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Characterization of Exopolysaccharides Produced by *Bacillus cereus* and *Brachybacterium* sp. Isolated from Asian Sea Bass (*Lates calcarifer*)

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ABSTRACT

Aims: EPS extracted from marine bacteria, which associated with Asian sea bass has potential antimicrobial activities.
Methodology and Results: Two marine Bacteria were isolated from Asian sea bass *(Lates calcarifer)* obtained from aquaculture farm, located at Johor bahru Malaysia. 16S rRNA analysis for bacteria identity revealed that bacteria ors1 had 99 % identity to *Bacillus cereus* and ors2 had 96 % identity with *Brachybacterium* sp. All bacteria shared many similarities and variation in terms of biochemical reactions and microscopic observation. Exopolysaccharides (EPSs) were extracted and purified from bacteria as they produced mucous colonies. Average analysis of EPS components showed 50 % carbohydrates, 26 % protein and 24 % fatty acids. The FTIR analysis confirmed the functional groups of the EPS. Screening for antimicrobial activities assays using Kirby-Bauer methods against both grams positive and negative had shown presence of inhibition zones.
Conclusion, significance and impact of study: This study recommends that bacteria isolated from Asian sea bass are having antimicrobial activities and could be used as a potential source for the development of marine drugs.

Keywords: Exopolysaccharide - *Bacillus cereus* - *Brachybacterium* sp. - *Lates calcarifer* –FTIR

INTRODUCTION

Nowadays, the study of bacteria from marine origin and their potential role in the production of bioactive compounds is becoming a new topic for research (Faulkner, 2001). The number of natural products isolated from marine organisms increases rapidly, and now exceeds 18,000, with hundreds of new compounds being discovered every year. The emergence of resistance of bacteria to antibiotics is a common phenomenon. Therefore, there has been a great concern from scientists to investigate marine microorganisms as new source of antibacterial compounds (Pabba *et al.*, 2011). A number of bacteria present in aquatic ecosystems inhibit growth of other microorganisms by producing antimicrobial substances (Verschuere *et al.*, 2000). Several compounds such as, alkyl amides, alkyl amines and phenolic compounds reported to have antimicrobial activities against both gram-positive and gram-negative organisms (Culler *et al.*, 1979, Kabara *et al.*, 1972, Maddox *et al.,* 2010).

Marine fishes are able to produce bioactive compounds with antibacterial activity to protect them from dangerous pathogens. Antibacterial compounds on fish epidermal are important in controlling fish diseases in aquaculture. Besides, hemolysins are exotoxins produced by bacteria that cause lysis of red blood cells in vitro. They are one of essential bacterial virulence factor (Tran *et al*., 2011). Bacteria adhere to solid surface of fish by extracellular polymeric substances in marine or fresh water environment. EPS is also known as exopolymer, exopolysaccharides and exoplymeric substances produced by archaeal, bacterial and eukaryotic microbes.

EPSs are high molecular weight polymers composed of saccharides subunits and are secreted by a microorganism into the surrounding environment. Microganisms synthesize large spectrum multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides (EPSs). Exopolysaccharides generally consist of monosaccharides and some non carbohydrate substituents (such as protein, nucleic acids, lipids, acetate, pyruvate, succinate, and phosphate). Microbial EPS plays an important task in interaction between bacteria and their environment. Moreover, it has many potential applications in a broad range of fields for instance adhesives, textiles, pharmaceuticals, food additives, oil recovery and metal removal in mining and industrial waste treatment (Decho, 1990). Furthermore, microbial EPSs are extremely susceptible to synthetic biodegradation in nature and less dangerous comparing polymer.

Asian sea bass are distributed within the tropical Indo-Pacific region (Greenwood, 1976) from Arabian Gulf through Southeast Asia to Papua New Guinea and Northern Australia. It is a demersal fish; survive successfully in a broad range of conditions on or near the

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bottom of the sea, lakes, creeks, rivers and estuaries in clear to turbid waters.

The aim of this study is to biochemically characterize EPS from Asian sea bass and to screen for its antimicrobial activities. *Bacillus cereus* and *Brachybacterium* sp. were isolated from Asian sea bass *(Lates calcarifer)*. EPSs test for inhibitory activities against two gram positive organism's *Lysinibacillus* sp. and *Paenibacillus* sp., and four gram negative *Pseudomonas* sp., *Ralstonia* sp., *Mesorhizobium* sp. *and Achromobacter insolitus.*

MATERIALS AND METHODS

Source of the sample

A fish sample was taken from an open sea aquaculture farm located at Gelang Patah Johor-Malaysia on June 2011. It was collected in a new sterile polythene bag and transported to the laboratory immediately for analysis.

Sample dissecting and processing

Weight and size of fish were recorded, general appearance was observed for presence of any abnormalities. All dissecting tools such as scissors, blades, scalpel, forceps, and tray were autoclaved. During dissecting, aseptic condition was performed. Samples from gut, eye gills, kidney and skin were obtained and kept in sterile microbiological tubes prior to inoculation into appropriate media. The unknown bacteria were isolated from fish organs, streaked on nutrient agar plates and incubated at 30 °C for overnight.

Screening for EPS production

Bacterial isolates were screened for EPS producing ability, by inoculation on nutrient agar medium with 5 % glucose as carbon source. The plates were incubated at 30 °C for 48 h and observed for mucoid colonies. *Bacillus cereus* strain ors1 and *Brachybacterium* sp. strain ors2, showed this type of morphology, and expected to produce EPSs. Further characterization and detailed studies were carried out to isolates.

Molecular analysis

Phenotypic identification was carried out according to Bergey's Manual of Systematic Bacteriology (Bergey, 1987). Biochemical and physiological tests, and further confirmed by genotypic identification through 16S rRNA sequencing. Morphological studies were achieved by microscopic observations of Gram stained cells under an oil immersion objective on a light microscope. For 16S rRNA sequencing, DNA of the isolates was extracted and purified. Extraction of DNA material was performed by using Promega Wizard® Genomic DNA Purification Kit. Universal PCR primers were used to amplify the 16S rRNA gene to provide phylogenetic information. The sequences of primers used are:
Forward "5'-AGA GTT TGA TCC TGG CTC AG-3' " and,
Reverse "5'-ACG GCT ACC TTG TTA CGA CTT-3' ".

The amplification reactions were achieved by PCR, followed by purification with QIAquick PCR purification kit (Qiagen, Hilden, Germany) prior sending for sequencing to 1st Base Laboratory, Selangor-Malaysia). The sequences were evaluated to the sequence in the public databases using BLAST search program on (NCBI) website (http://www.ncbi.nlm.nih.gov/) (Jing and Huyop, 2007).

Hemolytic activity

Blood Agar is a differential growth medium that can differentiate pathogenic from nonpathogenic bacteria. Inoculate organisms tested onto blood agar which is enriched with 5 % human blood and incubated the plate at room temperature for 48 h. Then, observed for the ability to lyse red blood cells. The types of hemolysis include alpha, beta and gamma. Alpha hemolysis represents bacteria that can partially break down the blood cells, causing green or brown discoloration of the agar around the colony. Beta hemolysis demonstrates bacteria that can completely lyse the blood cells casing a clear zone around the colony. Gamma hemolysis indicates bacteria cannot lyse the cells, thus causing no change in the agar (Faddin 1980., Lesmana *et al*., 2001).

Extraction and purification of exopolysaccharide (EPS)

Cold absolute ethanol extraction method has been applied in this project. 48 h grown bacterial cultures (100 mL) in LB broth for each were centrifuged at 8000 rpm for 20 minute. Pellets were resuspended in 300 µL EDTA solution (10 mM EDTA + 1.5 mM NaCl) and heated at 50 °C for 3 minutes in water bath in order to extract cells bound EPS. Suspensions were centrifuged, and supernatants were decanted and mixed with previous supernatants and pressure filtered through cellulose nitrate filters. EPS was precipitated by adding three volumes of chilled ethanol to filtrates and incubating overnight at 4°C. EPSs were recovered by centrifugation, and to remove impurities, it was dialysed for 48 h using 8 kDa MW cut-off dialysis bags against distilled water at 4 °C for 24 h. EPSs were lyophilised and stored at –20 °C until further analyses (Anju *et al.*, 2010).

Fourier-transformed infrared spectroscopy

FTIR is an instrument competent to detect the functional groups of purified EPSs. Pellets for infrared analysis were obtained by grinding 2 mg of EPSs with 200 mg of dry Potassium bromide the mixture was pressed into a 16 mm diameter mould. The FTIR spectra was recorded on a PERKIN ELMER instruments-spectrum One –FTIR Spectrometer (Abu *et al*., 1991).

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Characterization of EPS

Colorimetric analysis after dialysis, purified EPS was subjected to determination of total carbohydrate and protein contents. Total neutral carbohydrate contents were measured by phenol–sulfuric acid method by using D-glucose as a standard. (Dubois *et al.,* 1956) , and the protein contents were measured by Folin-Ciocalteau method (Classics Lowry *et al.,* 1951). Free fatty acid tests were determined by the method used by Novak, 1965 Antibacterial activities were achieved by using disk diffusion method. The zones of inhibition in discs were measured by millimeter scale (Bauer *et al*., 1999).

Antibacterial activities of exopolysaccharides

Antibacterial activities were achieved by using disk diffusion method by quantifying the clear zone of inhibition around the filter paper disk. In this method, the autoclaved filter paper discs were impregnated with 50 µl of the EPSs and positioned on nutrient agar plates seeded with the test organisms. Two gram positive organisms, *Lysinibacillus* sp., *Paenibacillus* sp. and six gram negative *Escherichia coli, Pseudomonas aeruginosa*, *Ralstonia* sp*., Mesorhizobium* sp. *Proteus vulgaris* and *Achromobacter insolitus* organisms were used in this study. Chloramphenicol was used as a positive control, while distilled water was used as a negative control. After firm placement of the discs the plates were incubated at 37 °C in inverted position for 1-2 days to allow different species of bacteria to grow. The zones of inhibition in discs were measured by millimeter scale (Bauer *et al*., 1999).

RESULTS

Identification of marine bacteria

Table 1 shows the morphological characteristics of the colonies which includes diameter, shape, margin and pigmentation of the colonies of the bacteria. Besides the analysis of Gram staining and biochemical analysis of ors1 and ors2 bacteria which includes catalase, oxidase, urease, citrate, motility, starch analysis, casein hydrolysis, Mackoncky Agar, hemolysis on blood Agar, H_2S fermentation, Voges Proskauer and Indole test.

Blood agar hemolysis

Bacillus cereus strain ors1 demonstrated a clear β hemolysis in forms of clear zone, which indicates presence of bacterial toxin. While *Brachybacterium* sp. strain ors2 showed α hemolysis, dark and greenish colonies on blood agar. Both bacteria were incubated at 30 °C for overnight.

Comparison components of EPS

The average of partial EPS components were calculated and summarized in one pie chart as in **Figure 1**, the chart confirming the early studies of polysaccharides that carbohydrates are major EPS components. Moreover, the pie charts also verifying that EPSs are a heterogeneous matrix of polymers. Similarly, the results of comparison in EPSs component showed the carbohydrates content, 50 % is higher than protein and fatty acid contents which represent 26 % and 24 % respectively.

Table 1: Basic biochemical analysis of ors1 and ors2

Characteristics	ors1	ors2
Colony diameter(mm)	0.5~1mm	1mm
Shape of colonies	circular	oval
Colony margin	undulate	raised
Colony pigmentation	red	yellow
Gram stain	Positive	Positive
Catalase	Positive	Positive
Oxidase	Negative	Negative
Urease	Negative	Negative
Citrate	Positive	Negative
Motility	Motile	Non Motile
Starch hydrolysis	Positive	Positive
Casein hydrolysis	Positive	Negative
Mackoncky Agar	Negative	Negative
Hemolysis on Blood Agar	Beta hemolysis	Alpha hemolysis
H_2S fermentation	Negative	Negative
Voges Proskauer	Positive	Negative
Indole	Negative	Negative

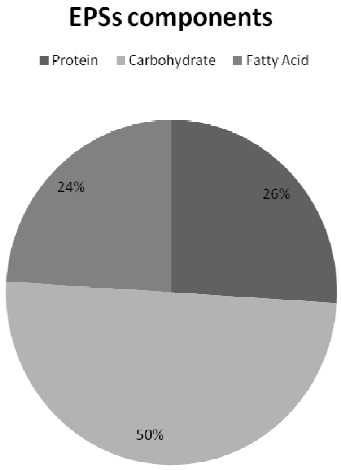

Figure 1: An average of partial components of EPS summarized in pie chart

Fourier-transformed infrared spectroscopy

The FTIR spectrum of the EPS revealed characteristics of functional groups (Figure not shown) such as N-H stretching peak of primary and secondary Amines and Amides group at 339369 m^{-1} and stretching peak of alkenes group at 164472 m^{-1}. Peak values recorded at

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2095.51 Absorption indicated alkenes, ketones, isocyanate and isothiocyanate groups. The value obtained between 1023.49-115939 m^{-1} indicates alcohols, ethers, esters carboxylic acids and phenols groups. Conspicuous absorption peaks at 77933 m^{-1} are assigned to phenyl rings and aromatic group (Anju *et al.*, 2010., Bragadeeswaran *et al*., 2011).

FTIR analysis of EPS of *Bacillus cereus* strain ors1 clearly revealed presence of many protein-related amine and amide groups indicating the possible presence of bacterial toxins. As a result, we can guess that EPS may considerably contribute in pathogenesis. In addition , the presence of some phenolic and carboxylic groups from FTIR analysis of the EPS may possibly account for antibacterial activity (Anju *et al*., 2010).

Antibacterial activity of EPS

EPS1 isolated from the strain ors1 showed inhibitory activities against *Lysinibacillus*, *Paenibacillus sp*, *Escherichia coli* and *Pseudomonas aeruginosa* but it did not show any inhibitory activities against *Ralstonia sp*. *Mesorhizobium sp. Achromobacter insolitus* and *Protrus vulgaris.* EPS2 isolated from the strain ors2 showed inhibitory activities against *Lysinibacillus*, *Paenibacillus sp.*, *Pseudomonas sp., Escherichia coli* and *Mesorhizobium sp.* but did not show any inhibitory activities against *Ralstonia sp., Achromobacter insolitus* and *Protrus vulgaris.*

DISCUSSION

The present study focused on *Lates calcarifer* and its associated microorganisms for the maintenance of antimicrobial defenses. Seawater typically contains bacteria, fungi, viruses and algae (Engel *et al*., 2002), including those which have been identified as causative agents in marine infectious diseases (Correa, 1997). Marine invertebrates and their symbionts are continuously exposed to a broad array of potentially harmful microorganisms, it is reasonable that the production of bioactive secondary metabolites could act as fundamental mechanisms of antimicrobial defence.

The discovery of new classes of antibiotics is necessary due to the increased incidence of multiple resistance among pathogenic microorganisms to drug that are currently in clinical use (Burgess *et al,*.1999). Johor, Malaysia enjoys a large coastline with diverse marine environment. The microbial variety was not greatly studied from Malaysian marine ecosystem in respect to bioactive compounds associated with Asian sea bass bacteria.

Hemolysins are one of the essential bacterial virulence factors that possible suggest the presence of bacterial toxin (Asao *et al*., 1984). β and α hemolysins were reported in ors1 and ors2 respectively. EPSs have adhesive prosperities so it could participate in the role of virulence of pathogenic bacteria, since it facilitates the interaction between the fish and bacteria. In addition, FTIR results showed presence of toxic compounds. Therefore, we can consider that EPSs also involve significantly in pathogenesis (Anju *et al*., 2010).

The presence of phenolic and carboxylic compounds which are obvious from stretching peaks in FTIR could suggest that EPSs involved in antibacterial activities against indicator organisms. Numerous antimicrobials compounds have been reported from marine microorganisms associated with fishes, attributable to their great role in their hosts. Furthermore, even pathogenic strains can be used as biological control agents alongside with other pathogens due to natural presence of various virulence factors, specifically hemolysin, and EPSs along with several antibacterial organic compounds. As a result, antibacterial metabolites synthesized by the test organism may serve as important drugs to control pathogenic bacterial strains causing fish and human diseases, and isolation of novel bacterial strains with antimicrobial activity recommends that marine ecosystem is a valuable foundation of antimicrobials.

Anju *et al*., 2010, isolated a pathogenic Aeromonas hydrophila strain An4 from marine catfish, which is able to produce β Hemolytic, and EPS, besides its antibacterial activity against marine bacterial Fish Pathogens. Shankar *et al*., 2010 isolated four biofilm bacteria (*Galionella* sp., *Alteromonas* sp*., S.aureus*, *Klebsiella* sp.) from the marine water. The EPS of the bacteria were isolated and tested for their antimicrobial activity. The results showed that higher activity against *Alteromonas* sp.

CONCLUSION

In conclusion, results of the present study recommend that bacteria isolated from Asian sea bass are having antimicrobial activities and could be used as a potential source for the development of marine drugs, as the emergence of resistance of bacteria to antibacterial drugs is a widespread phenomenon. Thus, there has been a great concern from researchers to explore marine microorganisms as novel source of antibacterial compounds. Further, exopolysaccharides produced by marine bacteria are reported to have various industrial, pharmaceutical and medical applications.

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