คุณสมบัติการยับยั้งเชื้อจุลินทรีย์ของสารโพลีแซคคาไรด์เจลจากเปลือกของผลทุเรียน

นางสาว นันทวัน นันทวนิช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเวชเคมี ภาควิชาชีวเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2544
ISBN 974-03-1642-5

ANTIMICROBIAL PROPERTY OF POLYSACCHARIDE GEL FROM DURIAN FRUIT-HULLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biomedicinal Chemistry
Department of Biochemistry
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2001

ISBN 974-03-1642-5

Thesis Title	Antimicrobial property of polysaccharide gel from durian fruit-hulls
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นันทวัน นันทวนิช: คุณสมบัติการยับยั้งเชื้อจุลินทรีย์ของสารโพลีแซคคาไรด์เจลจากเปลือกของผลทุเรียน. (ANTIMICROBIAL PROPERTY OF POLYSACCHARIDE GEL FROM DURIAN FRUIT-HULLS) อาจารย์ที่ปรึกษา: รศ. ดร. สุนันท์ พงษ์สามารถ, อาจารย์ที่ปรึกษาร่วม: รศ. ดร. วิมลมาศ ลิปิพันธ์, 93 หน้า. ISBN 974-03-1642-5

ได้ทำการศึกษาในหลอดทดลองเกี่ยวกับคุณสมบัติการต้านเชื้อจุลินทรีย์ของสารโพลีแซคคาไรด์เจลจากเปลือกของผลทุเรียนเพื่อค้นหาสารโพลีแซคคาไรด์ชนิดใหม่ที่มีฤทธิ์ต้านจุลินทรีย์ สารโพลีแซคคาไรด์เจลนี้ประกอบด้วยน้ำตาลชนิดต่างๆ ได้แก่ อราบิโนส แรมโนส ฟรุคโตส กลูโคส และกรดกาแลคทูโรนิค โดยทำการทดสอบกับเชื้อจุลินทรีย์มาตรฐานสายพันธุ์ต่างๆ เชื้อจุลินทรีย์ที่ใช้ในการทดสอบทุกสายพันธุ์มีความไวต่อยาปฏิชีวนะที่ใช้ในการทดลอง ผลการทดสอบพบว่าสารโพลีแซคคาไรด์เจลมีฤทธิ์ยับยั้งเชื้อแบคทีเรีย 7 สายพันธุ์ จากการทดสอบความไวของเชื้อจุลินทรีย์โดยเทคนิค agar diffusion สารโพลีแซคคาไรด์เจลที่ความเข้มข้นตั้งแต่ 0.32 เปอร์เซ็นต์ ให้ขนาดของวงใสมีขอบเขตที่คมและชัดเจนบนอาหารวุ้น ต้านการเจริญต่อเชื้อ *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9341, *Staphylococcus epidermidis* ATCC 12228, *Lactobacillus pentosus* ATCC 8041 และ *Escherichia coli* ATCC 25922 สำหรับสารโพลีแซคคาไรด์เจลที่ความเข้มข้นตั้งแต่ 0.625 เปอร์เซ็นต์ และ 1.25 เปอร์เซ็นต์ ขึ้นไปจะให้ขนาดวงใสต้านการเจริญต่อเชื้อ *Staphylococcus aureus* ATCC 6538P และ *Proteus vulgaris* ATCC 13315 ตามลำดับ การทดสอบโดยใช้เทคนิค broth macrodilution เป็นการทดสอบเพื่อหาปริมาณของสารโพลีแซคคาไรด์เจลที่ใช้ในการออกฤทธิ์ต้านเชื้อจุลินทรีย์ ค่าความเข้มข้นต่ำสุดของสารโพลีแซคคาไรด์เจลที่สามารถยับยั้งการเจริญของเชื้อ *B. subtilis*, *M. luteus*, *S. epidermidis*, *E. coli* และ *P. vulgaris* มีค่าเท่ากับ 6.4 มิลลิกรัม/มิลลิลิตร สำหรับ *S. aureus* และ *L. pentosus* นั้น ค่าความเข้มข้นต่ำสุดของสารโพลีแซคคาไรด์เจลที่สามารถยับยั้งเชื้อมีค่าเท่ากับ 12.8 และ 25.6 มิลลิกรัม/มิลลิลิตร ตามลำดับ การประเมินระยะเวลาของการทำลายเชื้อแบคทีเรียที่มีความไวต่อสารโพลีแซคคาไรด์เจลพบว่าสารโพลีแซคคาไรด์เจลที่ความเข้มข้น 25.6 มิลลิกรัม/มิลลิลิตร สามารถทำลายเชื้อ *B. subtilis* โดยเห็นผลของจำนวนโคโลนีที่ลดลงเหลือ 0 ในชั่วโมงที่ 12 และที่ความเข้มข้นของสารโพลีแซคคาไรด์เท่ากับ 25.6 มิลลิกรัม/มิลลิลิตร สามารถทำลายเชื้อ *M. luteus*, *E. coli* และ *P. vulgaris* ; *S. aureus* และ *S. epidermidis* ในชั่วโมงที่ 16 และ 20 ตามลำดับ สำหรับ *L. pentosus* นั้นจำนวนโคโลนีจะลดลงเหลือ 0 ในชั่วโมงที่ 8 เมื่อใช้สารโพลีแซคคาไรด์เจลความเข้มข้น 51.2 มิลลิกรัม/มิลลิลิตร การทดสอบเชิงคุณภาพของสารโพลีแซคคาไรด์เจลในการออกฤทธิ์ต้านจุลินทรีย์นั้นได้ทำการศึกษาโดยใช้โพลีแซคคาไรด์เจลที่เตรียมในรูปของแผ่นฟิล์ม ซึ่งสามารถอ่านผลการยับยั้งเชื้อจุลินทรีย์ได้จากความโปร่งใสของแผ่นฟิลม์ในบริเวณที่คลุมทับบนอาหารวุ้น การศึกษาภายใต้กล้องจุลทรรศน์อิเล็คตรอนแบบส่องกราดพบการเปลี่ยนแปลงบนพื้นผิวของเชื้อแบคทีเรียซึ่งบ่มในสารละลายน้ำเกลือที่มีสารโพลีแซคคาไรด์เจล สำหรับการทดสอบด้านการพัฒนาฤทธิ์ต้านสารโพลีแซคคาไรด์เจลของเชื้อ *S. aureus* และ *E. coli* โดยใช้เทคนิค repeated exposure ในอาหารเลี้ยงเชื้อที่มีโพลีแซคคาไรด์เจลในความเข้มข้นที่เป็นครึ่งหนึ่งของค่าที่ยับยั้งการเจริญของเชื้อ ผลการทดสอบพบว่า *S. aureus* และ *E. coli* ไม่ถูกชักนำให้มีฤทธิ์ดื้อต่อสารโพลีแซคคาไรด์เจลในระหว่างการทดสอบเป็นระยะเวลา 30 วัน ในการศึกษาครั้งนี้สารโพลีแซคคาไรด์เจลไม่มีผลยับยั้งการเจริญต่อเชื้อแบคทีเรีย 4 สายพันธุ์ คือ *Lactobacillus plantarum* ATCC 14917, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 10031 และ *Pseudomonas aeruginosa* ATCC 9721 และเชื้อยีสต์สองสายพันธุ์ที่ทดสอบคือ *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10230

ภาควิชา	.....ชีวเคมี.....	ลายมือชื่อนิสิต.....	
สาขาวิชา	.....ชีวเวชเคมี.....	ลายมือชื่ออาจารย์ที่ปรึกษา.....	
ปีการศึกษา	.....2544.....	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....	

4376583533: MAJOR BIOMEDICINAL CHEMISTRY

KEY WORD: ANTIMICROBIAL AGENT / DURIAN / *Durio zibethinus* / POLYSACCHARIDE GEL

NANTAWAN NANTAWANIT: ANTIMICROBIAL PROPERTY OF POLYSACCHARIDE GEL FROM DURIAN FRUIT-HULLS. THESIS ADVISOR: ASSOC. PROF. SUNANTA PONGSAMART, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. VIMOLMAS LIPIPUN, Ph.D., 93 page. ISBN 974-03-1642-5

Antimicrobial property of polysaccharide gel (PG) from fruit-hulls of durian (*Durio zibethinus* L.) was investigated to find a novel antimicrobial polysaccharide. Since PG was polysaccharide composed of sugars including arabinose, rhamnose, fructose, glucose and galacturonic acid. *In vitro* activity was performed to evaluate the susceptibility of microorganisms to PG. All standard strains of test microorganisms were susceptible to test antibiotic. PG showed inhibitory activity against 7 strains of bacteria. The susceptibility test was determined by agar diffusion method, inhibition zone of sharp and clear margin was observed on agar media with PG at concentration down to 0.32% against *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9341, *Staphylococcus epidermidis* ATCC 12228, *Lactobacillus pentosus* ATCC 8041 and *Escherichia coli* ATCC 25922, to 0.625% and 1.25% against *Staphylococcus aureus* ATCC 6538P and *Proteus vulgaris* ATCC 13315, respectively. Broth macrodilution method was used to determine a quantitative antimicrobial activity of PG. Minimal Inhibitory Concentration (MIC) of PG against *B. subtilis*, *M. luteus*, *S. epidermidis*, *E. coli* and *P. vulgaris* was 6.4 mg/ml and against *S. aureus* and *L. pentosus* were 12.8 and 25.6 mg/ml, respectively. Time-kill study demonstrated killing effect of PG against susceptible bacteria, the results showed that colony counts of bacteria were declined to zero with 25.6 mg/ml of PG at 12 hours against *B. subtilis*; at 16 hours against *M. luteus*, *E. co*li and *P. vulgaris;* at 20 hours against *S. aureus and S. epidermidis*. Whereas the colony count of *L. pentosus* was declined to zero with 51.2 mg/ml of PG at 8 hours. Preparation of PG as polysaccharide film was also qualitative assay for antimicrobial activity. Inhibitory effect of PG film against susceptible bacteria was demonstrated a transparency area on agar medium covered with PG film. Examination of bacterial cells exposed to PG in normal saline solution under scanning electron microscope appeared an alteration on cell surface of bacteria. The development of PG resistance in *S. aureus* and *E. coli* were studied by repeated exposure method in medium containing sub-inhibitory concentration of PG. The results demonstrated that *S. aureus* and *E. coli* were not inducible to obtain the PG resistance during 30 days. Inhibitory activity of PG was not found against 4 test bacteria, *Lactobacillus plantarum* ATCC 14917, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 9721, and 2 test yeast, *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10230 in this study.

Department ……Biochemistry…… Student's signature……..

Field of study ……Biomedicinal Chemistry….. Advisor's signature……..

Academic year……...2001…….…….… Co-advisor's signature……..

ACKNOWLEDGEMENTS

I wish to express my deepest appreciation and grateful thank to my thesis advisor, Associate Professor Dr.Sunanta Pongsamart of the Department of Biochemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University for her excellent instruction encouragement and guidance throughout this study.

I wish to express my grateful thank to my thesis co-advisor, Associate Processor Dr.Vimolmas Lipipun of the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for her valuable advice and encouragement throughout this study.

I would like to thank to the members of the department of Biochemistry and department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for their kindness and helps.

I would like to thank to my teachers and my friends at the department of Biochemistry and Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for their contribution and friendship.

I am very thankful Associate Processor Dr.Somboon Tanasupawat of the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for providing test microorganisms.

I would like to thank the Scientific and Technological Research Equipment Center, Chulalongkorn University for providing equipment.

I would like to thank the Faculty of Pharmaceutical Sciences Research Funds and the Ministry of University affairs for granting financial support in part to conduct this investigation.

Finally, I wish to express my infinite gratitude to my family for their love understanding and encouragement throughout my life.

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ABBREVIATIONS

ATCC	=	American Type Culture Collection, Maryland, USA
BHIA	=	brain heart infusion agar
BHIB	=	brain heart infusion broth
°C	=	degree celsius
CFU	=	colony forming unit
DMSO	=	dimethyl sulfoxide
g	=	gram
hr.	=	hour
hrs.	=	hours
kg	=	kilogram
L	=	liter
MBC	=	minimal bactericidal concentration
mg	=	milligram
MHA	=	mueller hinton agar
MHB	=	mueller hinton broth
MIC	=	minimal inhibitory concentration
min	=	minute
ml	=	milliliter
mm	=	millimeter
µg	=	microgram
µl	=	microliter
NCCLS	=	National Committee for Clinical Laboratory Standard
NSS	=	normal saline solution
SDA	=	sabouraud dextrose agar

SDB	=	sabouraud dextrose broth
S.E.M	=	standard error of mean
SEM	=	scanning electron microscope
TSA	=	tryptic soy agar
TSB	=	tryptic soy broth

CHAPTER Ⅰ

GENERAL BACKGROUND

1. Introduction

Polysaccharide gel (PG) isolated from fruit-hulls of durian (*Durio zibethinus* L.) have been found to be useful in preparation of food and pharmaceutical products such as jelly, tablet, suspension and emulsion (Pongsamart, Dhumma-Upakorn and Panmuang, 1989; Umprayn, Kaitmonkong and Pongsamart, 1990; Umprayn, Chanpaparp and Pongsamart, 1990). The composition of sugars and properties of PG were previously described (Pongsamart and Panmuang, 1998; Girddit *et al*.,2001). Toxicity test of PG was determined, a high oral dose (2g/kg) did not induced severe toxicity in male mice and rats (Pongsamart, Sukrong and Tawatsin, 2001 a). No toxic effects were observed in subacute treatment in male mice (Pongsamart, Jesadanont and Markman, 1989) and subchronic studies in male and female mice confirmed the consumptive safety of PG (Pongsamart, Sukrong and Tawatsin, 2001 b).

Some natural polysaccharides have antimicrobial properties, such as pectin (Petronio *et al*., 1997), acacia gum (Tokura *et al.*, 1999) and alginate (Song *et al*.,2001) showing antibacterial action. In addition, chitosan showing both antibacterial and antifungal action (Ibrahim, 1981; Jung *et al*., 1999). Chitosan has attracted much research attention in the last 20 years as a potentially important renewable resource that is both nontoxic and biodegradable (Goosen, 1997). After discovery of antimicrobial activities of chitosan, many researchers have continued studies in this field. For antimicrobial properties of chitosan are reportedly used in food, medical, pharmaceutical and industrial applications (Onsoyen and Skaugrud, 1990).

A novel natural polysaccharide from fruit hulls of durian have gelling property and sugar component similar to those of high polymeric carbohydrates, which is an

interesting substance for studying in order to discovery of new antimicrobial agent. Polysaccharide gel (PG) would possibly has advantage as a new type of water-soluble antimicrobial agent and give satisfactory results in utilizing as antimicrobial polysaccharides. Bactericidal activity of PG have brief reported recently (Lipipun, Nantawanit and Pongsamart, 2002).

In vitro antimicrobial susceptibility test is used to evaluate activity of new antimicrobial agents. Two basic techniques for determining the antimicrobial activity are agar diffusion and broth dilution method. *In vitro* test results will vary depending upon the test conditions such as, inoculum size, medium, test drug and incubation conditions (Collin, Lyne and Grange, 1995). Therefore, the susceptibility tests must be adequately standardized and controlled in order to give reproducible results.

In this investigation, we have studied about antimicrobial activities of polysaccharide gel from durian fruit-hulls against standard microorganisms and used the antibiotics as a positive control. The cell surface of microorganisms commonly composed of molecules of polysaccharide such as, lipopolysaccharide (LPS), capsular polysaccharide and cationic/anionic polymer (Sharon and Lis, 1993; McKane and Kandal, 1996). PG may bind and interact with these substances which are the cause to interfere normal activities of microorganisms. Therefore, the possible antimicrobial mechanisms of PG would be purposed by examining an alteration on cell surface of PG susceptible organisms under scanning electron microscope.

Resistance of test bacteria against PG have also determined in final study. The emergence of drug resistance is a major factor limiting long-term successful use of antimicrobial agent. Development of resistance in microorganisms is an increasing problem. We therefore embarked on study of development of antimicrobial resistance in microorganisms (Lorian, 1991). There are two techniques to study about the development of drug resistance, *in vivo* and *in vitro* study. Development of resistance will be found to be dependent upon these two basic factors, the character of the drug

and it's mechanism of activity (Schnizer and Grunberg, 1957). The main objective of this investigation is to develop the resistance to PG in PG susceptible microorganisms and evaluate the tendency of microorganism to become resistance to PG.

2. Review of literature

2.1 Structure and properties of polysaccharides

Carbohydrates are essential components of all living organisms and are the most abundant class of biological molecules. Many of natural polysaccharides participate in a variety of biochemical reactions. Current applications used many kinds of natural polysaccharide and their derivative in the pharmaceutical applications such as, chitin, pectin, gum and cellulose (Whistler and BeMiller, 1959).

Polysaccharides may be subdivided into two main categories. The first main group is termed *homopolysaccharide*. Members of this group are defined as containing only a single type of monosaccharide although linkages may vary. Probably satisfactory examples are chitin, starch, amylopectin and cellulose (Davidson, 1967).

The straight chain structures of homopolysaccharide include polymer such as cellulose and chitin. The most abundant animal polysaccharide is chitin. It is the major component of the exoskeletons of insects and crustacean, and the component is an N-acetyl-D-glucosamine $\beta(1\rightarrow4)$ homopolymer. Increasing interest in chitin as a specialty material has arisen as the result of some of its property. It is utilized in widely applications in pharmaceutical, food, medical and agriculture industries (Austin *et al*., 1981).

Cellulose is the most abundant polysaccharide on earth. It is linear polymer of glucose linked $\beta(1\rightarrow4)$ homopolymer and there are no branch point. Although cellulose is insoluble in water. Another is that cellulose can be converted into derivatives with useful solubility characteristics. They are widely used as additives for foods, cosmetics and pharmaceutical products (Dumitriu, 1998).

The second major group of polysaccharide is termed the *heteropolysaccharides*. These are defined as containing two or more different monosaccharide units, such as pectin, glucomannan and gum.

Pectin is a natural constituent of all terestrial plants. It is a heterogeneous complex of polysaccharide and its composition varies with the source and the conditions applied during isolation. Galacturonic acid is the major constituent of all natural pectins and polymerized by (1,4) bonds into linear chain. Pectins also contain varying quantities of neutral sugars, mainly arabinose, galactose, and rhamnose. The major outlet for pectin is through food applications (i.e. as thickening and gelling agent in jams and jellies, or as stabilizer in fruit and milk beverages) and pharmaceutical applications (Dumitriu, 1998).

The famous dietary polysaccharide is glucomannan, which composed of β (1,4)-linked-D-mannose and β(1,4)-linked-D-glucose residues. Among the benefits are improved glycemic control and decreased serum cholesterol (Hozumi *et al*., 1995). Therefore, they are widely used as diet food or food applications.

In additional, gums are substance present a high hydration capacity and in contact with water, they form a soluble colloidal glue of a gel with a very high water content. Gums are produced by pathogenic degradation of certain cells or whole plant tissues. The gum structure appears to consist of many types and ratios of monosaccharide residue, such as acacia gum that is made up of D-galactose, D-arabinose glucuronic acid, L-arabinose and L-rhamnose in a 3:3:1:1 ratio.

In recent years, more and more studies and applications of the antimicrobial activity of natural polysaccharide are being applied in the most medicinal and food industry (Rhoades and Roller, 2000). Hetland *et al*. (2000) reported that the antibacterial effect of a soluble pectin polysaccharide, PM II, isolated from the leaves of *Plantago major* was examined in mice experimentally infected with *Streptococcus*

pneumoniae serotype 6B. The data demonstrated that the pectin, PM II, protects against pnuemococcal infection in mice. In addition, *in vitro* study of antimicrobial properties of pectin shown that pectin was the agent with bactericidal effect on the most widely distributed pathogenic and opportunistic microorganisms and did not influence indigenic microflora. High concentration of pectin (> 2 percent) had and effect on the susceptible organisms (Men'shikov *et al*., 1997).

Maron *et al*. (2001) evaluated the antibacterial activity of mastic gum, a polysaccharides obtained from the *Pistacia lentiscus* tree, against clinical isolates of *Helicobacter pylori* by microdilution assay. The minimal bactericidal concentrations (MBCs) of mastic gum were demonstrated, the gum killed 50% of the strains tested at a concentration of 0.1325 mg/ml and 90% at concentration of 0.5 mg/ml. Maron *et al*. purposed about the influence of the gum on organisms by used of transmission electron microscopy (TEM) and the change of morphological abnormalities and cellular fragmentation in *H. pylori* cells induced by the gum may be the causes of cell dead. Furthermore, the study of Clark *et al*. (1993) demonstrated about the antibacterial activity of acacia gum against fresh isolates and reference strains of *Actinobacillus actinomycetescomitans*, *Capnocytophaga* spp., *Porphyromonas gingivalis*, *Prevoltella intermedia* and *Treponema denticola*. A fine aqueous suspension and a soluble fraction of acacia gum were incorporated into columbia agar at doubling concentrations in agar dilution test. Growth of *P. gingivalis* and *P. intermedia* cultures on the agar was inhibited by whole gum concentrations of 0.5-1.0 w/v.

Many studies that described above related to antimicrobial polysaccharide from plant, the following content is about the attractive antimicrobial animal homopolysaccharide, chitosan. Chitosan is a deacetylated derivative of chitin. It is polycationic at a pH less than 6 and interacts readily with negatively charged substances, such as proteins, anionic polysaccharides, fatty acid, bile acids, and

phospholipids, because of the high density of amino groups present in the polymer (Knorr, 1984). Most commercial chitosan have a certain degree of deacetylation that is greater than 70% and a molecular weight between 100,000 and 1.2 millionDa (Onsoyen and Skaugrud, 1990). Chitosan has attracted much research attention in antifungal, antiviral and antibacterial properties in the last 20 years. Wang *et al*. (1992) showed that the Gram-positive bacteria were more sensitive to chitosan than Gram-negative bacteria with MIC of 1 mg/ml, whereas the MIC for Gram-negative bacteria varied from 1 to 2 mg/ml. Chitosan may induced severe morphological alterations in Gram-positive bacterium, while in Gram-negative bacteria only retraction of the plasmamembrane was evident. Furthermore, the *in vitro* evaluation of Felt *et al*. (2000) showed that concentrations of chitosan as low as 0.0375% still exert a bacteriostatic effect against *Escherichia coli*. Minimal inhibitory concentration (MIC) values of chitosan were calculated to be as low as 0.375 mg/ml for *E. coli* and 0.15 mg/ml for *S. aureus*.

To improved antimicrobial property of chitosan, chitosan heteroxylan derivatives were prepared by Ebringerova, Belicova and Ebringer (1994). Antimicrobial susceptibility test of chitosan polymerized with xylan was determined by a dilution method, using mueller hinton broth for the bacteria and sabouraud dextrose broth for yeast. The inhibitory activities of xylan polymerized chitosan against bacteria, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*, and yeast, *Saccharomyces cerevisiae* were very high potency. The mechanism of antimicrobial action of polymerized chitosan is probably very complex. However, due to the cationic nature of derivatives, it is likely that they can interact and form polyelectrolyte complexes with acidic polymers present in the microbial cell wall surface as do other cationic antimicrobial agents. Also the neutral or acidic glycosyl side chains of quaternized

xylan polymer may be involved in the mechanism, contributing to the intermolecular interactions.

Because of the limited solubility of chitosan in water, synthetic of soluble sugar branched chitosan of Kurita *et al*. (1998) have proved to be a superb agent for antimicrobial. Kurita *et al.* described that sugar branched chitosan exhibited a higher activity against *Pseudomonas sp*. even at such low concentrations as 50 and 5 ppm. Because the branched chitosan are soluble in neutral water unlike linear chitosan, they would have advantages as a new type of water-soluble antimicrobial agent. This synthetic approach would be beneficial for diversification of the molecular design for developing polysaccharide based advanced materials.

Seo, Mitsuhashi and Tanibe (1990) evaluated antifungal activity of chitosan, the in vitro study by broth and agar dilution test demonstrated MIC values against many strains of fungi. The MIC values for *Fusarium oxysporum*, *Botrytis cinerea* and *Trichophyton equinum* were 1,000 ppm, 1,000 ppm and 2,500 ppm, respectively. The antimicrobial effects of chitosan were observed at less than 3% (w/w) of content for *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Antimicrobial activities of chitosan are believed to originate from the polycationic nature of chitosan that can bind with anionic sites in proteins or cellular walls, thus resulting in selective antimicrobial activities toward fungi or bacteria (Jung *et al*. 1998). The mechanism behind the antimicrobial activities of chitosan can be summarized as follow. (1) The cationic nature of chitosan binds with sialic acid in phospholipids, consequently restraining the movement of microbiological substance. (2) Oligomeric chitosan penetrates into cells of microorganisms and prevents the growth of cells by prohibiting the transforming DNA into RNA (Hadwiger *et al*., 1986).

Polysaccharide gel (PG) was extracted from fresh fruit-hull of durian (*Durio zibethinus* L.) (Pongsamart and Panmuang, 1998). The elemental compositions of PG extracts from dried fruit-hulls of durian are carbon, hydrogen and oxygen in molar ratio of 2.9:5.7:3.2 according the study of Girddit *et al*. (2001). The sugar composition of PG was also analyzed by technique of HPLC, PG composed of sugars including arabinose, glucose, fructose, rhamnose and galacturonic acid. Molecular weight of PG was 500-1,400 kDa. Powder of PG swelled in water giving viscous liquid with acid pH at 2.60 $\pm$ 0.14. Mineral composition of PG was also investigated. The major components were Na, K, Ca, and Mg.

The preliminary study to evaluate the susceptibility of bacteria and fungi to PG was demonstrated by Lipipun *et al*. (2002). The results showed that PG produced inhibitory activity against two bacterial strains tested, *Escherichia coli* and *Staphylococcus aureus* at concentration of 2.50% and 1.25%, respectively, in TSB medium. Two strains of fungus tested, *Candida albicans* and *Saccharomyces cerevisiae* were not susceptible to PG by this assays. The mechanism of PG against susceptible bacteria may be related to sugar composition of PG, especially its galacturonic acid composition. Also the neutral side chains of another sugar may be involved in the mechanism, contributing to the intermolecular interactions.

There are three important types of sugar acids (Figure 1): aldonic, aldaric and uronic acid. The aldoses are oxidized at the aldehydic carbon atom by weak oxidizing agents, e.g., sodium hypoiodite or by specific enzyme to from the corresponding carboxylic acid, which are called generally aldonic acids. D-Glucose, for example, yields D-gluconic acid, which in phosphorylated form in an important intermediate in carbohydrate metabolism.

If a stronger oxidizing agent is employed, e.g., nitric acid, both the aldehydic carbon atom bearing the primary hydroxy group are oxidized to carboxyl groups, yielding aldaric acids (also called saccharic acids). With D-glucose the product is called D-glucaric acid. Aldaric acids are sometimes useful for the identification of sugars, but they are of no great biological significance.

However, the third class of sugar acids, the uronic acids, are biologically very important. In uronic acids, only the carbon atom bearing the primary hydroxyl group is oxidized, to a carboxyl group. The uronic acid derived from D-glucose is D-glucuronic acid. Other important uronic acid are D-galacturonic acid and D-mannuronic acid. The uronic acids are components of many polysaccharides. (Lehniger, 1975; Roehrig, 1987)

To explain the mechanism of antimicrobial action of PG, it may be related to the anionic nature of PG likely in chitosan. The mechanism of the antimicrobial activity of chitosan is probably complex. However, given the polycationic nature of chitosan, it is likely that it can interact and form polyelectrolyte complexes with acidic polymers produced at the bacterial cell surface (e.g., lipopolysaccharides, teichoic and teichuronic, or capsular polysaccharides) produce disturbances of membrane or cell wall functions (Muzzarelli *et al*., 1990). Whereas, PG, a polyanionic polysaccharide may be interacted and form complex with cationic substance on cell surface of microorganisms and interfered normal function activity of cell surface.

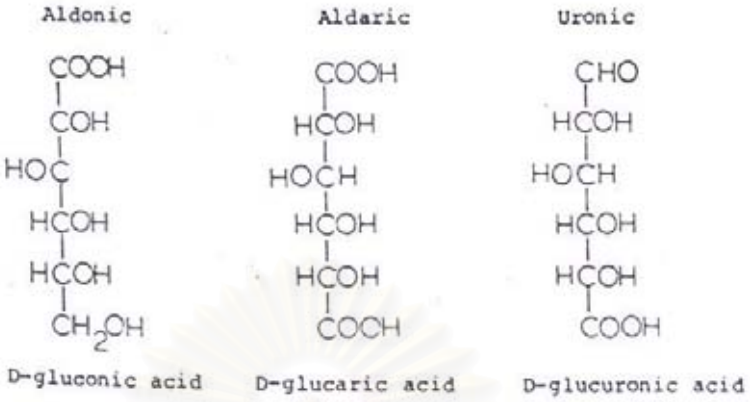


Figure 1 Chemical structure of sugar acids

Furthermore, the mechanisms of antimicrobial action of PG may be invloved the effect of "Osmotic pressure". If the concentration of solutes, such as sodium chloride, is higher in the surrounding medium than in the cell (hypertonic), then water to leave the cell. This osmotic loss of water causes plasmolysis or shrinkage of the cell's plasma (cytoplasmic) membrane. What is so important about this phenomenon is that the growth of cell is inhibited as the plasma membrane pulls away from the cell wall (Tortora, Funke and Case, 1995). Antibacterial activity of honey has been found to prevent growth of microorganism by this mechanism. As a result of solubility of PG in water giving viscous liquid, high concentration of PG might absorb water effecting to removing necessary water from microorganisms, that are present and thus prevent their growth.

2.2 Antimicrobial susceptibility tests

The primary purpose of antimicrobial susceptibility test is to guide the clinician the choice of appropriate agents for therapy. The test is also provided accumulateing data from which information on the suitable agents for empirical use can be derived. Antimicrobial susceptibility tests are use to evaluate an *in vitro* activity of new agents (Collin *et al*., 1995).

In vitro antimicrobial susceptibility tests are depended on two roles, diffusion and dilution. Laboratory procedures involving diffusion susceptibility tests are commonly performed in agar media called agar diffusion technique.

2.2.1 Agar diffusion susceptibility test

In general, agar diffusion tests are performed by inoculating a nutrient agar medium in a standardized manner and then applying the drug to be studied to the agar surface in some type of reservoir. The drug is allowed to diffuse into the surrounding medium. This exposes the test organism to a continuous gradient of drug concentrations, with concentration diminishing as distance from the reservoir increase. After an appropriate period of incubation, there should be a zone of inhibited growth around the reservoir. The size of zone may be measured to determine the degree of susceptibility of test organism (Lorian, 1991).

These tests depend on the ability of the antimicrobial agents to diffuse at predictable rates through the agar gel. The experiment work of Cooper (1964) provided a number of theoretical concepts that have led to a better understanding of variables that influence the formation of a zone inhibition in an agar medium. The more important variable influence diffusion test is described below.

2.2.1.1 Application of drug solution to agar medium

Solution of antimicrobial agents may be applied to surface of a seeded agar medium in several different ways as described by Lorian, 1991 (Figure 2). The convenient method uses filter paper disk that has been moisten with the drug solution and the applied directly to the agar while still wet. Disks may be prepared more accurately if a micropipette is use to load each disk with a measured volume of drug solution.

Alternatively, glass or metal cylinders may be applied to the surface of the seeded agar medium to facilitate application of drug solutions. Agar well may be cut from the seeded agar medium by using a hollow tube (4 to 6 mm in diameters and then be fill with the drug solutions.

About diffusion, the drug diffuses in a two-dimensional manner. In the relatively thin layer of agar normally poured into an agar plate, downward diffusion is quite limited and the concentration in the depth of medium soon approached that near the surface. The amount of drug available to diffuse outward is greater and consequently the zone of inhibition is likely to be larger (Barry and Fay, 1973). In a very thick pour plate, the three dimensional diffusion can result in a semicircular zone of inhibition, being small at the bottom and larger at the surface.

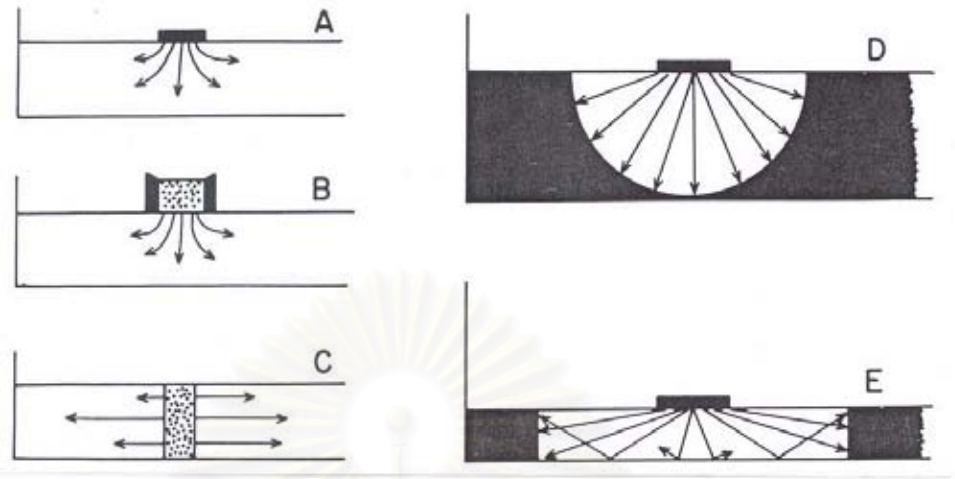


Figure 2 **Application of antimicrobial agent to seeded agar plates; (A) Filter paper disks may be saturated with the drug and applied to the surface of the agar; (B) Glass or metal cylinders may be applied to the agar surface and then filled with the drug solution; (C) Wells may be cut from the seeded agar and then filled with the drug solution; (D) In the latter case, diffusion is more nearly two dimensional. When disks are applied, diffusion is initially three-dimensional but become two-dimensional as the concentration at the bottom approaches that at the surface; (E) In a very thin layer of agar, outward diffusion is more rapid, since an equilibrium with the depths of the agar is quickly reached. Consequently, the zone of inhibition will be larger on thin agar plates. (From Lorian, 1991. p. 6)**

2.2.1.2 Inoculum density

Inocula are prepared by adding cells from four to five isolated colonies of similar colony morphology to a broth medium and then allowing them to grow to the log phase. Four or five colonies, rather than a single colony, are selected to minimize the possibility of testing a colony that might have been derived from a susceptible mutant. Inocula may also be prepared directly by inoculating colonies grown overnight or agar plate or slant into broth or saline. This direct inoculum suspension preparation technique, which does not require incubation, but the use of fresh (Mahon and Manuselis, 2000).

The number of organism tested must be standardizing regardless of the method used. The most widely use method of inoculum standardization involves McFarland turbidity standard and 0.5 McFarland standard (10^8 CFU/ml) is the most commonly used (Lorian, 1991). False susceptible results may be occur if too few or too many organisms. Increasing inoculum size reduce the susceptibility to agents in both diffusion and dilution test (Collin *et al*., 1995). In diffusion method, heavy inocula tend to give small zone of inhibition and when inoculum is light, organism often appear to be susceptible and give larger zone (Cooper, Linton and Sehgal, 1958). Consequently, minor changes in inoculum density result in extreme changes in the result of susceptibility test.

2.2.1.3 Visualization of the zone edge

Barry *et al*. (1979) demonstrated that zone of inhibition on susceptibility test plates can not be measured with extreme degrees of precision. In all situations it is very important to standardize the intensity and angle of light used to illuminate test plates when zone measurements are being determined.

2.2.1.4 Agar depth

The depth of agar medium is critically important in test systems that use very thin (2-3 mm) agar layers (e.g. bioassay system) (Lorian, 1996). In susceptibility testing, the agar medium is usually poured to a depth of about 4 mm (18-25 ml in a 9 cm petri-dish), and at that level fairly major variations in agar depth do not significantly affect the test results (Barry, 1976). With diffusion tests the size of the zone increase as the depth of the agar decrease but this effect is most marked with very thin plates. When working with very thin agar layers, extreme caution must be taken to avoid vary minor variations in the depth of the agar medium in different areas of the same plate.

2.2.1.5 Composition of the agar medium

The agar medium itself profoundly influences the zone sizes in three ways :

1. It affects the activity of different antimicrobial agents.
2. It influences the rate of diffusion of the antimicrobial agents.
3. It affects the growth rate of the test organism.

For susceptibility testing, the composition of test medium must be carefully standardized because the activity of antimicrobial agent is markedly influenced by many factors such as cation in the medium, pH of the medium and presence of various antagonistic materials.

The ideal medium should have sufficient nutrients to support growth of organism normally tested. Mueller-Hinton (MH) medium is the most widely recommended for bacterial susceptibility test (Barry *et al.,* 1978; Modugno *et*

al., 1997; Zarazaga *et al*., 1999). However, Zarazaga *et al.* (1999) investigated Lactobacillus species grew better in Brain heart infusion agar (BHIA) than in Mueller-hinton agar, therefore BHIA more suitable for Lactobacillus species.

Recently, Mueller-Hinton medium has been shown to be acceptable for a wide variety of antimicrobial susceptibility tests because of the relatively good reproducibility and simplicity being regulated to a reference medium (NCCLS, 1986). MH medium is well suited for standard rapid growing pathogen such as enteric gram-negative bacilli, *Pseudomonas* spp., *Staphylococci* spp. and *Enterococcus* sp. For bacteria that do not grow readily on this medium other supplements or alternative media may be required. Modugno *et al.* (1997) and Luh (2000) used MHA supplemented with 5% defibrinate blood sheep as described in NCCLS (1993) for *S. pneumoniae* and Haemophilis species for rapid grow in the susceptibility test medium.

The addition of various supplements of unknown chemical composition may be some instances alter antimicrobial activity. However, if adequate controls are incorporated and standards are developed for appropriate interpretation and if addition control strains are utilized in a testing protocol, media supplemented with varying compounds may be used successfully for the susceptibility test. Eliopoulos *et al.* (1989) reported on the effect of 5% sheep blood added to MHA on the activity of cefoxine and other cephalosporins (e.g. cefpirome, ceftazidime and ceftizoxime) against *E. faecalis* were enhanced by 5% sheep blood addition.

2.2.1.6 Growth characteristics of the test strain

The rate of growth on the test medium obviously affects the end result. In susceptibility testing, some strain to strain variability in growth rate is

unavoidable. Growth conditions have been standardized for optimal results with most of the common rapid-growing microorganisms. Microorganisms that demonstrate delayed growth under these standardized test conditions can not be test reliably. With such slow-growing strains, fairly large zones of inhibition may indicate susceptibility or may simply represent the effect of a delayed growth rate. On the other hand, a very small zone of all, can be interpreted reliably as indicating resistance (Lorian, 1991).

2.2.1.7 Preincubation and Prediffusion

In diffusion tests preincubation and prediffusion decrease and increase the sizes of zone, respectively (Collin *et al*., 1995). For susceptibility testing, the agar media is inoculated and then allowed to dry for a defined period of time before the disks are applied. This drying step is essential to prevent leaching of the antimicrobial agent from disk into the layer of moisture that may be left immediately after incubation of the agar medium. However, as the plates are allowed to dry, the microorganism are beginning the growth cycle, and the time allowed for the antimicrobial agent to diffuse through the agar medium will be diminished. Some procedures require a specific period of prediffusion at room temperature after the antimicrobial agent to diffuse through the agar gel before the critical cell mass is reached. This prediffusion period tends to prolong the lag phase of microbial growth and thus increase the critical time during which the position of the zone of inhibition is determined (Lorian, 1996). For the sake of standardization, the time intervals between inoculation of the plates, application of the antimicrobial disk, and incubation of the test plates must be carefully controlled.

2.2.1.8 Incubation

Susceptibility tests are normally incubated at 35-37°C for optimal growth of the common human pathogens. Use alternative conditions only, if essential for growth of the organisms such as, most fungi can grow well at temperature range 30-35°C. Woolfrey, Burns and Lally (1998) showed that exponentially growing cultures of *Staphylococcus aureus* in a susceptibility test will cease growing when the incubation temperature is increase a few degrees above that required for maximum exponential growth and that cell death will occur with further increase in temperature. The rate of growth will be prolonged at lower temperatures and thus the critical concentration of the antimicrobial agent has more time to diffuse further. To further complicate the situation, most antimicrobial agents diffuse more slowly at lower temperatures, partially because of the increased viscosity of the agar medium (Lorian, 1996). Consequently, low temperature during the critically important early hours of incubation have two effects, one tending to produce larger zones of incubation (decrease growth rate) and the other tending to produce smaller zones of inhibition decrease diffusion rate. Normally, the effect of decrease growth rates predominates and the zone increase with lower temperatures.

Most of *in vitro* tests with antimicrobial agents have been standardized with and "overnight" incubation period, usually defined as 16-18 hr (Isenberg, 1998). With the exception of some organisms, anaerobic bacteria most commonly incubated for 48 hr. (Chin and Neu, 1984; Lorian, 1991). The incubation zone may be come smaller with further incubation because of changes in the character of the growth at the edge of the zone. The character of the zone edge will change as a result of (a) the appearance of delayed growth, (b) better visualization of partially inhibited growth or (c) delayed appearance of resistant variants. Occasionally, the zones appear to increase in size because of change in character of the growth at the

zone edge or because of actual lysis of the initial growth within the inner ring of the zone.

With most of commonly organism pathogens, incubation recommended in an ambient-air incubator. Incubation in an atmosphere containing additional carbondioxide that required for growth in some organisms. Isenberg (1998) reported on the incubation atmosphere in disk diffusion test, *Haemophilus* spp., *Neisseria gonorrhoeae* and *Streptococcus* spp. were grown in CO_2 conditions.

For anaerobic bacteria, many others are killed by briefest exposure to oxygen. Fortunately, many important anaerobes including most clinic cal isolates, appear to be able to tolerate oxygen for the amount of time required for laboratory manipulations. They can be handle at the bench and then incubated in anaerobic jar, air tight containers from which oxygen has been removed by pumping out the air or by chemical reaction (Mckane and Kendel, 1996).

2.2.2 Dilution Method

Dilution antimicrobial susceptibility test methods are used to determine quantitatively in term of the minimum inhibitory concentration (MIC), the lowest concentration of antimicrobial agent required to inhibit the growth of an organism isolate or that which kill it, the minimum bactericidal (fungicidal) concentration (MBC, MFC) (Mahon *et al*., 2000). The NCCLS (1997) document described the details of performing MIC and MBC tests by broth macrodilution, broth microdilution and agar dilution.

2.2.2.1 Broth macrodilution (tube dilution) susceptibility test

Broth dilution tests performed in test tube are referred to as broth macrodilution or tube dilution susceptibility tests. The macrodilution method is most suitable for small numbers of tests (Collin *et al*., 1995). It is impractical for use as a routine method when several antimicrobial agents must be tested on an isolate or if several isolates must be tested. Some laboratories use broth macrodilution (Jackson and Finland, 1951; Washington and Sutter, 1980) when it is necessary to test drugs not include in their routine system of fastidious bacteria that require special growth media. Additionally, this method is often used when minimum bactericidal concentration (MBC) endpoints are to be subsequently determined.

Generally, a twofold serial dilution is prepared. Mueller-hinton broth is medium most commonly used for MIC test of fastidious bacteria, blood supplemented may be added to broth for fastidious organisms as for agar media and sabouraud dextrose broth is commonly used for fungi. A standardize suspension of test bacteria and yeast are added to each dilution to obtain final concentration of 5×10^5 CFU/ml (Mahon *et al*., 2000) and 10^5 CFU/ml (Lorian, 1996), respectively. A growth control tube (broth plus inoculum) and an uninoculated control tube (broth only) are used in each test. After incubation, the MIC is determine visually as the lowest concentration that inhibits growth, as demonstrate by the absence of turbidity (NCCLS, 1997).

2.2.2.2 Broth microdilution susceptibility test

Recently, microdilution method commonly used in the susceptibility test as recommended in many studies (Modugno *et al*., 1997; Ambaye *et al*., 1997; Salvatore *et al*., 1998; Zarazaga *et al*., 1999; Luh *et al*., 2000). The broth

macrodilution test has been adapted to multi-well microdilution trays. Polystyrene trays containing between 80 and 100 wells are filled with small volumes (usually 0.1ml) of two-fold dilution concentrations of antimicrobial agent in broth. Because of the large number of wells, several dilutions as many as 12 to 15 antimicrobial agents can be contained on a single tray, that will be subsequently inoculated with one organism isolate. In the microdilution method multiple plates may be conveniently produce by the use of apparatus for automatically diluting and dispensing solutions. The actual dilution factor used for preparation of the intermediate dilution depends on the volume of inoculum delivered to each well by the inoculating device. A growth control well is include on each tray. Growth may note as turbidity, a haze, or a pellet in the bottom of the well. After incubation, the tray is placed on one of several types of tray-reading devices (Mahon *et al*., 2000). The end point of MIC, as note by Lorian (1991), MIC is the lowest concentration of drug at which the microorganism tested does not demonstrate visible growth.

Several factors influence the outcome and reproducibility of broth susceptibility results. Most factor, media supplements, pH, incubation and inoculum size are likely effected as described in agar diffusion test. The other dilution method is agar dilution susceptibility test is discussed as follows.

2.2.2.3 Agar dilution susceptibility test

The agar dilution method for determining MICs has been accepted as the standard against which other methods are assessed (Ambaye *et al*., 1997; Hoellman *et al.*, 1998). It has advantages over broth dilution methods in that concentration is more easily seen and reisolation of the required organism is usually not a problem. However, this technique could not determined MBC or MFC likely in dilution techniques.

To determine the MIC for one or more organism isolates, the antimicrobial solution may be incorporated into a liquefied agar medium (45 to 50°C), which is then mixed, poured into standard petri-dishes, and allow to solidify (Barry, 1976; Synder *et al*., 1976). Mueller-hinton agar is generally used for testing aerobic bacteria (Ambaye *et al*., 1997), however, this can be used or supplement with other nutrients for other microbes. A series of petri-dishes are prepared with increasing concentrations of each antimicrobial agent and growth control plates without antimicrobial agent are prepared. After agar is allowed to solidify, then a standard number of test microbe (10^4 CFU/ml for aerobic bacteria) are spot inoculated into each plate using a replicating device, such as steer's replicator (Steer, Foltz and Graves, 1959). After overnight incubation the MIC is read as the lowest concentration of antimicrobial agent that inhibits the visible growth of the test microbe (one or two colonies are ignored (Mahon *et al*., 2000).

However, broth dilution tests are usually preferred for studying the antimicrobial activity of antimicrobial agents because subcultures can be made easily at different time intervals (Schoenknecht, Sabath and Thornsberry, 1985).

2.3 Variation and extension of dilution methods

2.3.1 Minimal bactericidal concentration (MBC) test

Minimum inhibitory concentration (MIC) tests identify the amount of antimicrobial agent required to inhibit the growth of multiplication of as organism isolate; the MBC test can be used for this purpose. The method is usually as extension of the broth dilution MIC. After reading the MIC, organisms are subculture from tubes of wells showing no growth to antimicrobial-free agar medium. After incubating the

plates the proportion of non-viable organism, compared with origin inoculum, is assessed.

MBC tests are subject to more technical pitfalls than MIC tests, and several variables must be rigidly controlled during MBC testing. The first involves inoculum. Because many antimicrobial agents exert a bactericidal effect only on growing cells, falsely elevated MBCs. The inoculum preparation method described for MIC tests that use stationary phase growth are unacceptable for MBC tests (Lorian, 1996).

Secondly, during inoculation for MIC tests care must be taken to ensure that all bacteria in the test inoculum are deposited directly into the antimicrobial solution. If this is not done, bacteria may stick to the wall of the tube or well above the meniscus of the antimicrobial solution and may remain viable during incubation of the MIC portion of the test. These cells (which have not been exposed to antimicrobial agent) may then be inadvertently transferred during the subculture step, ultimately resulting in falsely elevated MBCs.

Third, the volume subcultured following reading of the MIC test must be large enough to contain sufficient inoculum but small enough to prevent carry over of large amounts of antimicrobial agent to continue to exert an bacterial effect. Usually, 10μl (0.01ml) is recommended (Mahon *et al*., 2000).

For fungi strains, MFC determination was tested mostly by the same procedure as described for bacteria (Bartizal *et al*., 1997). The method may be adapted but have recommended by reference method, NCCLS (1995).

2.3.2 Time-kill analysis

Time kill assay is method which measures of the rate of the of killing of microorganism by an antimicrobial agent as determine by examining the number of viable organism remaining at various intervals after exposure to the agent. It is an extension of the MBC or MIC test. Test organism in mid-logarithmic growth phase is inoculated into several tubes of broth containing varying concentrations of antimicrobial agent and a growth control tube without drug. Most experiments are performed with a final inoculum of 10^5 to 10^7 CFU/ml. It is usually convenient first to adjust the overnight culture to match the 0.5 McFarland standard (NCCLS, 1997). These tubes are incubated. Then small aliquots are removed at specific time intervals (e.g. at 0,4,8,12 and 24 hours) diluted to obtain countable number of colonies, and plated to agar for colony count determination. The number of organisms remaining in each sample is plotted over time to determine the rate of antimicrobial agent killing (Bartizal *et al*., 1997; Hoelloman *et al*., 1998; Nilius *et al*., 2000). Generally, a three or more $\log_{10}$ reduction in organism count and the antimicrobial suspension as compared with growth control indicates and adequate bactericidal or fungicidal response (Hoelloman *et al*., 1998).

2.4 Antimicrobial resistance of microorganisms

Chemotherapeutic effectiveness depends upon the sensitivity of the pathogen to the agent. Some microbes respond predictably to certain antimicrobial agent, making selection of treatment easy. Other microbes may vary in their responses, and laboratory tests are usually required to ensure that the selected therapy is appropriate. Antimicrobial resistance, however, may develop in microbes with in population. A major problem associates with chemotherapy is the selection of resistant microorganisms may spontaneously mutate against a given trait in their environment

once in every 10^5 to 10^{10} cell divisions. Because of their rapid mutation rates, the chance of microbial mutations against a given antimicrobial agent is quite probable (Jensen, Wright and Robinson, 1997).

Antimicrobial agent resistance of microorganisms has been produced by exposure *in vitro*. In particular case, a change of virulence occurred in trypasome which were made resistance *in vitro* (Schnitzer *et al*., 1957, cited in Tobie and von Brand, 1953). Antimicrobial agent resistance of bacteria frequently offers a much more complicated aspect. The problem of bacteria antimicrobial agent resistance taking into consideration the multiplicity of biological changes which were observed in resistance bacteria in addition to the basis factor of reduced sensitivity (Schnitzer *et al*., 1957, cited in Morgenroth and Schnitzer, 1925). However, be mentioned here that antimicrobial agent-fast bacteria produce *in vitro* can frequently show changes of morphology and cultural appearance. In addition they may also exhibit reduction or complete loss of virulence for laboratory tests.

Development of resistance will be dependent upon these two basic factors. The character of the antimicrobial and its mechanism of activity. The basic technique to develop resistance in organisms can classify into two techniques, in vivo and in vitro study. The following content seems to cover the various methods which have been used to obtain antimicrobial agent resistance microorganisms and the emphasis is put on the repeated exposure *in vitro* test

Development of antimicrobial agent resistance *in vitro*, namely by :

(1) Repeated exposure in serial passages

(2) Continuous exposure

(3) Single exposure

(4) Indirect method

2.4.1 Resistance developed *in vitro*

2.4.1.1 Repeated exposure

Repeated exposure or serial passage method is the method which has first been used in 1913 to produce the first ethyhydrocupreine-fast *pneumococcus*. The strain became 200-flod resistance in 12 passages (Schnitzer *et al*., 1957).

Development of resistance is tested by sub-culturing organism in medium presence of sub-inhibitory concentration of antimicrobial agent and increasingly higher concentrations until more or less marked resistance was observed (van Zwet *et al*., 1994; Silverman *et al*., 2001). The potential for resistance development was determined by recording MICs after serial passages (Bartizal *et al*., 1997).

To induce and assess the level of test resistance that could be reached in broth and on agar medium. van Zwet *et al*. (1994) showed the development of resistance in *Helicobactor pylori* by simple subculture and tested the variability of MICs by agar dilution. Resistance isolate was obtained by three passages on agar plates containing sub-MIC of metronidazole. Whereas, Bartizal *et al*. (1997) induced resistance fungi by 40^{th} serial passage method in broth medium and test MICs by broth microdilution method.

If one review the numerous experiments in which antimicrobial agent resistance was obtained in many different genera and species of bacteria toward the great number of antimicrobial agents known at present, it is rather difficult to arrange the observations on the development of resistance entirely on the basis of the

method. It has been outlined before that one generally differentiates the various procedures according to the frequency of exposure. In the special case of repeated exposure in serial passages, one can furthermore describe the general technique used by all the different investigators with the following statement: in antimicrobial agent resistance experiments the bacteriostatic technique with graded concentration of the antimicrobial agent and light or moderately heavy inocula is used. The media are adapted to the nutritional requirements of the organisms and are in most instances standard media, either complex or synthetic. Additions to these media are sometimes made in order to study their influence on the development of resistance, or certain constituents of media are occasionally omitted for the same purpose. In rare instances media have been found in which the development of antimicrobial agent resistance is either delayed or even prevented. One example is the observations of Tobie *et al.* (1953) (Schnitzer *et al*., 1957, cited in Tobie *et al*., 1953) who obtained chloramphenicol resistance of *Staphelococcus aureus* in a complex medium but failed to do so in a synthetic medium. Also Merkel *et al*. (1951) mentioned that chloramphenicol resistance of *Escherichia coli* developed better in a complex brain-heart broth than in synthetic medium.

The actual technique of producing antimicrobial agent-resistance organisms was not change regardless of the media used. Antimicrobial agent resistance by serial passage method was carried out in liquid and on solid media, through sometimes with different results as describe in Table 1 (Schnitzer *et al*., 1957, cited in Murray *et al*., 1946).

There does not exist any rule as to the number of passages necessary to obtain resistance. In the study of earlier investigators it was customary to extend the duration of such experiments until maximal resistance was obtained or a reasonable number of passages with a partially resistant strain did not increase the drgree

Table 1 Influence of Liquid and Solid Media on the Rapidity of Development of Antimicrobial Agent Resistance [a]

Organism	MIC [b] of Organism (Units/ml)	Number of Transfers Required to Achieve Maximum Resistance (50,000 Units/ml)	
		Streptomycin Broth	Streptomycin agar
Acrobactor aerogenes	6.3	49	29
Acrobactor aerogenes	12.5	41	12
Acrobactor aerogenes	12.5	27	-
Klebsiella pneumoniae	12.5	44	19
Klebsiella pneumoniae	6.3	40	6
Pararcolon bacillus	25.0	-	29
Pseudomonas aeruginosa	12.5	13	5
Esherichia coli	25.0	44	19
Bacillus proteus	50.0	31	-
		Average 36	Average 17

[a] From Murray *et al*. (1946)

[b] MIC = minimum inhibiting concentration giving no growth on transfer to streptomycin-free broth.

of fastness. In more recent investigations, the tendency seems to be to limit the experiment to set number of passages, sometimes only 10, sometimes as many as 60. The limitation to a certain number of passages is not entirely arbitrary and is probably based on the experience that resistance toward some antimicrobial agents occur after comparatively few or only after a considerable number of passage (Schnitzer *et al*., 1957).

In the experiment with serial passages, transfers are generally made after 1 to 3 days of incubation. Silaratana (1982) discussed in incubation time before transfer to the passage that longer incubation time may be killed organisms and this is the one of important factor by which differences of the degree of the antimicrobial agent resistance might be obtained. However, the most decisive influence on the development of resistance is exerted by the antimicrobial agents and is obviously based on their mechanism of activity. Different strains of microbes may not only possess different sensitivity to an antimicrobial agent, but may also show a different response to attempts of inducing resistance. The reasons for this are unknown but open to many speculations of biochemical or genetic nature. Attempts to give a general interpretation of development of resistance frequently disregard these differences and the preoccupation of investigators with the establishment of a scientific dogma precludes the discussion of exceptions from an arbitrarily set rule. The study of other resistance strains, such as morphological characteristics, stability, and virulence, show further individual differences which make a simple interpretation of the observations difficult.

2.4.1.2 Continuous exposure

The term continuous exposure in the field of the technique of organisms antimicrobial agent resistance requires a definition. It is the counterpart of the exposure of short duration described in serial passages and signifies that the

organisms remain in contact with the antimicrobial agent for longer periods than necessary to obtain full growth. In the experiments with serial passages, transfers are generally made after 1 to 3 days of incubation unless one deal with organisms require a longer incubation time such as 60 days. In some of these experiments which were partly serial passages, very few transfers were performed during a comparatively long period (Mouton and Mulder, 1987). The degree of resistance obtained in the organisms by prolonged exposure in infrequent passages.

2.4.1.3 Single exposure

Single exposure experiment is a technique producing antimicrobial resistance organisms by a single exposure to the antimicrobial agent in liquid or solid medium in a period of incubation. Selection of resistance isolates can be carried out with the gradient plate or culture in test tubes with various concentration of the agents. The method of producing antimicrobial resistance strains by single exposure is based on the occurrence of individuals of different sensitivity in a mixed population. In 1948, Demerce showed that after a single contact with streptomycin, a great variety of surviving organisms was obtained which showed very different degrees of sensitivity. Strain were found which were only slightly resistant, a great number showed a moderate degree of resistance (Schnitzer *et al*, 1957, cited in Demerce, 1948). The methods have one requirement in common, the use of a large inoculum.

2.4.1.4 Indirect methods

Indirect methods by which antimicrobial agent resistance of microorganisms can obtained without exposure to an antimicrobial or a toxic agent. One of the reasons why the so called " indirect " method, namely the replicate plate

method of Lederberg and Lederberg (1952), can properly be call an indirect method of isolating resistant bacteria is the fact that the exposure of the organisms to the antimicrobial agent is used only to demonstrate resistance. The second reason is that the occurrence of the resistance is of secondary importance whereas the primary purpose is to prove the presence of less sensitive organisms before contact with the antimicrobial agent. Lederberg's technique is a most ingenious type of experiment consisting in the organisms under condition growth by an extremely simple device of replanting the organisms under conditions where their original location on the primary plate is maintained. This is achieved by carrying out the transplantation from one agar surface in a petri-dish to another by using a piece of sterile volveteen attached to a carrier which has the approximate diameter of the petri-dish. After transferred the organisms onto new agar surface of a plate which presence antimicrobial agent, only organisms resistant to this specific concentration of the agent will develop.

CHAPTER II

MATERIALS AND METHODS

1. Materials

The following substances were commercial available.

Tryptic soy agar, Tryptic soy broth, Sodium chloride, Potassium dihydrogen phosphate, Di-potassium hydrogen phosphate and Dimethyl sulfoxide were from E. Merck, Damstadt, Germany.

Gentamicin sulfate and Amphotericin B were from Sigma Chemical Co. Ltd., USA.

Mueller hinton Agar, Mueller hinton broth, Brain heart infusion agar, Brain heart infusion broth, Sabouraud dextrose agar, Sabouraud dextrose broth and Yeast were from Difco, Becton Dickinson and Company, France.

2. Equipments

- Incubator Model 6 (Thelco)
- Hot air oven (Memmert, Germany)
- Autoclave HA-3D (Hirayama Manufacturing Coorperation, Japan)
- Micropipette (Pipetman, Made in France)
- pH meter MP230 (Mettler Toledo, Switzerland)
- Centrifuge GS 200 (Clements, TMS, Thailand)
- Scanning electron microscope JSM 5410LV (JEOL)

3. Methods

3.1 Preparation of polysaccharide gel (PG)

3.1.1 PG isolation

A polysaccharide gel (PG) was isolated from dried fruit-hulls of durian (*Durio zibethinus* L.). Waste of fresh durian fruit-hulls was collected, washed, blended and dried in hot air oven at 70^0C. A process of PG isolation was performed based on the method previously described by Pongsamart and Panmuang (1998). PG was purified by reprecipitating of crude PG solution using acidic solution of ethanol.

3.1.2 Preparation of test solution of PG

Powder of polysaccharide gel (PG) was freshly prepared to make a series of two fold dilutions of various concentrations of polysaccharide gel in sterile distilled water and added to the agar and broth media for microbiological test.

3.2 Test microorganisms and preparations of inoculum

All microorganisms were obtained from the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Eleven bacteria, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Lactobacillus plantarum* ATCC 14917 and *Lactobacillus pentosus* ATCC 8041 were used as test gram positive bacteria and *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumoniae* ATCC

10031 and *Pseudomonas aeruginosa* ATCC 9721 were used as test gram-negative bacteria.

All test bacteria were cultivated overnight on tryptic soy agar (TSA) slant at 37^0C except for Lactobacillus species that was cultivated on brain heart infusion agar (BHIA) with 0.3% yeast. The bacteria were washed from surface agar slant with sterile normal saline (NSS). The culture was then adjusted to match turbidity of standard Mcfarland no. 0.5 before used.

Two yeast strains, *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC10230 were used and cultivated on sabouraud dextrose agar (SDA). The yeast suspension was prepared by the same procedure as described for bacteria cell cultures.

3.3 Preparation of agar and broth media

All agar and broth media were dispensed in water and sterilized in autoclave for 15 min at 15 pounds pressure (121 ^{0}C).

Mueller hinton agar (MHA), brain heart infusion agar (BHIA) and sabouraud dextrose agar (SDA) were media used for agar diffusion susceptibility test. All test bacteria were tested on MHA except for Lactobacillus species that was cultivated on BHIA with 0.3% yeast. Two yeast strains were cultivated on SDA. For broth macrodilution susceptibility test, Mueller hinton broth (MHB), brain heart infusion broth (BHIB) and sabouraud dextrose broth (SDB) were used as test media.

3.4 Determination of antimicrobial activity of polysaccharide gel

3.4.1 Agar diffusion susceptibility test

Susceptibility determinations were made as described in the standard guideline technique (Lorian, 1991). Agar diffusion testing was performed as follows: serial two-fold dilutions of various concentrations of PG 5, 2.5, 1.25, 0.625 and 0.32% in sterile distilled water were freshly prepared.

Plates with internal diameter of 100 mm containing 25 ml of enriched agar media were inoculated with 1% microorganism suspension by seed layer method (Lorian, 1991). Sterile stainless steel cylinders (6 mm internal diameter and 10 mm height) were placed on the inoculated agar surface. The various concentrations of PG were filled into the cylinders (300 μl/cylinder). After pre-diffusion at room temperature for 1 hour, the plates were incubated at 37^0C overnight. The results were obtained by measuring the diameters of inhibition zone. The NSS filled in the cylinder was used as control and the determination were carried out in triplicate.

3.4.2 Broth macrodilution susceptibility test

3.4.2.1 Determination of MIC and MBC

Broth macrodilution testing was carried out using media containing 4.5 ml of broth with various dilution of PG and 0.5 ml of microorganism suspension. Media without PG were used as a culture control.

MIC of PG testing was performed using broth media with various dilutions of PG and the inoculated media were incubated at 37^0C overnight. MIC was defined as the lowest concentration of PG that inhibited visible growth of microorganisms. MBC was determined by subculturing from tubes showing no turbidity onto PG-free agar media and observed the lowest concentration of PG showing no growth further incubation for 24 hours.

3.4.2.2 Time-kill analysis

Time-kill analysis is an extension of the MBC and give information on the rates at which organisms are killed. The inoculated media with PG were incubated at 37^0C for 24 hours. At the indicated time, the culture were removed at 0, 2, 4, 8, 12, 16, 20 and 24 hrs and the number of viable colonies were counted by drop plate method (Lorian, 1991) on agar media after serial dilutions in NSS. Viable colonies were calculated to give colony forming unit (CFU) per milliliter and survival were plotted with time against the logarithm of the viable count. Cultivation of bacteria in NSS and media without PG were used as culture control to see bacterial survival pattern in comparison to media with PG.

3.4.3 Determination of antimicrobial activity of polysaccharide gel film

Polysaccharide gel was found to have film-forming property (Girddit *et al*., 2001). The films prepared from PG was investigated to determine their antimicrobial activities. This method was a qualitative and empirical assay (Muzzarelli *et al*., 1990) based on the used as a PG film (solid content of PG, 4.42 mg/cm^2; thickness 41$\pm$6 μm). PG films had been and prepared according to procedure as described by Nantawanit *et al*.(2001) and Girddit *et al*.(2001). A strip

of PG film 1.5 cm^2 was deposited on a surface of an agar plate which previously inoculated 0.1 ml suspension of microorganisms (10^8 CFU/ml) by spread plate technique (Murray *et al*., 1995). PG film became transparent in a few minutes after moisten on agar surface. Sterile filter paper (1.5 cm^2) was use as control. The plates were incubated at 37^0C overnight. Microbial growth under the thin PG film was observed in comparison with the growth of microbial under the sterile filter paper. Inhibitory effect was denoted by the observation of transparency area of PG film covered on surface of the agar medium. No inhibitory effect was determined by the observation of an opaque layer of full growth of bacteria under the PG film, no transparency area was observed.

3.5 Determination of antibiotic susceptibility

Antibiotic susceptibility tests were determined as a positive control to ensure that the test system is functioning properly. Determination of the antimicrobial activity of antibiotic was performed by the same procedure of agar diffusion and broth macrodilution susceptibility test (MIC determination) as described for PG testing.

Gentamicin sulfate was used as antibacterial agent against all test bacteria. Gentamicin sulfate powder was completely dissolved in sterile water and serial doubling dilutions were prepared in sterile 0.1M potassium phosphate buffer, pH 7.4 to obtain concentrations ranging from 32 to 0.015 μg/ml on the day of use. (Modugno *et al*., 1997)

For determination of the antimicrobial activity of antibiotic against test yeast, amphotericin B (AMB) was used as the antifungal agent. AMB was dissolved in 25% dimethyl sulfoxide (DMSO) and prepared according to the

method recommended by Lorian (1991) to obtain the same concentrations ranging as described in gentamicin sulfate.

3.6 Examination the effect of PG on cell surface of microorganism under scanning electron microscopy

Preliminary survival study by Lipipun *et al*. (2002) of *S. aureus* and *E. coli* in NSS in the presence of the lowest concentration at 0.02%PG indicated that the colony count at 37^0C of both bacteria were declined to zero within 24 hrs, whereas those bacteria normally survived in NSS for several days.

Suspension of PG susceptible microorganisms in NSS with various concentrations of PG were incubated at 37^0C for 24 hrs and the remaining cells were filtered and used to determine an appearance of the cell surface under scanning electron microscope. The microorganism cultivated in NSS without PG was used as a culture control. Before preparation of the culture sample for microscopy, the survival of the cultivated microorganisms were tested by inoculating the cultures on agar media and incubated using the same condition previously described.

Sample preparation for microscopy was performed using standard operating technique and viewed on JEOL JSM 5410LV scanning electron microscope under standard operating conditions (Prateepsain, 1981)

3.7 *In vitro* development of resistance to PG in microorganisms

The development of PG resistance in microorganisms was studied by repeated subculture in the presence of PG that called serial passage (repeated

exposure) method (Schnitzer *et al*., 1957). Selected PG susceptible microorganisms to cultivated in broth media containing sub-inhibitory concentration (sub-MIC) of PG and incubated overnight at 37^0C. After the first incubation, the bacterial cells were centrifuged and then washed in sterile NSS and adjusted the turbidity to match a 0.5 Mcfarland standard before subculture in new broth media containing sub-MIC of PG. Subculturing was continued for 30 days by the same method as previously described. After the last passage, reculturing the test culture in free-PG medium for three passages, and then the resistance of the microorganisms was tested. The resistance was showed by abundant growth of bacteria in the presence of PG concentrations at which the parent strain was susceptible. MIC determination was used for examining the resistance, resisted microorganisms would illustrate an increasing of its MIC value.

CHAPTER III

RESULTS AND DISCUSSION

The *in vitro* activity of polysaccharide gel (PG) extracted from durian fruit-hull was determined against 11 bacterial and 2 yeast strains. Of the six strains of gram-positive bacteria, *S. aureus* and *S. epidermidis* is the cause of skin infection and pus; *L. pentosus* and *L. plantarum* can be found in gastrointestinal tract as normal flora; *M. luteus* and *B. subtilis* can be found in skin as normal flora and in environment. Five strains of gram-negative bacteria; *E. coli* and *P. vulgaris* can be found in gastrointestinal tract as normal flora; *S. typhimurium* is the cause of food poisoning; *K. pneumoniae* and *Ps. aeruginosa* can cause infection in immunocompromised individuals. Two yeast strains, *S. cerevisiae* can be found in the environment, whereas *C. albicans* can cause infection in healthy individuals. (Tortora *et al*., 1995). PG showed inhibitory activity against 7 of bacteria tested, *B. subtilis, M. luteus, S. aureus, S. epidermidis, L. pentosus, E. coli* and *P. vulgaris* by agar diffusion and broth macrodilution tests.

1. Determination of antimicrobial activity of polysaccharide gel

1.1 Agar diffusion susceptibility test

The inhibition zone was observed on agar media with PG at concentration down to 0.32% against *B. subtilis*, *M. luteus*, *S. epidermidis*, *E. coli* and *L. pentosus*; to 0.625% and 1.25% against *S. aureus* and *P. vulgaris*, respectively. According to viscosity of PG, the highest allowable concentration of 5%PG was used in this study. Inhibition zone of sharp and clear margin was obtained. An increment of inhibition zone diameter was found with respect to increasing

Table 2 Antimicrobial activity of polysaccharide gel (PG) on growth of microorganisms by agar diffusion method. nz = no inhibition zone, S.E.M = Standard Error of Mean

%PG	Diameter of inhibition zone, mm (mean±S.E.M)						
	B. subtilis	*M. luteus*	*S. aureus*	*S. epidermidis*	*L. pentosus*	*E. coli*	*P. vulgaris*
5	12.96±0.14	16.58±1.31	11.86±0.49	13.09±0.65	18.72±1.23	13.44±0.49	11.64±0.22
2.5	12.45±0.25	14.28±0.33	10.21±0.62	12.73±0.33	16.26±0.56	12.86±0.35	10.03±0.57
1.25	11.67±0.51	13.74±0.73	9.33±1.15	11.89±1.10	14.12±0.81	12.52±0.86	8.35±0.12
0.625	11.05±0.22	12.91±0.49	8.41±0.17	10.88±0.88	12.98±1.22	11.52±0.45	nz
0.32	9.86±1.16	10.92±0.51	nz	10.13±0.20	11.84±0.69	10.49±0.62	nz
NSS	nz	nz	nz	nz	nz	nz	nz

Table 2 (continued)

%PG	Diameter of inhibition zone, mm (mean±S.E.M)					
	L. plantarum	*S. typhimurium*	*K. pneumoniae*	*Ps. aeruginosa*	*S. cerevisiae*	*C. albicans*
5	nz	nz	nz	nz	nz	nz
2.5	nz	nz	nz	nz	nz	nz
1.25	nz	nz	nz	nz	nz	nz
0.625	nz	nz	nz	nz	nz	nz
0.32	nz	nz	nz	nz	nz	nz
NSS	nz	nz	nz	nz	nz	nz

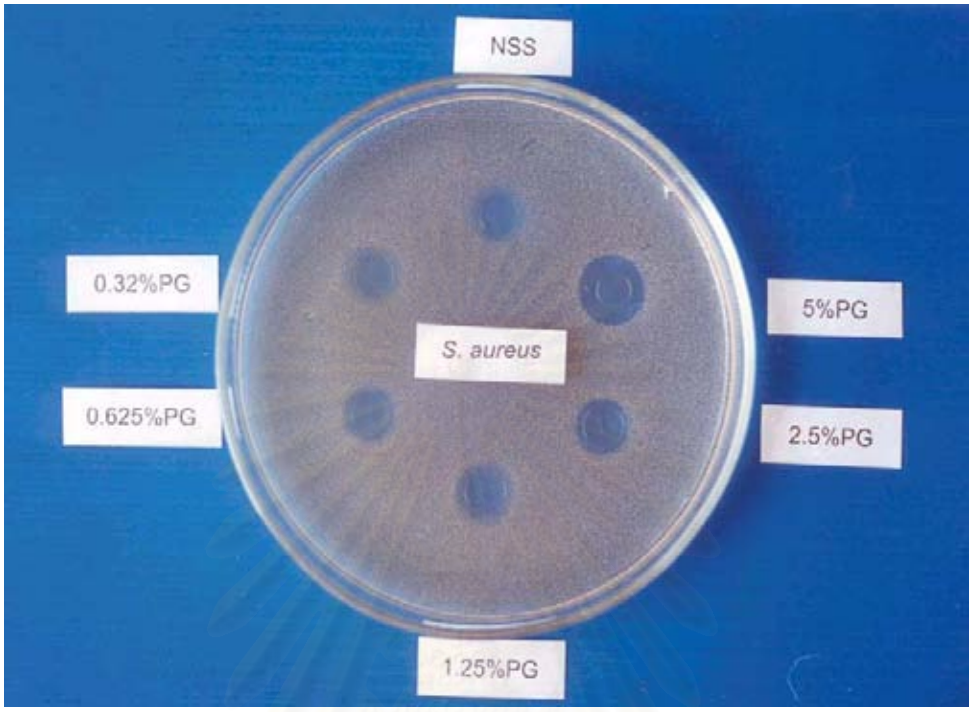


Figure 3 Microbiological assay plate for *S. aureus* ATCC 6538P on medium MHA. Cups contain various concentrations of PG and control filled with NSS.

concentrations of PG as indicated in Table 2 and Figure 3. Four strains of test bacteria, *L. plantarum* that represented as gram-positive bacteria and as gram-negative bacteria, *S. typhimurium*, *K. pneumoniae* and *Ps. aeruginosa* were not inhibited by PG according to this assay. Two strains of test yeast, *S. cerevisiae* and *C. albicans*, appeared no inhibition zone as shown in Table 2.

1.2 Broth macrodilution susceptibility test

1.2.1 Determination of MIC and MBC

MIC and MBC values are shown in Table 3, the values showed a complete inhibition and bactericidal activity of PG against bacteria. The results indicated that 7 test bacteria, *B. subtilis*, *M. luteus*, *S. aureus*, *S. epidermis*, *L. pentosus*, *E. coli* and *P. vulgaris* were susceptible to inhibit by polysaccharide extracted from durian fruit-hulls. Four strains of test bacteria and two strains of test yeast were not susceptible to PG by this assay, as shown in Table 3, no MIC or MBC values was obtained. Figure 4 demonstrated MIC of PG against *S. epidermis*, MIC of 6.4 mg/ml which was the lowest concentration of PG that inhibit bacterial growth, an appearance of visible growth was not obtained.

1.2.2 Time kill analysis

Bactericidal activity of PG against 7 strains of PG susceptible bacteria was demonstrated by time-kill analysis. Bacterial cells were cultivated in NSS, media without PG, media with 1.6 mg/ml of PG, media with MIC of PG and media with MBC of PG. Bacterial survival pattern were compared to 5 conditions as previously described. All test bacteria showed the same survival pattern in time-kill study. In NSS, bacteria were normally survived in the static level after

Table 3 MICs and MBCs of polysaccharide gel (PG) against microorganisms

Microorganisms	MIC (mg/ml)	MBC (mg/ml)
B. subtilis	6.4	25.6
M. luteus	6.4	25.6
S. aureus	12.8	25.6
S. epidermidis	6.4	25.6
L. pentosus	25.6	51.2
E. coli	6.4	25.6
P. vulgaris	6.4	25.6
L. plantarum	-	-
S. typhimurium	-	-
K. pneumoniae	-	-
Ps. aeruginosa	-	-
S. cerevisiae	-	-
C. albicans	-	-

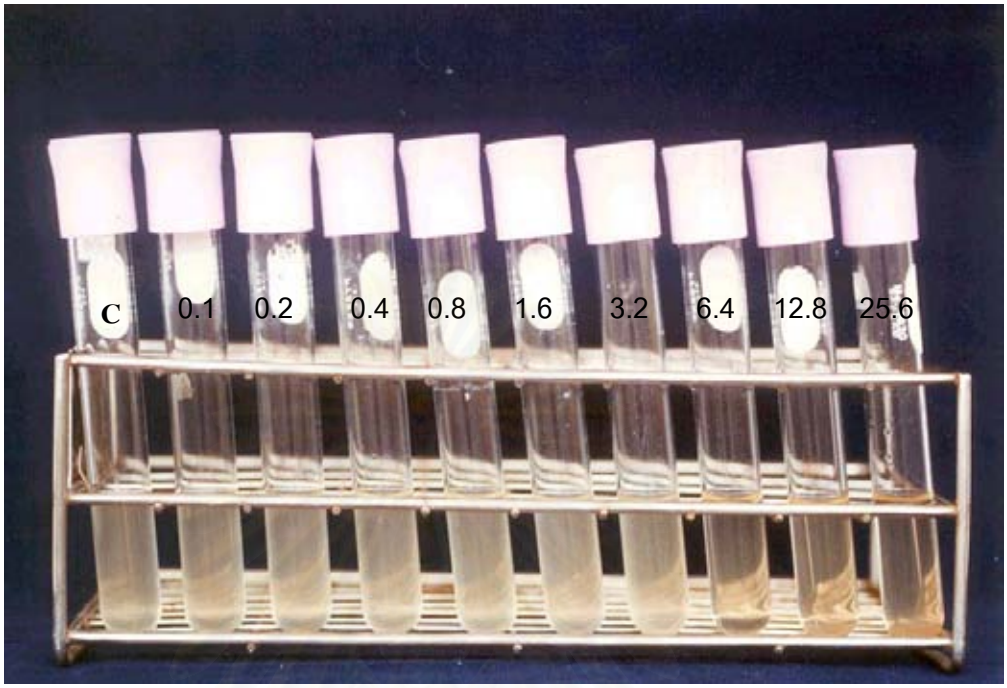


Figure 4 MIC of PG at 6.4 mg/ml in mueller hinton broth against to *S. epidermidis* ATCC 12228, C = Control(PG = 0 mg/ml)

Figure 5 Time-kill analysis of polysaccharide gel (PG) for *B. subtilis* in mueller hinton broth

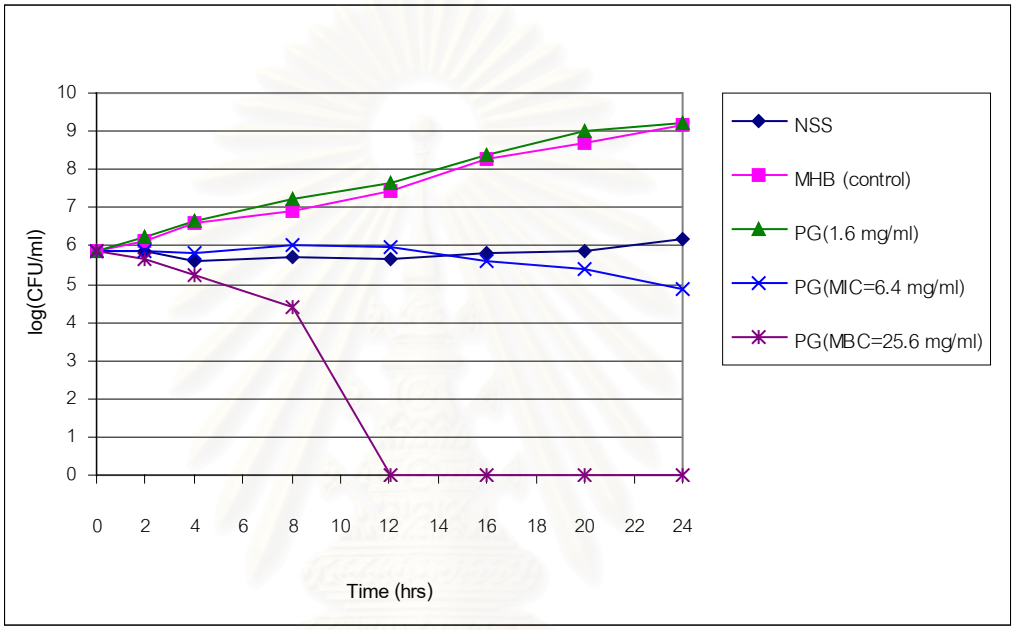


Figure 6 Time-kill analysis of polysaccharide gel (PG) for *M. luteus* in mueller hinton broth

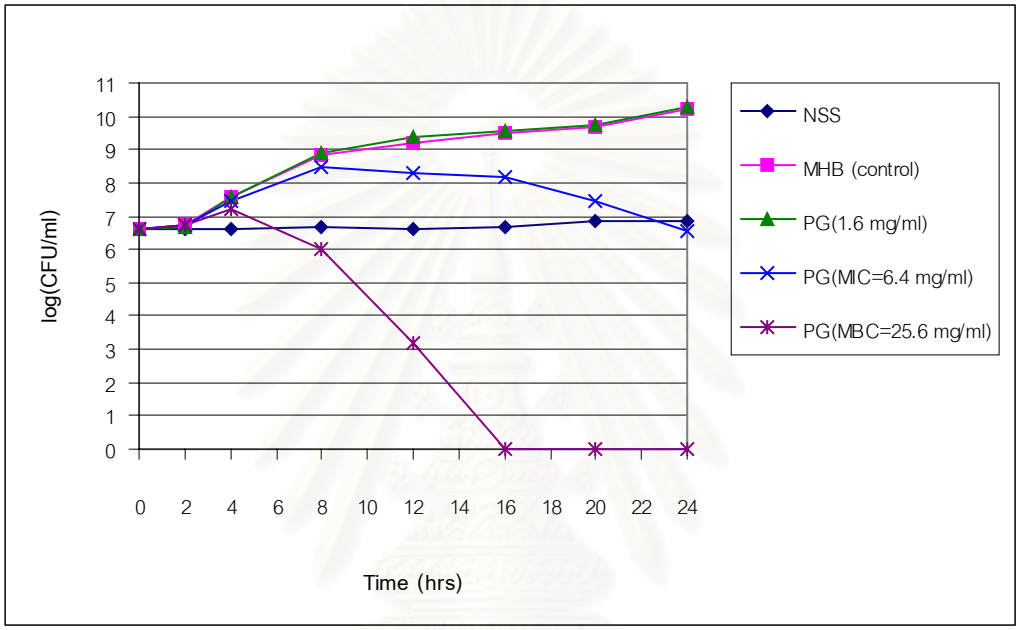


Figure 7 Time-kill analysis of polysaccharide gel (PG) for *S. aureus* in mueller hinton broth

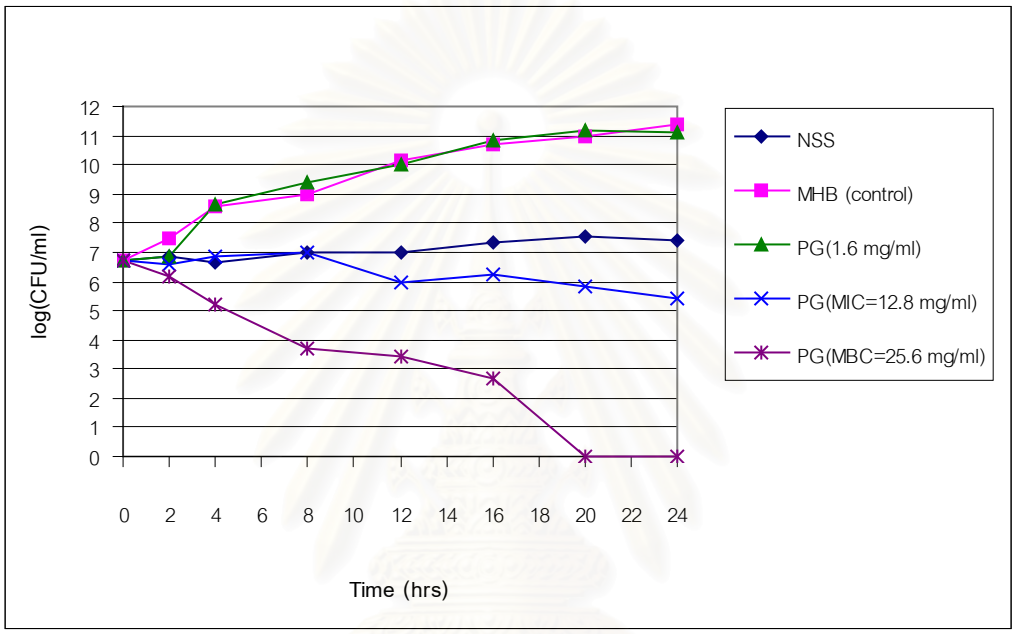


Figure 8 Time-kill analysis of polysaccharide gel (PG) for *S. epidermidis* in mueller hinton broth

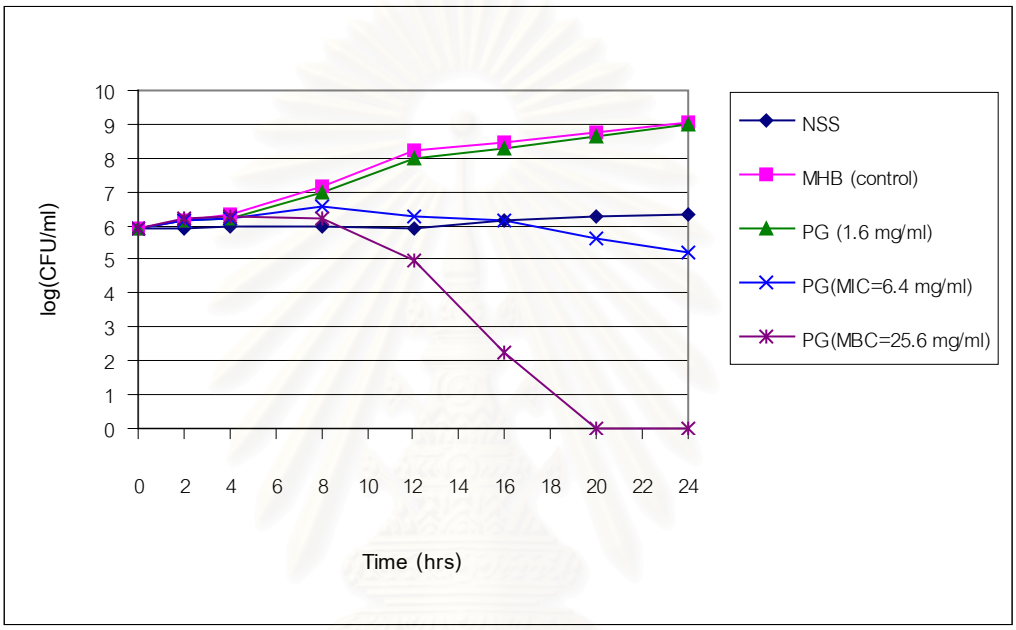


Figure 9 Time-kill analysis of polysaccharide gel (PG) for *E. coli* in mueller hinton broth

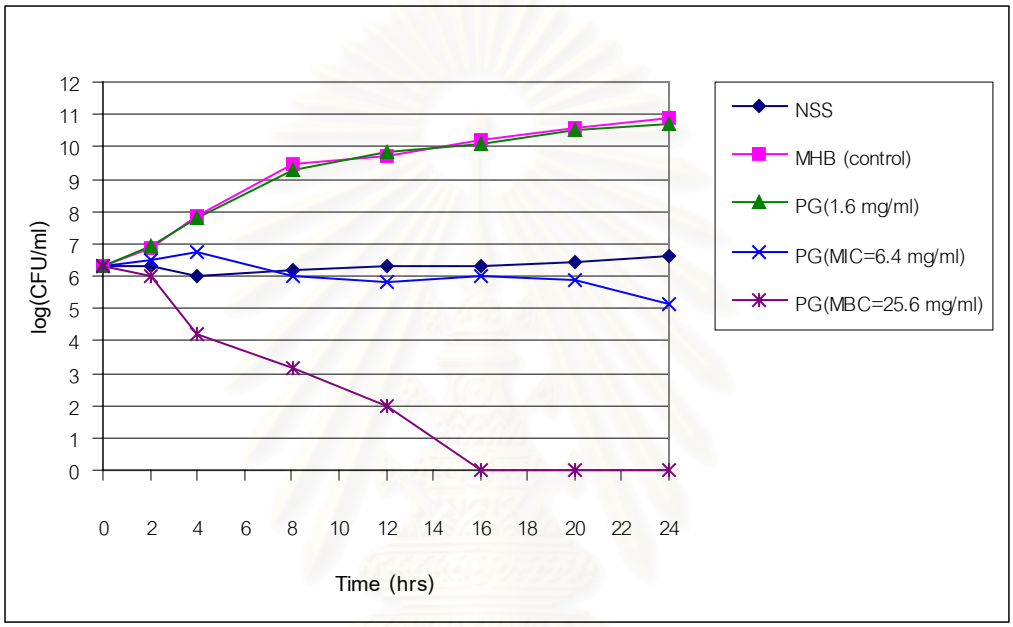


Figure 10 Time-kill analysis of polysaccharide gel (PG) for *P. vulgaris* in mueller hinton broth

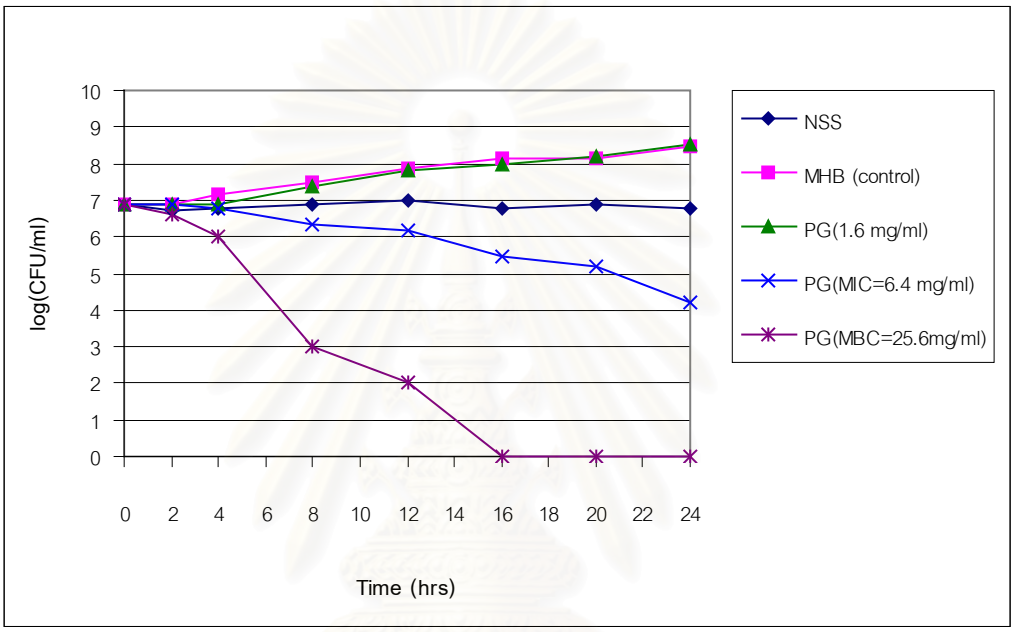
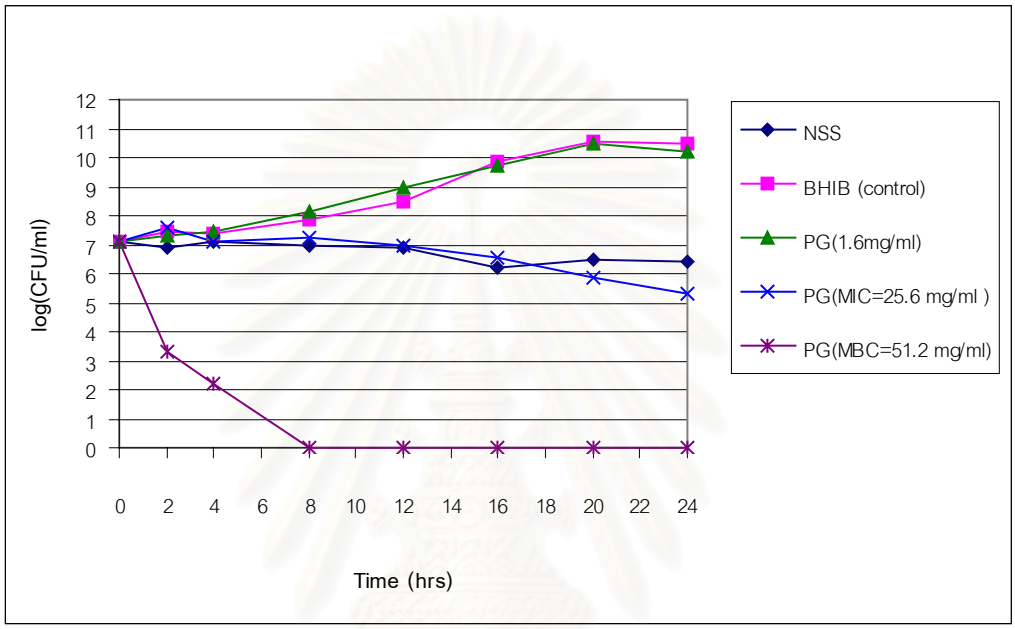


Figure 11 Time-kill analysis of polysaccharide gel (PG) for *L. pentosus* in brain heart infusion broth



incubation, whereas they were grew better in PG free media (control) and media with low concentration of PG (1.6 mg/ml). The colony counts of bacteria that cultivated in media with MIC of PG were slowly declined throughout the incubation time. While, the colony counts of bacteria that cultivated in media with MBC of PG were declined to 0% within 24 hours of incubation. Time-kill analysis illustrated that PG at the concentration of 25.6 mg/ml produced bactericidal activity in MHB medium against *B. subtilis*, *M. luteus*, *E. coli*, *P. vulgaris*, *S. aureus* and *S. epidermis*, the colony counts were declined to 0% at 12, 16, 16, 16, 20 and 20 hours, respectively (Figure 5, 6, 9, 10, 7 and 8).

PG at the concentration of 51.2 mg/ml produced bactericidal activity in BHIB with 0.3%yeast medium against *L. pentosus,* the colony count was declined to 0% at 8 hours (Figure 11).

1.3 Determination of antimicrobial activity of polysaccharide gel film

PG film was used to assay an antimicrobial activity of PG against microorganisms. PG film showed inhibitory activity against 7 strains of bacteria, *B. subtilis*, *M. luteus*, *S. aureus*, *S. epidermis*, *L. pentosus*, *E. coli* and *P. vulgaris* as indicated in Table 4. Inhibitory effect of PG film was illustrated by an observation of transparency area of the film covered on the surface of agar medium, only small number of colony was appeared under the film (Figure 12A), bacteria were fully grew under the sterile filter paper which was used as a control. However inhibitory effect of PG film did not showed transparence surface of inhibition completely. The result may explain according to the fact that PG film covered on agar surface was not enough dissolved to make fully inhibit the susceptible bacteria. Whereas, insusceptible bacteria, *L. plantarum*, *S. typhimurium*, *K. pneumoniae* and *Ps. aeruginosa* and 2 yeast strains, *S. cerevisiae*

Table 4 Antimicrobial activity of polysaccharide gel film against various bacteria and yeast

Microorganisms	Inhibitory activity of PG film
B. subtilis	+
M. luteus	+
S. aureus	+
S. epidermidis	+
L. pentosus	+
E. coli	+
P. vulgaris	+
L. plantarum	-
S. typhimurium	-
K. pneumoniae	-
Ps. aeruginosa	-
S. cerevisiae	-
C. albicans	-

+ : Inhibitory effect of PG film

- : Lack of inhibitory effect of PG film

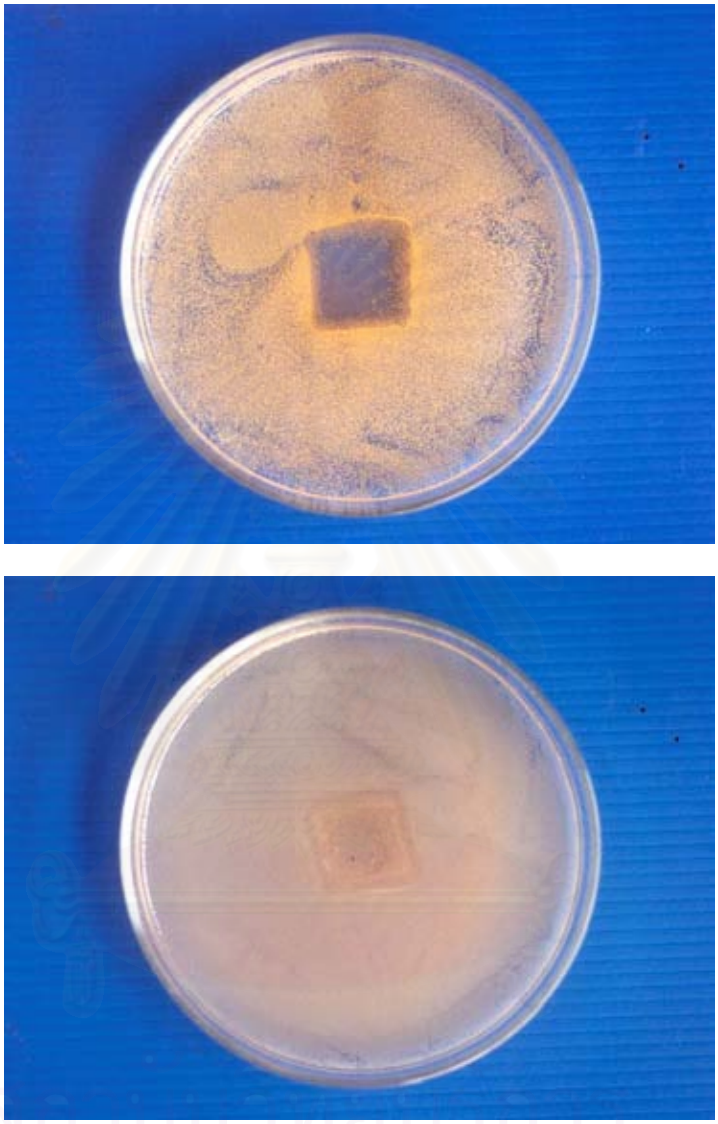


Figure 12 Empirical assay used to assess qualitatively the antimicrobial activity of PG film covered on agar media at the central part of an inoculated plate incubated at 37^0C for 24 hours; (A) Inhibitory effect of PG film against *S. aureus*; (B) No inhibitory effect of PG film against *S. typhimurium*.

and *C. albicans* were fully grew under the film and transparency appearance were not obtained at the film area on the agar medium as shown in Figure 12B.

The results from this investigation were correlated to previous susceptibility test, agar diffusion and broth macrodilution method. The same susceptible strains of bacteria to PG were demonstrated after using both assay. The data suggested that antibacterial activity of PG film have potential application as dressing film, and it should be further studied to a suitable preparation for wound dressing.

2. Determination of antibiotic susceptibility

Determination of antimicrobial activity of antibiotic was performed by agar diffusion and broth macrodilution susceptibility test. Table 4 and 5 showed inhibition zone of gentamicin sulfate and amphotericin B at concentration raging from 32 to 0.5 μg/ml against test bacteria and yeast, respectively. While, Table 6 and 7 illustrated the MIC and MBC values of both antibiotics against test bacteria and yeast.

Inhibition zone and MIC values that obtained from both of susceptibility test demonstrated that all standard strains of test microorganisms were susceptible to all test antibiotics within the acceptable range of standard method. Gentamicin sulfate resistant strains was showed MIC values more than 16 μg/ml of gentamicin sulfate (NCCLS, 1990). In case of amphotericin B, the resistant strains were showed MIC values more than 2 μg/ml of amphotericin B (Collier, Balows and Sussman, 1998).

Table 5 Antibacterial activity of gentamicin sulfate on growth of bacteria by agar diffusion method.

nz = no inhibition zone, S.E.M = Standard Error of Mean

Gentamicin sulfate (μg/ml)	Diameter of inhibition zone, mm (mean±S.E.M.)					
	B. subtilis	*M. luteus*	*S. aureus*	*S. epidermidis*	*L. pentosus*	*L. plantarum*
32	17.51±0.19	16.93±0.30	15.65±0.29	14.62±0.13	21.59±0.30	22.50±0.15
16	15.76±0.21	14.60±0.44	13.86±0.12	13.44±0.24	16.40±0.31	19.82±0.22
8	13.79±0.14	12.76±0.27	11.93±0.16	11.79±0.17	14.53±0.24	17.93±0.58
4	10.15±0.64	11.92±0.20	10.80±0.12	10.65±0.34	13.51±0.26	16.44±0.19
2	8.21±0.18	10.74±0.29	9.12±0.07	10.15±0.28	12.08±0.40	14.86±0.12
1	nz	8.31±0.28	nz	9.23±0.21	10.66±0.15	12.47±0.21
0.5	nz	nz	nz	nz	8.86±0.28	10.16±0.28
NSS	nz	nz	nz	nz	nz	nz

Table 5 (continued)

Gentamicin sulfate (μg/ml)	Diameter of inhibition zone, mm (mean±S.E.M.)				
	E. coli	*P. vulgaris*	*K. pneumoniae*	*Ps. aeruginosa*	*S. typhimurium*
32	17.34±0.16	15.12±0.23	13.58±0.20	11.34±0.29	13.26±0.24
16	15.46±0.25	12.53±0.45	11.39±0.23	10.78±0.25	11.40±0.30
8	13.58±0.13	10.96±0.18	11.40±0.16	10.33±0.56	10.12±0.25
4	10.23±0.24	9.88±0.15	10.52±0.28	9.89±0.30	9.34±0.48
2	9.14±0.14	9.02±0.12	9.65±0.20	9.41±0.31	8.22±0.28
1	nz	8.06±0.29	8.18±0.19	8.85±0.36	nz
0.5	nz	nz	nz	nz	nz
NSS	nz	nz	nz	nz	nz

Table 6 Antifungal activity of amphotericin B (AMB) on growth of yeast by agar diffusion method. nz = no inhibition zone, S.E.M = Standard Error of Mean

AMB (μg/ml)	Diameter of inhibition zone, mm (mean±S.E.M.)	
	S. cerevisiae	*C. albicans*
32	16.11±0.21	18.58±0.64
16	14.82±0.46	15.31±0.22
8	13.16±0.24	13.74±0.18
4	11.40±0.25	12.20±0.25
2	10.87±0.19	11.15±0.50
1	9.94±0.32	10.47±0.36
0.5	8.76±0.40	9.12±0.29
NSS	nz	nz

Table 7 MICs and MBCs of gentamicin sulfate against bacteria

Microorganisms	MIC (μg/ml)	MBC (μg/ml)
B. subtilis	1	1
M. luteus	0.25	4
S. aureus	4	32
S. epidermidis	1	16
L. pentosus	0.25	1
L. plantarum	0.25	1
E. coli	2	8
P. vulgaris	0.5	4
S. typhimurium	4	8
K. pneumoniae	8	64
Ps. aeruginosa	0.5	1

Table 8 MICs and MBCs of Amphotericin B against yeast

Microorganisms	MIC (μg/ml)	MBC (μg/ml)
S. cerevisiae	0.5	1
C. albicans	0.5	2

3. Examination of the effect of PG on cell surface of microorganism under scanning electron microscopy.

Four bacterial strains which were susceptible to PG, *S. aureus* and *S. epidermis* representing a gram-positive bacteria and *E. coli* and *P. vulgaris* representing a gram-negative bacteria were used to demonstrate an appearance of cells under scanning electron microscope.

Survival test of microorganism was examined before the culture of bacterial cells were filtered and the cells were gold coated to determined cell surface using scanning electron microscope. It was demonstrated that all strains of test bacteria were killed within 24 hours in the presence of the 0.32% of PG in NSS, although, bacteria were normally survived in NSS.

An appearance under scanning electron microscope showed the alteration on bacterial cell surface as shown in Figure 13B-16B, whereas, this phenomenon was not observed in culture incubated in NSS without PG of control (Figure 13A-16A). The appearance in culture control showed the smooth surface of bacterial cells, while the bacteria which exposed to PG in NSS were observed the adhesion of fragments that may be PG covered on the outer surface. In addition, scanning electron micrographs of *E. coli* showed an appearance of surface structure of pili or fimbriae (Figure 15A), whereas pili of *E. coli* in NSS in the presence of 0.32% and 0.64% of PG were disappeared (Figure 15B).

The mechanisms of action of PG, while not yet elucidated, seems to relate to nature and sugar compositions of PG as well as the sugars component produced inhibitory activity in chitosan (Muzzarelli *et al*., 1990). PG composed of sugars including arabinose, rhamnose, glucose, fructose and galacturonic acid. About half

of PG composition contained galacturonic acid (Girddit *et al*., 2001) which is an acid sugar producing carbonyl anionic charge. In addition, the polyanionic polymer of PG, may effectively bind and form polyelectrolyte complex with the cationic side chain of lipopolysaccharide present on the bacterial cell surface thus interfere and disturbance of cell wall or cell membrane function, therefore, normal function of bacteria was inhibited (Neu *et al*., 1992).

Pili are hair-like structures that project out from the cell wall presented on some bacterial species, this structure attached to the cytoplasmic membrane by a small hook at the end of structure (Figure 19). Disappearance of pili as shown in case of *E. coli* after incubation in NSS in the presence of PG (Figure 15B) may suggest that PG attachment effect to damage cell membrane of bacteria.

Antimicrobial susceptibility test showed antibacterial effect of PG against 7 bacteria, most of all represented as gram-positive bacteria, whereas the most insusceptible bacteria to PG represented as gram-negative bacteria. This main point may based on the structure of bacterial cell envelope. The cell envelope of gram-positive cells is relatively simple, consisting of two to three layers which are the cytoplasmic membrane, a thick peptidoglycan layer, and in some bacteria an outer layer called the capsule. Whereas the cell envelope of gram-negative is highly complex, multilayered structure (Figure 19) (Jawetz *et al*., 1995). A gram-negative bacteria has an outer membrane of lipopolysaccharide and other materials over a comparatively thin layer of peptidoglycan (Tortora *et al.,* 1995). The effect of PG on inhibition against bacteria seems to have more susceptible to gram positive bacteria than gram negative bacteria in this study.

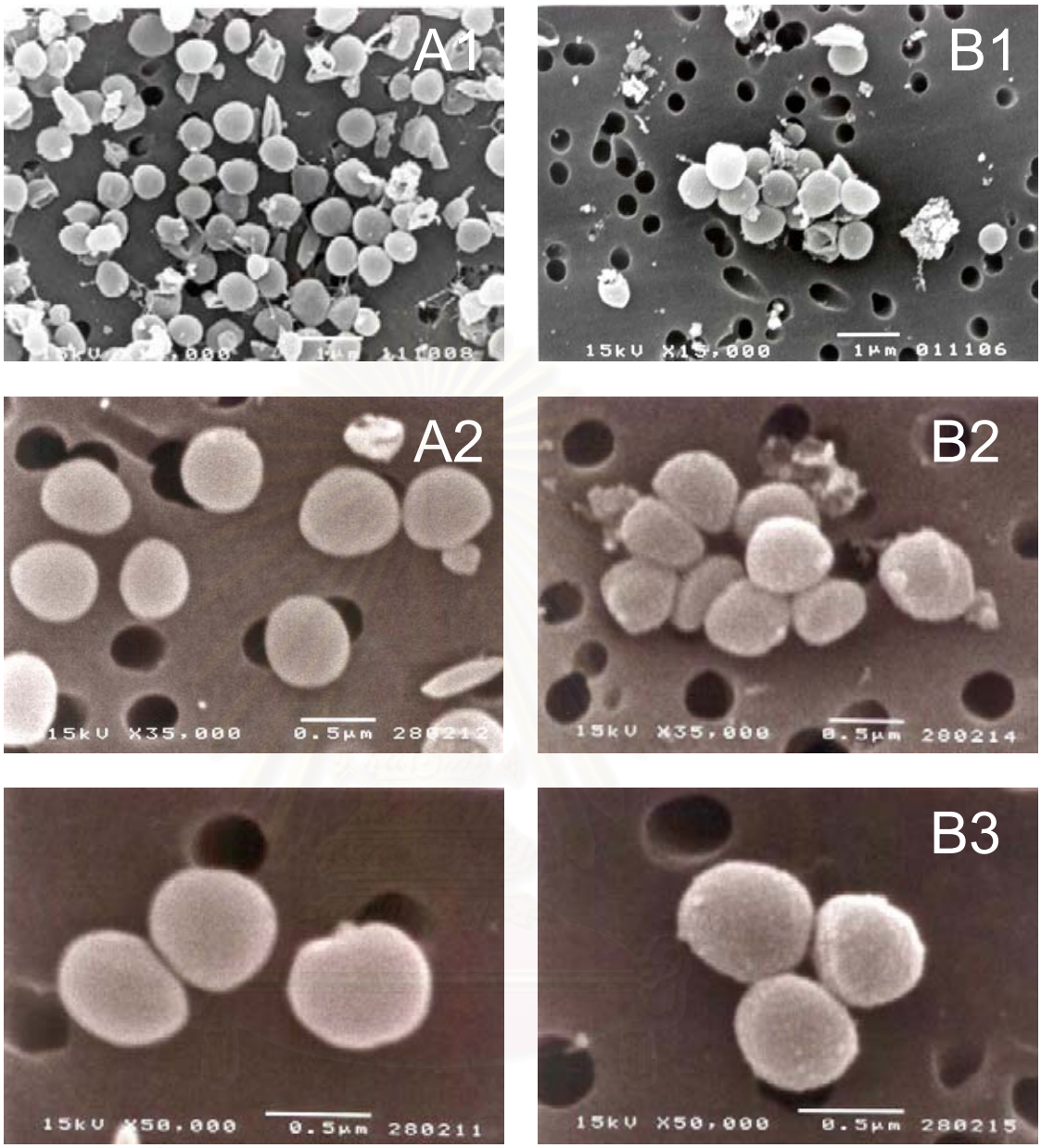


Figure 13 Scanning electron micrographs of *S. aureus* ATCC 6538P after incubation for 24 hrs. at 37^0C; (A1,A2,A3) *S. aureus* in NSS without PG; (B1) *S. aureus* in NSS with 0.64% PG; (B2,B3) *S. aureus* in NSS with 0.32% PG.

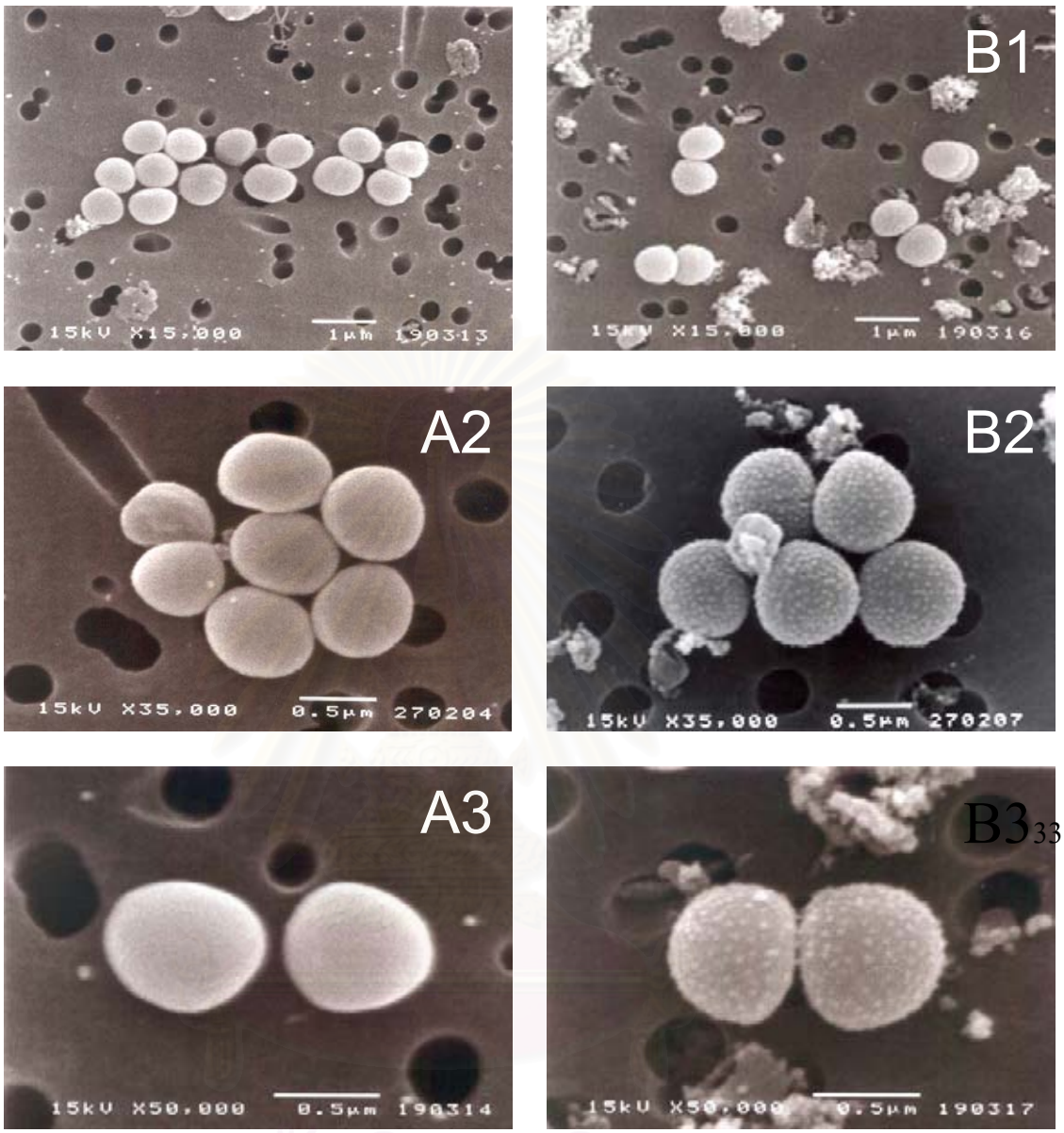


Figure 14 Scanning electron micrographs of *S. epidermidis* ATCC 12228 after incubation for 24 hrs. at 37^0C; (A1,A2,A3) *S. epidermidis* in NSS without PG; (B1,B2) *S. epidermidis* in NSS with 0.32% PG; (B3*)* *S. epidermidis* in NSS with 0.64% PG.

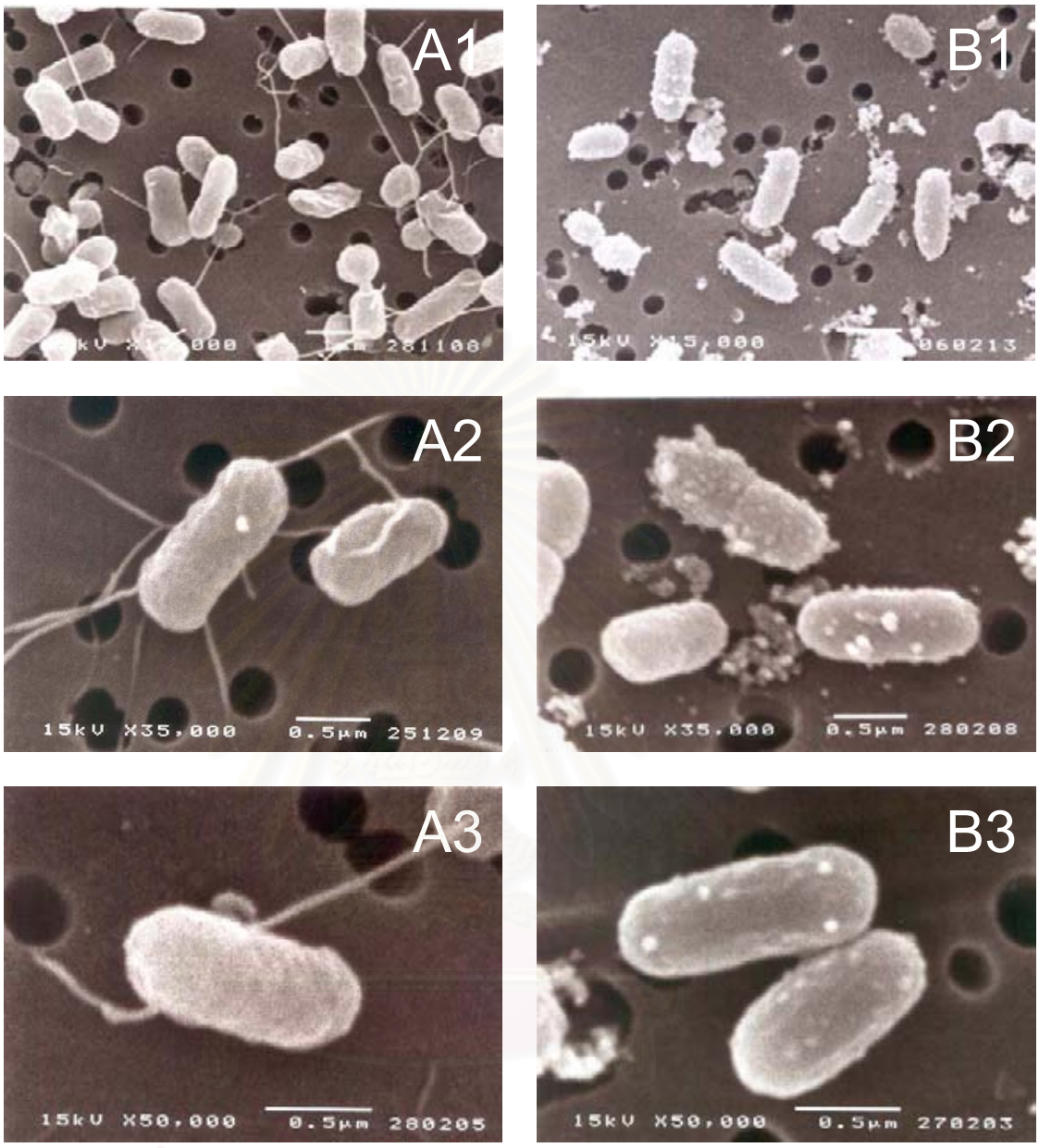


Figure 15 Scanning electron micrographs of *E. coli* ATCC 25922 after incubation for 24 hrs. at 37^0C; (A1,A2,A3) *E. coli* in NSS without PG; (B1,B3) *E. coli* in NSS with 0.32% PG; (B2) *E. coli* in NSS with 0.64% PG.

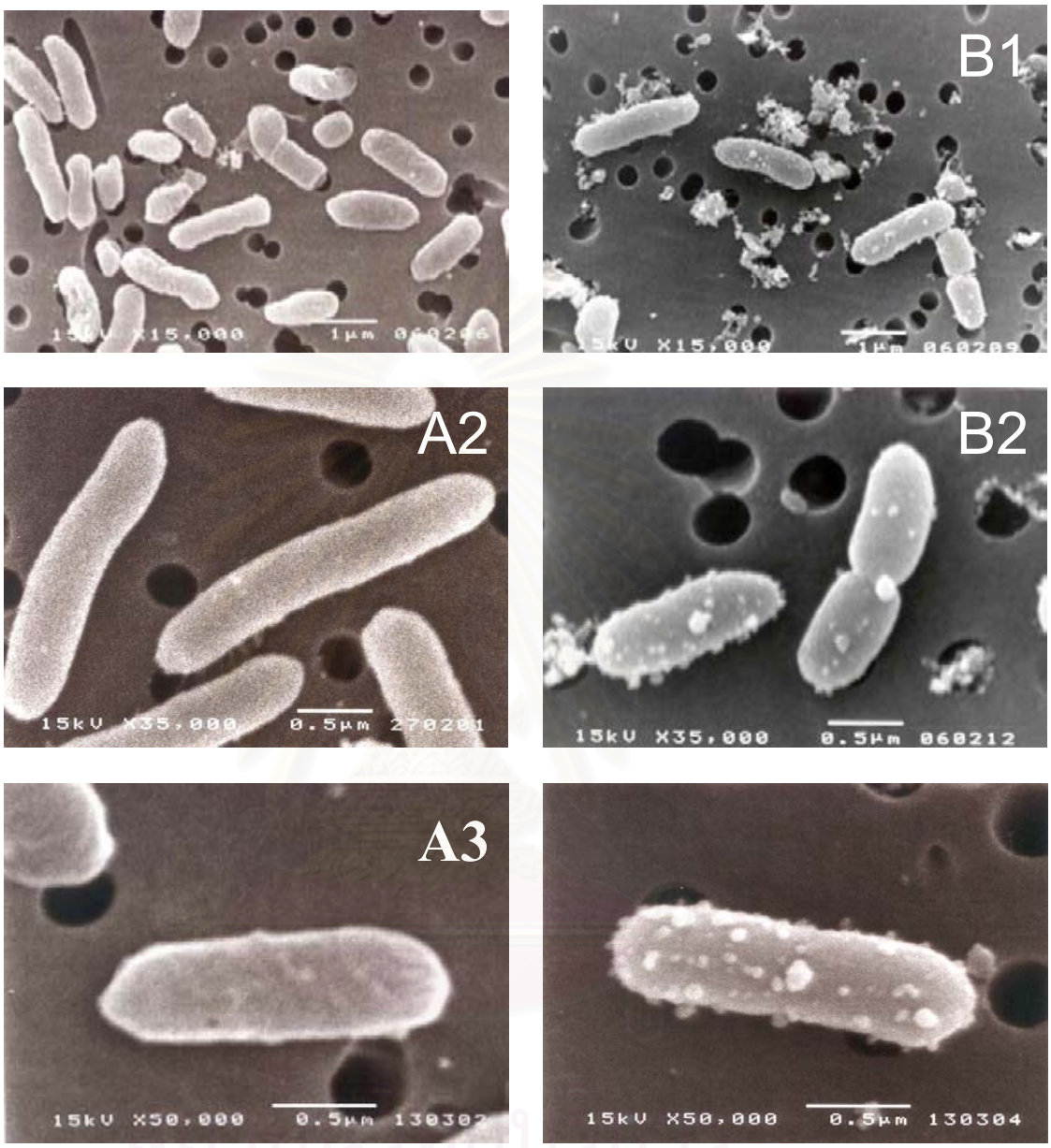


Figure 16 Scanning electron micrographs of *P. vulgaris* ATCC 13315 after incubation for 24 hrs. at 37^{0}C; (A1,A2,A3) *P. vulgaris* in NSS without PG; (B1,B2,) *P. vulgaris* in NSS with 0.32% PG; (B3) *P. vulgaris* in NSS with 0.64% PG.

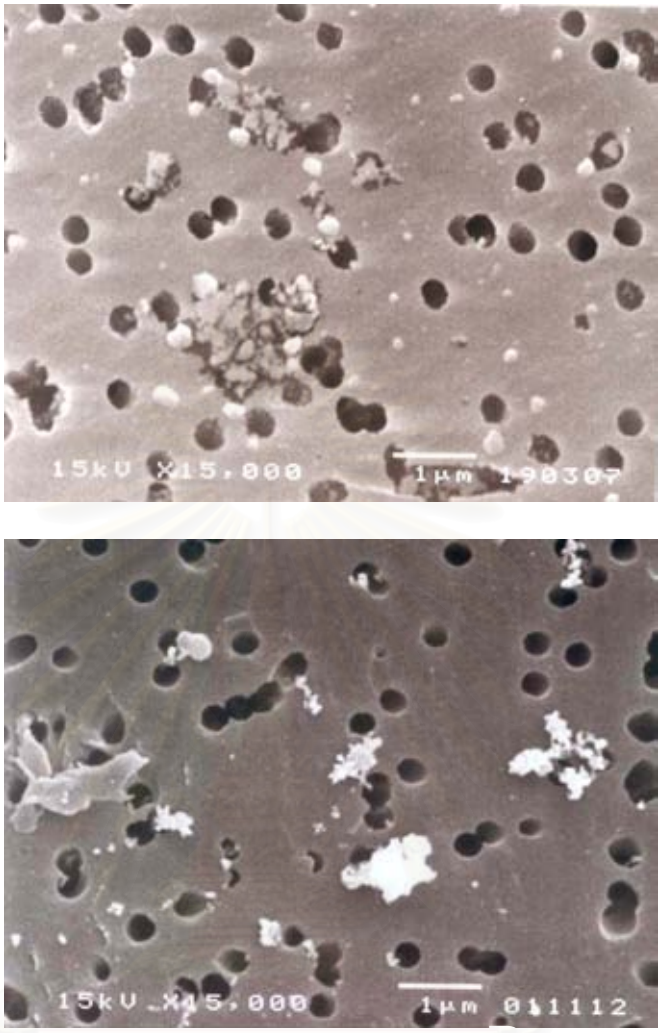


Figure 17 Scanning electron micrographs of 0.32%PG in NSS.

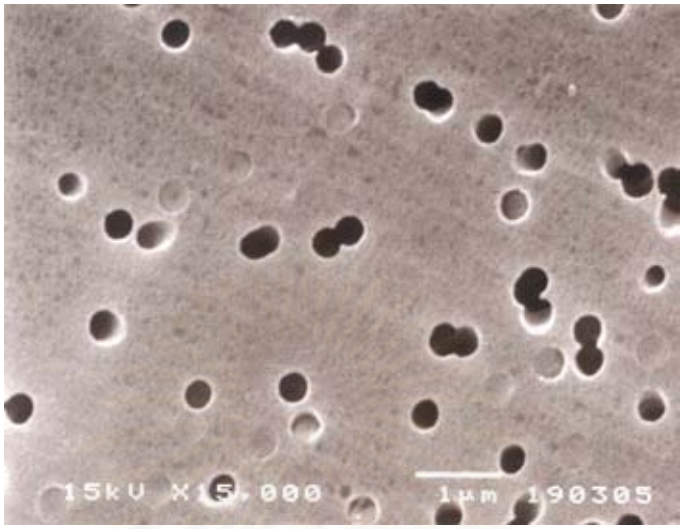


Figure 18 Scanning electron micrograph of NSS.

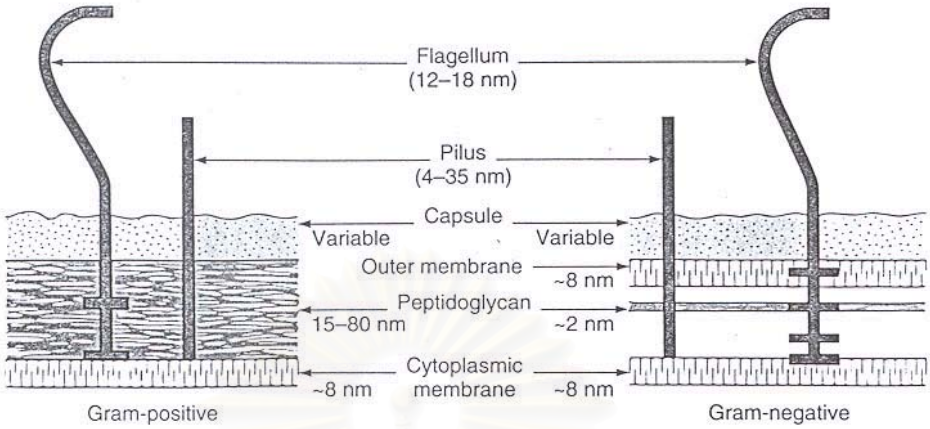


Figure 19 Comparison of the structure of gram-positive and gram-negative cell envelopes. The region between the cytoplasmic membrane and the outer membrane of the gram-negative envelope is called the periplasmic space. (From Jawetz, Melnick and Adelberg, 1995, p. 14)

4. *In vitro* development of resistance to PG in microorganisms

The development of PG resistance in bacteria by repeated exposure method was studied in two representative bacteria which was susceptible to PG. *S. aureus* and *E. coli* were used to evaluate the tendency of microorganisms resist to PG in this study.

After 30 passages, MIC values of PG against *S. aureus* and *E. coli* were not increased, whereas MIC value of *S. aureus* was decreased to two-fold as shown in Table 8. The results from induction studies indicated that the susceptibility of the both test bacteria were not increased after the exposure to sub-inhibitory of 6.4 and 3.2 mg/ml of PG for *S. aureus* and *E. coli*, respectively during 30 days. Growth of microorganisms at the lower than and at MIC breakpoint interpreted as susceptibility, while growth at higher than doubling-dilution of MIC value interpreted as resistance (Murray *et al*., 1995). Therefore, it was then possible to conclude that *S. aureus* and *E. coli* were not inducible to acquire the PG resistance for 30 days.

The emergence of drug resistance is a major factor limiting long-term successful use of antimicrobial agent. Development of resistance in microorganisms is increasing problem (Lorian, 1991). PG resistance bacteria were not observed in this investigation, therefore it would have advantages to used PG for antibacterial applications such as, film dressing for healing wounds. The results in Table 9 seem to indicate that *S. aureus* became more susceptible to PG during 30 passages, whereas *E. coli* was not effected. These results support the advantage of PG for an application as topical antibactericide as well as medical diet food.

Table 9 MICs of PG against microorganisms at the baseline and after repeated exposure experiment

Microorganisms	MIC (mg/ml)	
	Base line	After 30 passages (30 days)
S. aureus	12.8	6.4
E. coli	6.4	6.4

CHAPTER IV

CONCLUSION

Polysaccharide gel (PG) extracted from fruit-hulls of durian (*Durio zibethinus* L.) is carbohydrate composed of sugars including arabinose, rhamnose, fructose, glucose and galacturonic acid. Antimicrobial activity of PG was investigated to find a novel antimicrobial polysaccharide. The results showed that PG produced antibacterial activity against 7 strains of test bacteria, *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228, *Lactobacillus pentosus* ATCC 8041, *Escherichia coli* ATCC 25922 and *Proteus vulgaris* ATCC 13315.

1. Determination of antimicrobial activity of polysaccharide gel

Determination of antimicrobial activity of polysaccharide gel by agar diffusion susceptibility test showed sharp and clear margin inhibition zone against the susceptible bacteria. Inhibition zone was observed on agar media at concentration down to 0.32% of PG against *B. subtilis*, *M. luteus*, *S. epidermis, E. coli* and *L. pentosus*; to 0.625% and 1.25% of PG against *S. aureus* and *P. vulgaris*.

Broth macrodilution susceptibility test showed MIC and MBC values which demonstrated the complete inhibition and bactericidal activity of PG against bacteria. MIC and MBC of PG against *B. subtilis*, *M. luteus*, *S. epidermis*, *E. coli* and *P. vulgaris* were 6.4 and 25.6 mg/ml, respectively. Whereas, MIC and MBC of PG against *S. aureus* and *L. pentosus* were 12.8, 25.6 and 25.6, 51.2 mg/ml, respectively. Time-kill analysis showed killing effect of PG, which kill bacteria completely in 24 hours. The results showed that PG at the concentration of 25.6

mg/ml produced bactericidal activity against *B. subtilis*, *M. luteus*, *E. coli*, *P. vulgaris*, *S. aureus* and *S. epidermis*, the colony counts were declined to 0% at 12, 16, 16, 16, 20 and 20 hours, respectively. The colony counts of *L. pentosus* was declined to 0% at 8 hours with 51.2 mg/ml of PG.

PG film was used to qualitative assay for antimicrobial activity of PG against test microorganisms. PG film produced bactericidal activity against 7 strains of bacteria, *B. subtilis, M. luteus, S. aureus, S. epidermidis, L. pentosus, E. coli* and *P. vulgaris*. The results from this investigation correlated to previously susceptibility test, agar diffusion and broth macrodilution method which reported the same susceptible strains of bacteria to PG.

2. Determination of antibiotic susceptibility

Gentamicin sulfate and amphotericin B was used as a positive control against test bacteria and yeast, respectively in this study. Inhibition zone and MIC values of antibiotics against microorganisms which obtain from agar diffusion and broth macrodilution susceptibility test demonstrated that all standard strains of test microorganisms were susceptible to both test antibiotics within the acceptable range of standard method.

3. Examination of the effect of PG on cell surface of microorganism under scanning electron microscope

Survival test of microorganisms demonstrated that all strains of test bacteria, *S. aureus, S. epidermidis, E. coli* and *P. vulgaris* were killed within 24 hours in NSS in the presence of PG, however, they were normally survived in NSS. Examination of bacterial cells surface after 24 hrs. exposed to PG in NSS

under scanning electron microscope appeared an alteration on the outer surface of bacterial cells and pili of *E. coli* were disappeared. This examination concluded that killing effect of PG against bacteria may be involved the interaction of PG and outer surface of bacterial cells.

4. *In vitro* development of resistance to PG in microorganisms

The development of PG resistance in microorganisms by repeated exposure method was studies in two representative bacteria which susceptible to PG, *S. aureus* and *E. coli*. MIC values of PG against *S. aureus* and *E. coli* which exposed to PG for 30 days were not increased. The results demonstrated that *S. aureus* and *E. coli* were not inducible to obtain the PG resistance during 30 days.

This study demonstrated that no inhibitory activity of PG was appeared against 4 test bacteria, *Lactobacillus plantarum* ATCC 14917, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 9721, and 2 test yeast, *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10230.

This investigation discovered a novel polysaccharide antibacterial agent from fruit hulls of durian. PG is an interesting product from the viewpoint of its remarkable solubility and antibacterial activity. The data from the studied are of importance in defining the suitability applications. PG may thus have a high potential for practical applications in various fields including medicine, cosmetics and food. Water soluble film dressing for healing wound is the most interesting preparation to be further study.

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APPENDIX

APPENDIX

1. Potassium phosphate buffer, 0.1M; pH 7.4

Solution A : 27.2 g KH_2PO_4 per liter (0.2M)

Solution B : 45.6 g K_2HPO_4 per liter (0.2M)

Mix solution A 19 ml and B 81 ml, then dilute with H_2O to 200 ml.

2. Media

All media were dispensed and sterilized in autoclave for 15 min at 15 pounds pressure (121^0C). They were prepared in distilled water.

Media composition (g/L)

2.1 Tryptic soy broth (TSB)

Peptone from casein	17.0	g
Peptone from soymeal	3.0	g
D(+) glucose	2.5	g
Sodium chloride	5.0	g
Di-potassium hydrogen phosphate	2.5	g

pH 7.3±0.2

2.2 Tryptic soy agar (TSA)

Peptone from casein	17.0	g
Peptone from soymeal	3.0	g
Sodium chloride	5.0	g

Agar	15.0	g

pH 7.3±0.2

2.3 Mueller hinton broth (MHB)

Beef extract powder	2.0	g
Casein hydrolysate	17.5	g
Soluble starch	1.5	g

pH 7.4±0.2

2.4 Mueller hinton agar (MHA)

Beef extract powder	2.0	g
Casein hydrolysate	17.5	g
Soluble starch	1.5	g
Agar	13.0	g

pH 7.4±0.2

2.5 Brain heart infusion broth (BHIB)

Nutrient substrate		
(extracts of brain and heart and peptone)	27.5	g
D(+) glucose	2.0	g
Sodium chloride	5.0	g
Di-potassium hydrogen phosphate	2.5	g

pH 7.4±0.2

2.6 Brain heart infusion agar (BHIA)

Nutrient substrate		
(extracts of brain and heart and peptone)	27.5	g
D(+) glucose	2.0	g
Sodium chloride	5.0	g
Di-potassium hydrogen phosphate	2.5	g
Agar	15.0	g

pH 7.4±0.2

2.7 Sabouraud dextrose broth (SDB)

Dextrose	20.0	g
Enzymatic digest of casein	10.0	g

pH 5.6±0.2

2.8 Sabouraud dextrose agar (SDA)

Dextrose	20.0	g
Enzymatic digest of casein	10.0	g
Agar	15.0	g

pH 5.6±0.2

MIC interpretive standards for dilution susceptibility testing[a]

Antimicrobial agent and organism tested	MIC (μg/ml)			
	Susceptible	Moderately susceptible	Intermediate	Resistant
Penicillins				
Penicillin G				
Staphylococci, *Branhamella catarrhalis*	≤0.12			≥0.25
Neisseria gonorrhoeae	≤0.06	0.12–1		≥2
Enterococci		≤8		≥16
Listeria monocytogenes	≤2			≥4
Streptococcus pneumoniae	≤0.06	0.12–1.0		≥2
Other streptococci	≤0.12	0.25–.20		≥4
Oxacillin/nafcillin	≤2			≥4
Methicillin	≤8			≥16
Ampicillin				
Enterobacteriaceae	≤8	16		≥32
Staphylococci, *B. catarrhalis*	≤0.25			≥0.5
L. monocytogenes	≤2			≥4
Enterococci		≤8		≥16
Other streptococci	≤0.12	0.25–2		≥4
Ampicillin/sulbactam	≤8/4	16/8		≥32/16
Amoxicillin/clavulanic acid				
Staphylococci	≤4/2			≥8/4
Other organisms	≤8/4	16/8		≥32/16
Carbenicillin				
Pseudomonas aeruginosa	≤128	256		≥512
Other gram-negative bacilli	≤16	32		≥64
Ticarcillin				
P. aeruginosa	≤64	128		≥128
Other gram-negative bacilli	≤16	32–64		≥128
Ticarcillin/clavulanic acid				
P. aeruginosa	≤64/2	128		≥128/2
Other gram-negative bacilli	≤16/2	32/2–64/2		≥128/2
Mezlocillin				
P. aeruginosa	≤64			≥128
Other gram-negative bacilli	≤16	32–64		≥128
Piperacillin				
P. aeruginosa	≤64			≥128
Other gram-negative bacilli	≤16	32–64		≥128
Azlocillin for *P. aeruginosa*	≤64			≥128
Cephalosporins				
Cephalothin	≤8	16		≥32
Cefazolin	≤8	16		≥32
Cefamandole	≤8	16		≥32
Cefonicid	≤8	16		≥32
Cefaclor	≤8	16		≥32
Cefuroxime sodium	≤8	16		≥32
Cefuroxime axetil[b]	≤4	8–16		≥32
Cefoxitin	≤8	16		≥32
Cefotetan	≤16	32		≥64
Cefixime	≤1	2		≥4
Cefotaxime	≤8	16–32		≥64
Ceftazidime	≤8	16		≥32
Ceftriaxone	≤8	16–32		≥64
Ceftizoxime	≤8	16–32		≥64
Cefoperazone	≤16	32		≥64
Moxalactam	≤8	16–32		≥64
Other β-lactams				
Imipenem	≤4	8		≥16
Aztreonam	≤8	16		≥32
Aminoglycosides				
Gentamicin	≤4		8	≥16
Tobramycin	≤4		8	≥16
Netilmicin	≤8		16	≥32
Amikacin	≤16		32	≥64

(*Continued on next page*)

- *Continued*

Antimicrobial agent and organism tested	MIC (µg/ml)			
	Susceptible	Moderately susceptible	Intermediate	Resistant
Miscellaneous antibiotics				
Clindamycin	≤0.5		1–2	≥4
Erythromycin	≤0.5		1–4	≥8
Vancomycin	≤4		8–16	≥32
Enterococci		≤4	8–16	≥32
Other gram-positive organisms	≤4		8–16	≥32
Rifampin	≤1		2	≥4
Ciprofloxacin	≤1	2		≥4
Enoxacin	≤2	4		≥8
Ofloxacin	≤2	4		≥8
Trimethoprim/ sulfamethoxazole	≤2/38			≥4/76
Norfloxacin[c]	≤4		8	≥16
Nitrofurantoin[c]	≤32		64	≥128
Tetracycline[c]	≤4		8	≥16
Nalidixic acid[c]	≤16			≥32
Cinoxacin[c]	≤16			≥64
Sulfonamides[c]	≤256			≥512
Trimethoprim[c]	≤8			≥16

[a]Adapted from NCCLS (1990), which should be consulted for further details concerning recommended interpretive standard for dilution testing.

[b]The moderately susceptible category for this drug applies only to gram-negative bacilli isolated from the urinary tract.

[c]For use against urinary tract isolates only.

VITA

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