia* were detected in unfermented coconut. BLAST followed by Multiple Sequence Alignment enabled to construct phylogenetic tree which helped to study the similarity and evolutionary relationship among the bacteria isolated from coconut.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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