The Potential of Sea Grapes (*Caulerpa Lentilifera*) Extracted Polysaccharide as Prebiotics on Inhibiting Pathogenic Bacteria *Vibrio Parahaemolyticus*

Anek Sopon, Ekthida Thongdet, Porntep Punnarak, and Sucharat Suksai

***Abstract*—Sea grapes or green cavier (*Caulerpa lentilifera*), a common tropical green seaweed, consisted of polysaccharides to be used as a prebiotic precursor for prevention of pathogens in aquatic animals. The efficiency of polysaccharides extracted from sea grapes for prebiotic properties was conducted by comparing the growth of probiotic bacteria *Bacillus subtilis* at different concentrations in co-cultured with pathogenic bacteria *Vibrio parahaemolyticus*, a gram-negative motile bacterium that inhabits marine and estuarine environments throughout the world, causes of violent diseases outbreak in aquatic animals. The experimental research was 4 treatments with 4 replications including control group (LB broth), extracted sea grape enrichment in LB broth at 0.5, 1.0 and 2.7 mg.C/l. The result showed that the highest growth of probiotic bacteria appeared significantly at extracted sea grape in LB broth with 0.5 mg.C/l ($1.64\times10^7 \pm 6.04\times10^6$ cfu/ml). The said concentration was used as benchmark to clarify the pathogenic resistance. The comparison between monoculture of probiotic bacteria and co-culture of probiotic bacteria plus pathogenic bacteria indicated that there was non significantly different in growth of the bacterias. Hence extracted polysaccharides from sea graps (*C. lentilifera)* had potential to be utilized not only as a growth enrichment of probiotic bacteria but also inhibiting pathogenic bacteria.**

***Index Terms*—Sea grape, seaweed, polysaccharide, prebiotic, probiotic bacteria.**

I. Introduction

One of the important sources of polysaccharides is seaweed due to the high content aside from being a source of protein increased food nutrition for animals. Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic linkages, and on hydrolysis give the constituent monosaccharides or oligosaccharides.

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Both types of polysaccharide through fermentation processes can be a source of carbon for growth of all types of bacteria.

Polysaccharides are therefore considered as prebiotics and valuable in inhibiting pathogens. As prebiotic, polysaccharide provides carbon source for probiotic which is a group of useful bacteria. In aquaculture *Bacillus* sp., *Lactobacillus* sp. and Bifidobacterium are interested and commonly applied bacterial group.

The polysaccharides extracted from red seaweed *Grateloupia filicina* and *Eucheuma spinosum* can be counted as growth promoter of the best probiotic bacteria *Bifidobacterium*. [1], green seaweed *Cladomorpha linum* nourished growth of bacteria up to 70 percent. [2], brown seaweed *Padina australis, P. minor, Sargassum polycystum* raised bacterial growth. [3]

Pathogenic bacteria commonly produce a thick, mucous-like, layer of polysaccharide [4] and produce signal communication process between bacterial populations called quorum sensing mechanism. The role of polysaccharide, bioactive and antimicrobial compound extracted from seaweed in interruption of this communication process can be defined as pathogenic bacterial inhibitor. The mechanism is interaction in pathogenic bacterial cell and cell membrane and interrupt intracellular substance uptake and their transportation [5] such as the antimicrobial activity of water extracted compound of red seaweed *Polysiphonia lanosa* on pathogenic bacteria *Salmonella aureus*, *Escherichia cloacea* and *Clostridium perfringens.* Due to this interested function, seaweed extracts have used as alternative effective and disease controlling methods. [5] [6]

Pathogenic bacteria *Vibrio parahaemolyticus* is usually found in a free-swimming state; with its motility conferred by a single polar flagellum affixed to inert and animate surfaces including zooplankton, fish, shellfish or any suspended matter underwater [2] and cause of the mass mortality of aquaculture production.

Sea grapes or green cavier (*Caulerpa lentilifera*) is a green seaweed found commonly both in the Gulf of Thailand and Andaman sea. It contains 33.8 percent carbohydrate. [5] The extraction of polysaccharides from 2 percent weight sea grapes per volume has ability to supplement the growth of bacteria *Lactobacillus* and *Escherichia coli*. [6] The utilization of polysaccharides extracted from sea grapes as prebiotics and pathogenic resistant are not well recognized in Thailand. Therefore, it is interesting to clarify how to extract polysaccharide from sea grapes at optimal condition suited for growth of prebiotic bacteria and pathogenic resistance.

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II. Materials and Method

A. Preparation of Polysaccharide Extracted from Sea Grapes

Fresh sea grapes were cleaned by flow through tap water and dried at 60°C in hot air oven until had a constant weight. Dried seaweeds were calculated for percentage dried weight, then stored in a hygroscopic container.

Dried sea grapes were extracted using hot water extraction modified method. [7] The ratio of sea grape content to water was 1: 125 g/ml. The extracted condition was controlled at 80 °C throughout the extraction process for 45 minutes.

B. Preparation Bacillus subtilis and Vibrio parahaemolyticus and Culture Media

Bacillus subtilis was transferred [8] from the stocked in the LB agar (Luria-Bertani) plate into the LB broth tube, incubated at 30 °C for 24 hours and used for the further experiments.

Vibrio parahaemolyticus was transferred from the stocked in the TCBS and TSA Agar plate into TSA broth, incubated at 30 °C for 24 hours and used for the further experiments.

C. Study on the Growth of B. subtilis Cultured in Polysaccharide Extracted from Sea Grape

This experiment, 3 treatments of LB broth mixed with polysaccharide extraction from sea grape at 0.5, 1.0, and 2.7 mg.C/l were prepared as treatment group and LB broth without polysaccharide extraction was used as control.

B. subtilis was inoculated into 3 treatments of LB broth mixed with polysaccharide extraction and 1 treatment of control. Each treatment was conducted in triplication. The initial inoculation of *B. subtilis* was 10^5 cfu/ml. The inoculated tubes were incubated at 30°C for 48 hours. Then growth of *B. subtilis* in each treatment were investigated by total plate count technique and compared at 0 (initial), 24, and 48 hours of incubation. The treatment which provided the highest *B. Subtilis* growth were further study on the potential of probiotic *B. subtilis* on inhibiting pathogenic bacteria *V. parahaemolyticus*.

D. Study on the Potential of Probiotic B. subtilis on Inhibiting Pathogenic Bacteria V. parahaemolyticus

This experiment, growth of bacteria in co-culture of *B. subtilis* and *V. parahaemolyticus* in LB broth mixed with 0.5 mg.C/l of polysaccharide extraction from sea grape (treatment) and LB broth without mixing of polysaccharide extraction (control) were investigated for clarifying the ability of probiotic bacterial *B. subtilis* on inhibition of pathogen bacteria *V. parahaemolyticus*. Each treatment was done in triplication.

Both bacteria were inoculated with initial amount of 10^4 cfu/ml, incubated at 30 °C and then compared growth by total plate count technique at 0 (initial), 24, and 48 hours after incubation with LB Agar for *B. Subtilis* and TCBS Agar and TSA Agar for *V. parahaemolyticus*.

E. Statistical Data Analysis

Growth of *B. subtilis* cultured in the different concentration of polysaccharide extraction was compared to investigate the potential of adding polysaccharide extraction as prebiotic in culture media.

Growth of *B. Subtilis* co-cultured with *V. parahaemolyticus* in LB broth mixed with polysaccharide extraction treatment was compared with control to investigate the potential of probiotic *B. subtilis* on inhibiting pathogenic bacteria *V. parahaemolyticus*.

Comparison of growth between treatment were done using One-way ANOVA of SPSS program and represent by mean ± SD.

III. Results

A. The Growth of B. subtilis Cultured in Polysaccharide Extracted from Sea Grape

Growth of *B. subtilis* cultured in LB broth media with 3 concentrations of polysaccharide of extracted from sea grape (0.5, 1.0, 2.7 mg.C/l) and 0 mg.C/l of extracted polysaccharide as control were compared at 0, 24, and 48 hours after incubation using total plate count technique, initial inoculation varied between $2.50\times10^4 \pm 3.79\times10^4$ to $1.55\times10^5 \pm 1.06\times10^5$ cfu/ml (Table I). The results showed that after 24 hours of incubation, the amount of *B. subtilis* cultured in LB broth with 0.5 mg.C/l extracted polysaccharide was $1.64\times10^7 \pm 6.04\times10^6$ cfu/ml, significantly ($p < 0.05$) higher than amount of *B. subtilis* cultured in LB broth with 1.0 and 2.7 mg.C/l ($3.40\times10^5 \pm 4.98\times10^5$ cfu/ml and non-detected, respectively) and tent to higher than control ($4.57\times10^6 \pm 3.38\times10^6$ cfu/ml) as showed in Table I and Fig. 1.

After 48 hours of incubation (Table I and Fig. 1), amount of *B. subtilis* cultured in LB broth with 0.5 mg.C/l extracted polysaccharide was $5.44\times10^6 \pm 3.45\times10^6$ cfu/ml. This amount still higher than that of treatments with 0, 1.0 and 2.7 mg.C/l extracted polysaccharide ($1.57\times10^6 \pm 6.07\times10^5$, $1.42\times10^6 \pm 1.64\times10^6$ cfu/ml, and non-detected, respectively).

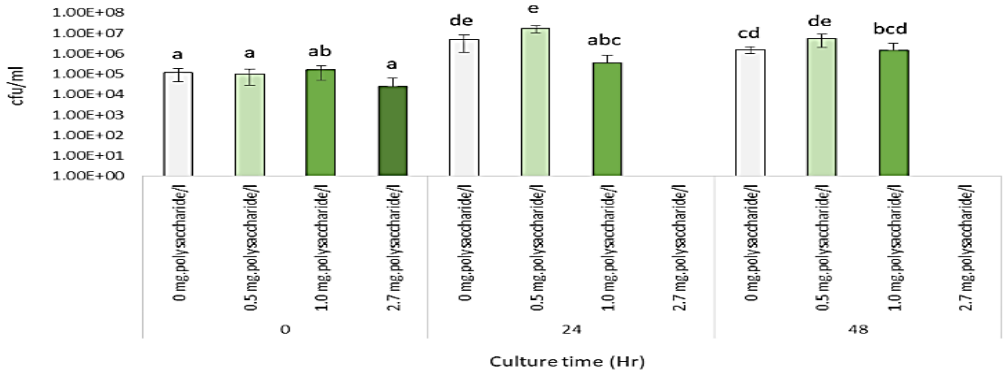
Due to the highest growth of *B. subtilis* was found in treatment of LB broth with concentration of extracted polysaccharide at 0.5 mg.C/l. So, this concentration of polysaccharide was used for the study of potential of probiotic *B. subtilis* on inhibiting pathogenic bacteria *V. parahaemolyticus*.

B. Potential of Probiotic B. subtilis on Inhibiting Pathogenic Bacteria V. parahaemolyticus

This experiment, growth of *B. subtilis* and *V. parahaemolyticus* which had co-cultured in LB broth mixed with 0.5 mg.C/l of polysaccharide extraction from sea grape (treatment) and LB broth without mixing of polysaccharide extraction (control) were compared at 0, 24, and 48 hours after incubation using total plate count technique, initial inoculation of *B. subtilis* and *V. parahaemolyticus* varied between $1.00\times10^5 \pm 1.00\times10^5$ to $6.00\times10^5 \pm 2.00\times10^5$ and $8.33\times10^4 \pm 7.64\times10^4$ to $1.50\times10^5 \pm 1.32\times10^5$ cfu/ml, respectively. (Table II). The results showed that there were no different of growth of *B. subtilis* and *V. parahaemolyticus* in control ($6.43\times10^6 \pm 1.49\times10^6$ and $5.33\times10^6 \pm 1.67\times10^6$ cfu/ml, respectively) after 24 hours of incubation. While, the amount of *B. subtilis* in LB broth mixed with 0.5 mg.C/l of polysaccharide extraction was $5.33\times10^6 \pm 1.67\times10^6$ cfu/ml

and *V. parahaemolyticus* was not founded as showed in Fig. 2.

After 48 hours of incubation, the amount of *B. subtilis* and *V. parahaemolyticus* in control were $2.15\times10^{6} \pm 1.44\times10^{6}$ and $1.38\times10^{6} \pm 8.96\times10^{5}$ cfu/ml, respectively. While, amount of *B. subtilis* and *V. parahaemolyticus* in LB broth mixed with 0.5 mg.C/l of polysaccharide extraction were $7.03\times10^{6} \pm 2.92\times10^{6}$ and $8.12\times10^{6} \pm 1.32\times10^{5}$ cfu/ml, respectively. There were no significantly different between amounts of both bacteria after 48 hours of incubation. But amount of *B. subtilis* cultured in 0.5 mg.C/l of polysaccharide extraction seeming higher compared to control and increasing of *V. parahaemolyticus* had investigated (Table II).

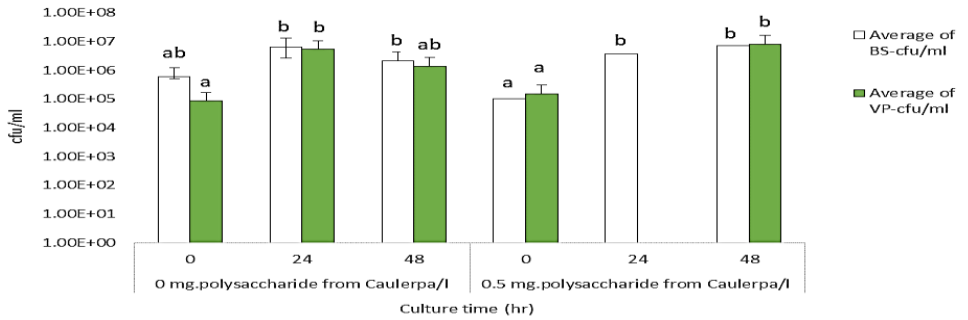


Different letters indicate significant different at $p<0.05$

Fig. 1. The amount of *B. subtilis* (cfu/ml) cultured in LB broth containing polysaccharide extract from sea grape at various concentrations.

TABLE I: THE AMOUNT OF *B. SUBTILIS* (CFU/ML) CULTURED IN LB BROTH CONTAINING POLYSACCHARIDE EXTRACT FROM SEA GRAPE AT VARIOUS CONCENTRATIONS

Incubation time	**Concentration of polysaccharide extracted from sea grape (mg.C/l)**			
(h)	**0**	**0.5**	**1**	**2.7**
0	$1.15\times10^{5} \pm 7.37\times10^{4a}$	$1.00\times10^{5} \pm 7.12\times10^{4a}$	$1.55\times10^{5} \pm 1.06\times10^{5ab}$	$2.50\times10^{4} \pm 3.79\times10^{4a}$
24	$4.57\times10^{6} \pm 3.38\times10^{6de}$	$1.64\times10^{7} \pm 6.04\times10^{6e}$	$3.40\times10^{5} \pm 4.98\times10^{5abc}$	nd
48	$1.57\times10^{6} \pm 6.07\times10^{5cd}$	$5.44\times10^{6} \pm 3.45\times10^{6de}$	$1.42\times10^{6} \pm 1.64\times10^{6bcd}$	nd



Different letters indicate significant different at $p<0.05$

Fig. 2. The amount of *B. subtilis* and *V. parahaemolyticus* (cfu/ml) cultured in LB broth containing polysaccharide extracts and control.

TABLE II: THE AMOUNT OF *B.* SUBTILIS AND *V. PARAHAEMOLYTICUS* (CFU/ML) CULTURED IN LB BROTH CONTAINING POLYSACCHARIDE EXTRACTS AND CONTROL

Incubation	***B. subtilis***		**V. *parahaemolyticus***	
time (h)	**0.0 mg.C of polysaccharide/l**	**0.5 mg.C of polysaccharide/l**	**0.0 mg.C of polysaccharide/l**	**0.5 mg.C of polysaccharide/l**
0	$6.00\times10^{5} \pm 2.00\times10^{5ab}$	$1.00\times10^{5} \pm 1.00\times10^{5a}$	$8.33\times10^{4} \pm 7.64\times10^{4a}$	$1.50\times10^{5} \pm 1.32\times10^{5a}$
24	$6.43\times10^{6} \pm 1.49\times10^{6b}$	$3.75\times10^{6} \pm 2.03\times10^{6b}$	$5.33\times10^{6} \pm 1.67\times10^{6b}$	nd
48	$2.15\times10^{6} \pm 1.44\times10^{6b}$	$7.03\times10^{6} \pm 2.92\times10^{6b}$	$1.38\times10^{6} \pm 8.96\times10^{5ab}$	$8.12\times10^{6} \pm 1.32\times10^{5b}$

IV. DISCUSSION

The properties of extracted polysaccharides as carbon source for probiotic bacteria is interested. The composition of polysaccharides derived from seaweed considered as a sulfated polysaccharides, which is a type of polysaccharides that are different from terrestrial plants and also provide biological properties whether it is an antioxidants including immunological and inhibition effects on infections and tumours as well. [9] The composition of polysaccharides in *Caulerpa racemose* consisted of glucose, arabinose, galactose, mannose xylose and ramnose which correspond to

the composition of the polysaccharides in *C. lentillifera* and *C. sertulariodes.* [10]-[12]

It had been reported that *C. prolifera* had a high ratio of sugar to sulfate and had more than 50 percent resistance to viral infections. While *C. sertularioides* shown the ability to inhibit free radicals more than 10 percent compared to red algae and brown algae. [13] In addition, the oligosaccharide extracted from *C. racemose,* was also found in the form of α-(1→4) glucose after being hydrolyzed, and have been classified as gluco-oligosaccharides which can be a good source of carbohydrates for bacteria to grow. [10], [14] Furthermore, it had been reported that *Bacillus* spp. isolated from digestive tract of turbot at the weaning stage had higher growth when used Raftilose P5 which is the oligofructose as carbon source compared with glucose [15]. This result revealed the importance of oligosaccharide on growth of probiotic such as *B. subtilis* in aquaculture.

Comparing the growth of *B. subtilis* cultured in LB broth (control) and LB broth mixed with polysaccharide extract from sea grape revealed that the medium mixed with polysaccharide extracted from sea grape 0.5 mg.C/l promoted significantly highest amount of bacteria *B. subtilis* ($1.64x10^7 \pm 6.04x10^6$ cfu/ml) compared to control an another concentration of extracted polysaccharide after 24 hours of incubation. This may be due to obtaining oligosaccharide as additional carbon sources for bacterial growth. Oligosaccharide probably was hydrolysed from polysaccharide during sea grape extraction process according to the study of *C. racemose* polysaccharide extraction, oligosaccharide in α-(1→4) glucose form was found after hydrolyzation of polysaccharide [11], [14].

In contrast, *B. subtilis* cultured in only polysaccharide extraction which is contained 2.7 mg.C/l did not promote the growth of bacteria. Therefore, there was a possibility that, the polysaccharide extracted from sea grape may inhibit the growth of *B. subtilis* or insufficient carbon sources for bacterial growth.

A possibility in inhibition on growth of *B. subtilis* by seaweed extraction have been reported in the study between green seaweed *C. prolifera, C. racemosa and C. webbiana* and growth of *B. subtilis* MB964. But seaweeds were extracted by methanol and growth of bacteria was investigated using the inhibition zone. However, there was the interesting explanation about the inhibition process of seaweed extract on growth of bacteria which the anticoagulant and antioxidant activities. The 0.01 – 2.00 mg/ml polysaccharide from *C. prolifera* had antiproliferative efficacy on HeLa cell proliferation (36.3 – 58.4%) and the result showed the positive correlation between sulfate content and antiproliferative efficacy. The sulfated polysaccharides extracted from *C. lentilifera* at concentration 2.7 mg.C/l in this study may had ability of antioxidant and antiproliferation against the growth of *B. subtilis*. [16], [17]

In addition, those two possibilities may showed in the result of lesser growth of *B. subtilis* cultured in control and LB broth mixed with 1 mg.C/l of polysaccharide extraction compared with significantly higher growth of *B. subtilis* cultured in LB broth mixed with 0.5 mg.C/l of polysaccharide extraction. Higher concentration of 1 mg.C/l of polysaccharide extraction in LB broth may slightly inhibit the growth of bacteria. While, lower carbon source in control compared to treatment with 0.5 mg.C/l of polysaccharide result in lower growth of *B. subtilis* as well. However, the inhibition of this seaweed extract on growth of bacteria still need the further study.

The results of the studying on the potential of probiotic *B. subtilis* on inhibiting pathogenic bacteria *V. parahaemolyticus* showed that growth of *B. subtilis* and *V. parahaemolyticus* when cultured together in LB broth containing with polysaccharide 0.5 mg.C/l at 24 hr of the cultivation were significantly difference due to growth of *V. parahaemolyticus* were low and could not be detected. This result was similar with variation of *Vibrio* sp. and *Bacillus* sp. in intestinal tract of weaning turbot. Before feeding with oligofructose *Vibrio* spp. represented 96% of the isolation. But the proportion of *Vibrio* sp. was reduced, and *Bacillus* sp. was increased after fed with oligosucrose. In addition, *Bacillus* spp. have been reported and evaluated as probiotic and biocontrol due to their abilities of water quality improvement, reducing the incidence of pathogen especially *Vibrio* sp. [18].

However, the growth of both *B. subtilis* and *V. parahaemolyticus* were not showed the significantly difference after the 48 hr of cultivation in LB broth containing with polysaccharide 0.5 mg.C/l. This result may due to the co-utilization of two bacteria.

V. Conclusion

The supplementation of polysaccharides from sea promoted the best *B. subtilis* growth at the concentration of 0.5 mg.C/l when compared to the control and the other concentration treatment of supplemented polysaccharides. When culture *B. subtilis* together with *V. parahaemolyticus* in culture media containing polysaccharide supplemented for 0.5 mg.C/l of seaweed in both varieties, the growth of *B. subtilis* was significantly higher than growth of *V. parahaemolyticus* after 24 hours of cultivation, but there was not significantly different after 48 hours of cultivation. In consequence extracted polysaccharides from sea graps (*C. lentilifera*) had potential to be utilized not only as a growth enrichment of probiotic bacteria but also inhibiting pathogenic bacteria.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Anek Sopon, Ekthida Thongdet and Sucharat Suksai work in the laboratories; Porntep Punnarak analyzed data statistic; Anek Sopon wrote the paper; all authors had approved the final version.

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