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TAXONOMICAL IDENTITY AND POLYSACCHARIDE PRODUCED BY *Bacillus* SPECIES ISOLATED FROM OLD AGED MEDICINAL DECOCTIONS

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Abstract: In Malay folk medicine, bacterial broth are used in treating diseases such as sore throat and fever. However, the characterisation and identification of the bacterial are not know. In the present study, the bacterial broth used as a decoction in Malay folk medicine was investigated. The polysaccharide-producing bacteria were isolated and characterised based on a series of morphological and biochemical assays. A total of 14 isolates tentatively identified as *Bacillus* spp. were further characterised using a PIBWin bacterial identification program. The identification revealed that the cocktail of bacteria belongs to species of *Bacillus subtilis* ssp. *subtilis, Bacillus pumilis*, *Bacillus coagulans* and *Bacillus lincheniformis*. Five out of 14 isolates, *B. coagulans, B. subtilis* ssp. *subtilis*, *B. lincheniformis,* and two *Bacillus* spp. were found to produce exo-polysaccharide under static culture conditions. *B. coagulans* produced the highest amount of polysaccharide followed by *B. lincheniformis* and *B. subtilis* ssp. *Subtilis*. Types of monosaccharide were tentatively identified using paper chromatography. *B. coagulans* possesses almost all types of monosaccharides, whereas in the other strains, lactose, raffinose, galactose, glucose, mannose, xylose and/or rhamnose were identified. The existence of monosaccharide between these isolates showed that the exo-polysaccharide produced by *Bacillus* spp. could possibly be utilised for medicinal purposes. In addition, the identified bacterial polysaccharide may also have a role in anti-bacterial responses to the traditional treatment approach.

KEYWORDS: *Bacillus* spp., Paper Chromatography, Monosaccharide, Malay Traditional Medicine, Poly-saccharide

Introduction

Bacteria are often maligned as the cause of human and animal diseases. Despite this characterisation, certain bacteria such as *Acetobacter* sp. and lactic acid bacteria were found to produce polysaccharides, function as antibiotics and find use as food additives (Sutherland, 2001). Over the past 20 years, a new class of microbial product – microbial polysaccharide – has been grown for industrial importance (Sutherland, 2001; De Vuyst *et al.,* 2001). These microbial polysaccharides can be divided into capsular polysaccharides (CPS) and slime Exo-polysaccharides (EPS) (De Vuyst et al. 2001) CPS were found to attach to the cell wall while EPS were found to be secreted into the extracellular medium. EPS secreted from the cells were shown to form a layer over the surface of the organism with known functions as a general physical barrier, neutralizing bacteriophage, preventing desiccation and avoiding antibody responses (De Vuyst *et al.,* 2001). Nevertheless, bacterial polysaccharides such as dextran, xantan, gellan, pullulan and sclerglucan have also been used widely in various industries (Sutherland, 2001; De Vuyst *et al.,* 2001; De Vuyst and Degeest, 1999). Moreover, the EPS produced by lactic acid bacteria are widely used as probiotics such as in *Lactobacillus rhamnosus*, which

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produces a LMM microcin-like compound. This compound has an anti-bacterial activity toward *Bacteoides, Bifidobacterium, Clostridium, E. coli, Pseudomonas, Salmonella,* and *Streptococcus* (Looijesteijn *et al.,* 2001; yang, 2000). On the other hand, EPSs produced by these lactic acid bacteria were also reported to have cholesterol-lowering activities and anti-tumour activities (Nakajima *et al.,* 1992).

In Malay folk medicine, a broth made of sucrose syrup or boiled tea inoculated with bacteria is drunk as a decoction to cure fever and sore throat. However, the compound that is responsible in such activities is unknown. It was observed that the bacteria broth kept at room temperature produced slimy polysaccharides-like morphology over time. Investigation towards these morphological changes in solution led to identification of some *Bacillus* spp. that might be responsible for the polysaccharide production. In this study, *Bacillus* species that may have a role in anti-bacteria or anti-inflammatory responses to treatment were identified and characterised. This is probably the first report on characterisation of bacteria used as decoction in Malay traditional medicine.

Materials and Methods

Isolation of the bacteria

The decoction of bacterial broth was obtained from Dr. Aziz Ahmad, UMT. 250µl of the broth was pipetted onto the surface of nutrient agar (NA) plates. The plates were then incubated at 37°C overnight. A single bacterial colony with different morphological characteristics such as colony elevation, colour, shape, margin and surface texture were isolated and transferred onto fresh NA plates. A single colony of the purified isolates was then cultured into one ml of nutrient broth (Buck, 1982; Oppenheimer and Zobell, 1952) and further incubated overnight at room temperature (28ºC) with gentle agitation at 100 rpm. For maintenance and storage of bacterial isolates, the broth was then diluted with a sterile glycerol solution to a final concentration of 60% glycerol and kept frozen at -21°C.

Morphological Characterisation

Bacterial cultures grown on nutrient agar were examined based on their Gram reaction by conventional staining techniques (Buck, 1982). Selective media such as MacConkey and Acetobacter-Gluconobacter agar were used to characterise these isolates. Motility test was performed using modified SIM medium (Kalimutho et al., 2007).

Phenotypic Characterisation

Isolated strains were characterised by conventional microbiological methods (Schiraldi *et al.,* 2005; Marshall *et al.,* 1995) such as: Catalase Test; Oxidase Test; Methyl Red Test; Voges-Proskauer Test; degradation of starch, urea, casein, gas and acid production from D-lactose, D-sucrose, D-glucose, D-maltose, D-fructose; Triple Sugar Iron Test; growth temperature (37°C and 50°C) and presence of NaCl (10%). In these assays, the bacteria were grown on the specific medium according to the standard preparation protocol with minor modifications as appropriate. All media used were filter-sterilised using nitrocellulose membrane 0.2µm pore size. The pH was adjusted according to the type of media used. *Escherichia coli* and *Bacillus* sp. were used as an internal control.

Antibiotic Resistance

Response of the isolates to different antibiotics was tested on nutrient agar medium. Nutrient agar plates were surface-seeded with 200 µL bacterial broth. Antibiotic discs: Chloramphenicol, Erythromycin, Streptomycin and Polymyxin B (Becton Dickinson, USA) with effective concentrations or resistance concentration (Table 1) were placed over the plates. Inhibition of growth depicted by a clear zone formation around the discs indicated sensitive reaction, otherwise the isolates was resistant to the antibiotic. Diameter of the inhibition zone was measured with an antibiotic zone scale (Michela *et al.,* 1999).

Isolation of Polysaccharide

The identified bacteria were then inoculated in peptone-yeast extract (PYE) medium added with

Table 1. Morphological and biochemical characterisation of bacteria isolated from decoction of Malay traditional medicine

Isolates	UMT 1	UMT 2	UMT 3	UMT 4	UMT 5	UMT 6	UMT 7	UMT 8	UMT 9	UMT 10	UMT 11	UMT 12	UMT 13	UMT 14
Morphological Characteristics Tests														
Gram Reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Shape	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Spore	+	+	+	+	+	+	+	+/-	+	+	+	+	+	+
Motility	+	+	+	+/-	-	+	+/-	+/-	+	+	+/-	+/-	+	+
Cultural Characteristics Tests														
MacConkey Medium	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetobacter Medium	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic Growth	-	-	-	-	-	-	-	+/-	-	+	+	+	+	+
Chloramphenicol Resistance (30µg)	-	-	-	-	-	-	-	+/-	-	-	-	-	-	-
Erythromycin (Resistance 15µg)	-	-	-	-	-	-	-	+	-	-	-	-	+/-	-
Streptomycin Resistance (10µg)	-	-	-	-	-	-	-	-	-	+/-	-	-	-	-
Polymyxin (Resistance 300IU/IE)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Biochemical Test														
Catalase Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase Test	-	-	-	-	-	-	-	-	-	+/-	+	+	+	+
Starch Hydrolysis	+	+	-	-	+	-	-	+	+	+	+	+	+	+
Casein Hydrolysis	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Mannitol Salt Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl Red Test	+/-	+/-	+/-	+	+	+	+	+/-	+/-	+	+	+	+	+
Voges-proskaur Test	+	+	+	+	+	+	+	+	+	+/-	-	-	-	+
Simmon Citrate Test	+	+	+	+	-	+	+	+	+	+	-	-	-	-
Triple Sugar Iron	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A
Urea Hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10% NaCl	+	+	+	+	+	+	+	+	+	-	+	+	+	+
50^{0}C	+	+	+	+	-	+	+	+	+	+	+	+	+/-	+/-
Sucrose acid	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-
Glucose acid	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-
Lactose acid	-/-	-/-	-/-	ND	ND	ND	-/-	-/-	-/-	-/-	ND	-/-	-/-	-/-
Fructose acid	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-	A/-
Galactose acid	-/-	-/-	A/-	A/-	A/-	A/-	A/-	-/-	ND	ND	A/-	A/-	A/-	ND

Key: (+): Positive result; (-): negative result; (+/-); intermediate result; R: Rod shape; K/A: Glucose fermentation and peptone catabolism occur; A/-: Acid/No gas; -/-: not acid production/no gas; NaCl: Sodium Chloride; ND: Not determine

3 % (w/v) sucrose to enhance the production of exopolysaccharide (Sudhamani et al., 2004). The flasks were incubated for five days of cultivation with vigorous shaking, 100rpm at 30°C during the late-log phase in which the optical density (600nm) was 0.7 to 0.8 at. The cultures were harvested by centrifugation at 4500rpm for 25min; the pellet was discarded and the supernatant was filtered with Whatman GF/F filter. The filtered supernatant was precipitated with 90% ethanol (v/v) in a 1:2 ratio. The diluted supernatants were then dialysed for 48h under running tap water, followed by de-ionised water at 10°C for 48h and the dialysed solution was then freeze-dried for further analysis.

Identification of Monosaccharide

Types of monosaccharide were identified using a paper chromatography (Moonmangmee et al., 2000). The polysaccharide was hydrolysed with 2 ml of 2M-trifluoroacetic acid (TFA) at 100°C for 12h in a sealed glass tube. The hydrolysed product was then concentrated using rotary evaporator at 40°C until dried. The precipitate obtained was dissolved in 1ml of acetonitril: de-ionised water (1:1) prior to spot on Whatman paper No.1. Monosaccharides such as xylose, glucose, sucrose, maltose, mannose, galactose, arabinose, trehalose, raffinose, and rhamnose were used as standard controls. The sugars were separated by mixture of ethyl acetate: acetic acid: formic acid: water (18:3:1:4, v/v) as a top solvent for 18h. Separated sugar was dried and stain-dipped in alkaline silver nitrate reagent. Retention factors (R_f) were calculated by comparing the R_f value with standard and the samples.

Identification of the Isolates

The biochemical test was used to aid in definitive identification and to differentiate the closely-related bacterial species. The bacteria were identified according to Bergey's Manual of Determinative Bacteriology (Holt, 1994) and Probabilistic Identification of Bacteria for Windows (PIBWinProgram), which can be accessed from http://www.som.soton.ac.uk/staff/tnb/pib.htm.

Results

***Bacillus* spp. Phenotypic Characteristics and Drug Sensitivity**

The bacteria existing in broth used as decoction in treatment of sore throat and fever in Malay folk medicine was successfully isolated and characterised. Morphological observation and gram-staining reaction showed that the strains were clustered in Gram-positive category. With regards to the systematic position, the bacteria are of a single genus and were tentatively identified as *Bacillus* spp., (Table 1). Moeller's spore staining (Fontaine et al., 1991) and confirmatory test (Yun and Park, 2000) strongly suggested that these isolates produce spore after 24h incubation, except the strain of UMT08. Further analysis of phenotypic characteristics showed that isolates UMT11, UMT12, UMT13 and UMT14 resulted positive in the reaction for both catalase and oxidase tests. All the isolates were shown negative for mannitol salt test. Only isolate UMT009 could not hydrolyse casein. Isolate UMT03, UMT04, UMT06 and UMT07 resulted negative for the starch hydrolysis test. All the isolates except isolate UMT05 were grown at 50°C. All the obtained isolates were shown to be salt-tolerant even in the presence of 10% NaCl (Table 1).

Carbohydrate utilisation test such as utilisation of different sugars i.e. sucrose, glucose, lactose, fructose and galactose were carried out. The entire *Bacillus* spp. could not utilise lactose (Table 1). Most of the isolates utilised galactose as a sole carbon except isolates UMT11, UMT12 and UMT13. Drug sensitivity analysis showed that these isolates are not sensitive to most of the antibiotics tested except a minor sensitivity was observed with polymyxin B, further suggesting that these isolates might be derived from the same genus. The rest of the biochemical assays are reported in Table 1.

Identification of Possible Species of *Bacillus.*

The phenotypic results somehow revealed that the species from genus *Bacillus*. Isolates UMT01, UMT02 and UMTS09 were tentatively identified as *Bacillus subtilis,* whereas isolates UMT03, UMT004, UMT06 and UMT07 were

tentatively identified as *Bacillus pumilis*. The isolate UMT05 was suspected to be *Bacillus mycoides*. The remaining isolates of UMT08 and UMT10 were tentatively identified as *Bacillus coagulans* and *Bacillus licheniformis*, respectively (Table 2).

Table 2. Species of the isolated bacteria from decoction of Malay traditional medicine.

Isolates	Tentatively identified Species
UMT 01	*Bacillus subtilis ssp. subtilis*
UMT 02	*Bacillus subtilis ssp. subtilis*
UMT 03	*Bacillus pumilis*
UMT 04	*Bacillus pumilis*
UMT 05	*Bacillus mycoides*
UMT 06	*Bacillus pumilis*
UMT 07	*Bacillus pumilis*
UMT 08	*Bacilluscoagulans*
UMT 09	*Bacillus subtilis ssp. subtilis*
UMT 10	*Bacilluslicheniformis*
UMT 11	*Bacillus* sp.
UMT 12	*Bacillus* sp.
UMT 13	*Bacillus* sp.
UMT 14	*Bacillus* sp.

Identification of Monosaccharides

A thick pellicle-like morphology was observed on the fermentation culture and five isolates were thus chosen for further monosaccharide identification as indicated in Table 3. Different types of monosaccharides were identified through the selected strain, suggesting that it may play a role in this medicinal broth. The mean polysaccharide produced by each strain varied in quantity (mg/ml). The mean polysaccharide value across these five isolates was 130.72 ± 85.65 mg/ml. The identified monosaccharides are reported in Table 3 based on the Rf. value of the detected spot in reference to standard sugars (Table 4).

Discussion

A total of 14 isolates belonging to genus *Bacillus* demonstrated the possibility of polysaccharide-producing bacteria on the basis of biochemical and paper chromatogram profiles. In this systematic study, we demonstrated that this *Bacillus* spp. produced various types of monosaccharides, under static fermentation conditions. Most *Bacillus* spp. are versatile chemoheterotrophs capable of respiration by using a variety of simple organic compounds. In some cases, they can also ferment carbohydrates in a mixed reaction that typically produces glycerol and butanediol (Sutherland, 2001). A few species, such as *Bacillus megaterium*, does not require any organic growth factors whereas others may require amino acids, B-vitamins, or both. Moreover, the majority of the *Bacillus* spp are mesophiles, with temperature optima between 30 and 45 ^{o}C. However, it was observed that the isolated species grew at temperatures up to 50^{o}C, further suggesting that these isolates may belong to a thermophilic group. The genus *Bacillus* also contains a number of thermophilic species with optima as high as 65^{o}C (Oppenheimer and Zobell, 1952).

Bacillus species are easily isolated and readily grown in the bacteriology laboratory. The most accurate way to distinguish the member of genus *Bacillus* is by placing them into ecophysiological groups, such as nitrogen-fixers, denitrifying insect pathogens, animal pathogens, thermopiles and antibiotic producers. Moreover, antibiotic producers of genus *Bacillus* include *B. brevis* (e.g. gramicidin, tyrothricin), *B. cereus* (e.g. cerexin, zwittermicin), *B. circulans* (e.g. circulin), *B. laterosporus* (e.g. laterosporin), *B. licheniformis* (e.g. bacitracin), *B. polymyxa* (e.g. polymyxin, colistin), *B. pumilus* (e.g. pumulin) and *B. subtilis* (e.g. polymyxin, difficidin, subtilin, mycobacillin) (Yang, 2000; Marshall *et al.,* 1995). In addition, EPS usually consists of three to eight monosaccharides (De Vuyst *et al.,* 2001) and the identified species of monosaccharides from the sample were more than three (Table 3). EPS from lactic acid bacteria were galactose, glucose and rhamnose (Moonmangmee *et al.,* 2000) and our results are in concordance with the study conducted by Michela *et al*. (1999), who suggested that bacteria derived from the same species produce the same type of monosaccharides. Only isolates UMT08, UMT 11 and UMT12 contain rhamnose while other isolated strains contain galactose

Table 3. Different amount and species of monosaccharide produce by selected *Bacillus* spp.

Isolates	Mean amount of polysaccharides produced (mg/ml)	Identified Monosaccharides
UMT 08	270	Lactose, Raffinose, Galactose, Glucose Mannose, Xylose, Rhamnose
UMT 09	87.5	Galactose, Mannose, Arabinose
UMT 10	156.3	Raffinose, Maltose, Trehalose, Galactose, Mannose, Xylose
UMT 11	66.3	Lactose, Trehalose, Galactose, Arabinose, Xylose, Rhamnose
UMT 12	72.5	Lactose, Raffinose, Glucose, Arabinose, Xylose, Rhamnose

Table 4. R_f value for standard sugars and selected spot of monomers from the paper chromatography using two different solvents.

Standard sugars		Rf Value for isolated monosaccharide						
		UMT 08 (HCl)	UMT 08 (TFA)	UMT 09 (HCl)	UMT 10 (HCl)	UMT 10 (TFA)	UMT 11 (HCl)	UMT 12 (HCl)
Lactose	~ 0.10	-	~ 0.10	-	-	-	~ 0.15	~ 0.10
Raffinose	~ 0.30	-	~ 0.33	-	-	~ 0.30	-	~ 0.27
Maltose	~ 0.43	-	-	-	-	~ 0.40	-	-
Trehalose	~ 0.46	-	-	-	~ 0.50	-	~ 0.46	-
Galactose	~ 0.90	~ 0.90	-	~ 0.70	~ 0.90	~ 0.90	~ 0.92	-
Glucose	~ 1.00	-	~ 1.00	-	-	-	-	~ 1.00
Mannose	~ 1.10	~ 1.10	~ 1.15	~ 1.10	~ 1.20	~ 1.10	-	-
Arabinose	~ 1.40	-	-	~ 1.40	-	-	~ 1.35	~ 1.37
Xylose	~ 1.70	-	~ 1.86	-	~ 1.70	-	~ 1.80	~ 1.70
Rhamnose	~ 2.40	-	~ 2.30	-	-	-	~ 2.50	~ 2.30

Key: TFA: Trifluoroacetic acid; HCl: Hydrochloric acid

and mannose as the major sugar identified. This condition may indicate that the bacteria from the same genus may produce polysaccharides of the same type of monosaccharide.

According to Sudhamani *et al*., (2004), when the bacteria used lactose as a primary carbon source, the EPS production in media will contain sucrose, maltose, glucose, galactose, fructose and xylose and may contain rhamnose, arabinose and mannose. However, in our experimental conditions under YPE fermentation, the entire isolates were able to produce EPS with various sugars. During incubation time, the isolates were found to produce pellicles on the YPE medium surface. This pellicle is only produced by bacteria with novel fragile polysaccharides (Marshall *et al.,* 1995). In our experiment, no pellicles were observed in the cultures with non–polysaccharide-producing bacteria. *B. subtilis* UMT09 produced a thick pellicle within a few hours of static incubation. The crude polysaccharide isolated from this *B. subtilis* showed fragile characteristics. The monosaccharides from this bacterium were galactose, mannose and arabinose. Studies by other groups showed that the *B. subtilis*

polysaccharide contain monosaccharides such as glucosamine, galactosamine, mannosamine, glucose, galactose, glucuronic acid, (Iwasaki et al., 1989) and a repeating unit of hexasaccharide (Fontaine et al., 1991). The *Bacillus* sp. polysaccharide was reported to have much higher flocculating activity than xanthan and guar gum (Yun and Park, 2000).

Conclusions

Phenotypic studies of the microbial communities of the Malay traditional medicine have provided valuable information on the existence of potential polysaccharides-producing bacteria. There are still many opportunities for new discoveries in this Malay traditional medicine and to understand the function beyond the production of particular polysaccharides which may act in the immune responses. Further studies are in progress to identify potential strains for pharmaceutical and biomedical applications.

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