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FULL PAPER

Growth promotion of some lactic acid bacteria by crude extract of *Spirogyra* sp., *Cladophora* sp., *Caulerpa lentillifera* and *Caulerpa corynephora*

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Abstract

The aim of this research was to evaluate the growth promotion of two freshwater macroalgae (*Spirogyra* sp., *Cladophora* sp.) and two saltwater macroalgae (*Caulerpa lentillifera* and *Caulerpa corynephora*) crude extracts to some lactic acid bacteria using *in vitro* fermentation. Total soluble carbohydrates of macroalgae were obtained after extraction with 75°C of water for 1 hr. The concentration of total sugar and reducing sugar were 0.764 and 0.197 mg/g in *Spirogyra* sp., 0.368 and 0.082 mg/g in *Cladophora* sp., 0.484 and 0.055 mg/g in *C. lentillifera*, and 0.253 and 0.037 mg/g in *C. corynephora*, respectively. Degree of polymerization (DP) that refers to the size of oligosaccharide, were 3.8, 4.5, 8.8 and 6.8, respectively. The prebiotic activity was assessed by the change of the bacterial population. Crude extracts from macroalgae were tested for growth stimulation effect on lactic acid bacteria, *Lactobacillus plantarum* TISTR862 and *Escherichia coli* TISTR073. Results demonstrated that the population of *L. plantarum* TISTR862 and *E. coli* TISTR073 were higher in crude extracts from freshwater than saltwater macroalgae. The prebiotic activity score was calculated based on the change of growth in probiotic and pathogen after 24 hrs of incubation time. The highest score was obtained from *C. corynephora* extracts (1.10) follow by *C. lentillifera* extracts (0.77), *Cladophora* sp. extracts (0.173) and *Spirogyra* sp. extracts (0.07). In comparison with commercial culture (*Lactobacillus casei, Lactobacillus lactis, Streptococcus thermophilus* and *Bifidobacterium*), the addition of 3.5% *C. lentillifera* extracts resulted as prebiotic activity score value as 3.5% FOS. Preliminary study demonstrated that crude extract of *C. lentillifera* could be a prebiotic substance.

1. Introduction

The interest in probiotics and prebiotics are increasing due to the important role played in human nutrition. Probiotic are defined as live microorganisms that confer a health benefit on the host (Cao *et al*., 2020) that can protect humans from diseases, modulated the immune system and have anticancer properties (Konuray and Erginkaya, 2018). Prebiotics are defined as non-digestible food ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of one or a limited number of bacterial in the colon (Gibson and Roberfroid, 1995).

Spirogyra sp., Tao in Thai common name, is the freshwater macroalgae in northern Thailand (Figure 1a). Tao was found in stagnant water and reaching abundance during hot dry and before entering the rainy season. *Cladophora* sp. is known as Kai, is also freshwater macroalgae (Figure 1b). It is abundant in Nan province, the Northern part of Thailand (Khuantrairong and Traichaiyaporn., 2011). Their identity character is the light rough. *Spirogyra* sp. and *Cladophora* sp. are freshwater macroalgae in Division in Chlorophyta (Sakulpong *et al*., 2015). *C. corynephora* or Konnok is the saltwater macroalgae and found in southern of Thailand (Figure 1c). *C. corynephora* is an edible and treasure house of novel healthy food ingredients and biological active compounds (Fithriani, 2015). Also *C. lentillifera* shown in Figure 1d, also known as Sea Grapes, are saltwater macroalgae eaten raw as salad and cultivated in different parts of the world, particularly in the Indo-Pacific region (Yap *et al*., 2019). Macroalgae is a source of food for human and feed for animal. Many kinds of research were focused on the nutritional value of macroalgae such as protein, lipid, fiber and carbohydrate. From nutrition point, macroalgae are rich

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in carbohydrates. Polysaccharides are polymeric molecules of carbohydrates. Nowadays, the interest in the development of prebiotics has been focused on the use of nondigestible oligosaccharides and polysaccharides which cannot be digested but are readily fermented by colonic bacteria (Zhang *et al*., 2003). Many polysaccharides from various sources have display prebiotic properties both *in vitro* and *in vivo*. There are few studies on the use of macroalgae as prebiotics substances. So, the growth promotion of some lactic acid bacteria by two freshwater macroalgae (*Spirogyra* sp., *Cladophora* sp.) and two saltwater macroalgae (*C. lentillifera* and *C. corynephora*) crude extracts using *in vitro* fermentation were evaluated.

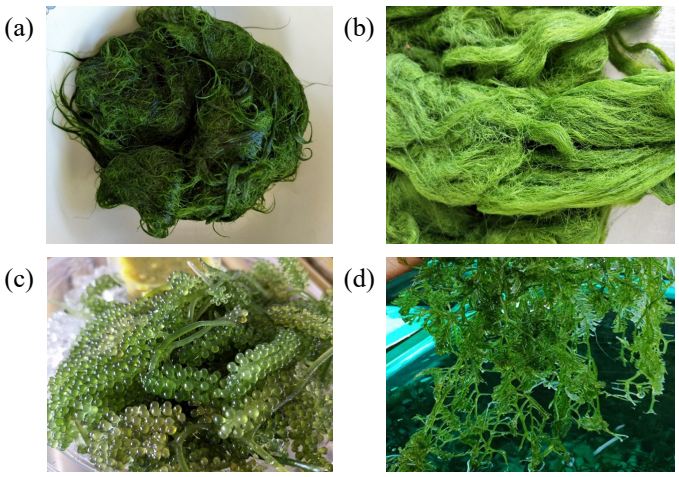

Figure 1. Freshwater and saltwater macroalgae; freshwater macroalgae (a) *Spirogyra* sp. or "Tao" (b) *Cladophora* sp. or "Kai", (c) *Caulerpa lentillifera* or "Sea Grape" and (d) *Caulerpa corynephora* or "Konnok".

2. Materials and methods

2.1 Chemicals, reagents and microorganisms

De Man, Rogosa and Sharpe Agar or MRS agar (HIMedia, India), Eosin Methylene Blue Agar or EMB agar (HIMedia, India), M17 agar (HIMedia, India) and Bifidobacterium agar (HIMedia, India) were used in the isolation and selective enumeration of *Lactobacillus* species, *E. coli*, *Streptococcus* sp. and *Bifidobacterium*, respectively.

Lactic acid bacteria used in this research was *L. plantarum* TISTR082 and enteric strain used were *E. coli* TISTR073 were purchased from the Thailand Institute of Scientific and Technological Research (TISTR), Pathumthani Province, Thailand. Commercial culture of probiotics (*L. acidophilus, L. casei, S. thermophiles* and *B. animalis*) was purchased from Sacco, Italy.

2.2 Materials

Freshwater macroalgae (*Spirogyra* sp., *Cladophora* sp.) and two saltwater macroalgae (*C. lentillifera* and *C. corynephora*) were studied. *Spirogyra* sp., *Cladophora* sp. were collected from Phare and Nan Province, Thailand, *C. lentillifera* and *C. corynephora* were collected from Phetchaburi and Krabi Province, Thailand, respectively. All impurities were removed by the water. Macroalgae were dried at 70°C for 12 hrs.

2.3 Extraction

The samples of macroalgae were air-dried and grounded. Crude macroalgae carbohydrates were extracted with 70°C of water at the ratio of 1:10 (powdered macroalgae: distilled water) and shaken with rotary shaker for 1 hr. Subsequently, crude extracts were filtrated, evaporated and concentrated by the rotary evaporator at 45°C. Finally, the resultant samples were stored at -20^{0}C until use.

2.4 Analysis of the amount of carbohydrate

The total sugar and reducing sugar in each crude extracts were determined by using Phenol-Sulphuric method and DNS method, respectively (DuBois *et al*., 1956; Miller 1972; Li *et al*., 2017). An average degree of polymerization (DP) was calculated as the value of the amount of total sugar divided by the amount of reducing sugar (Wongputtisin *et al*., 2015).

2.5 In vitro prebiotic activity

2.5.1 Prebiotic property study by single culture test

The crude extracts of macroalgae were added as a carbon source at a concentration of 3.5% (w/v) in the basal medium (K_2HPO_4 0.3, KH_2PO_4 0.1, yeast extract 1.0, peptone 1.0, $MgSO_4$ 0.2 and $(NH_4)_2SO_4$ 2.5 g/L, pH 7.0). The media were sterilized. The individual tubes were inoculated with approximately 10^6 CFU of 12 hour-old inoculum of *L. plantarum* and *E. coli*. The cultivations were incubated at 37°C for 60 hrs in anaerobic condition. Samples of the fermentation broth were withdrawn from each tube at 0, 12, 24, 36, 48 and 60 hrs for cell counting. Sampling of viable cell enumeration was performed on MRS agar and EMB agar for *L. plantarum* and *E. coli*, respectively. Moreover, the growth of tested strains in basal medium with glucose and without carbon source was also studied as a control treatment. Data were reported as the ratios of the log CFU/mL counts of the bacteria.

2.5.2 Prebiotic property study by commercial culture test

The evaluation growth promotion of crude macroalgae extracts was performed as described above. Varying concentration of each carbon source ranging from 1.5 – 3.5% w/v was tested. Commercial cultures were used in the experiment and represented as probiotic. This commercial culture was mixed with *L.*

casei, L. lactis, S. thermophiles and *B. animalis*. FOS was used as a positive control due to its well-established prebiotic properties, whereas basal medium without carbon source was used as a negative control. Inoculum of commercial culture was transferred to a sterilized basal medium. The cultivations were conducted at 37°C for 24 hrs in anaerobic condition. Samples of the fermentation broth were withdrawn at 0 and 24 hrs for cell counting. Sampling of viable cell enumeration was performed on MRS agar, M17 agar, Bifidobacterium agar and EMB agar for *L. casei, L. lactis, S. thermophiles*, *B. animalis and E. coli*, respectively.

2.6 Prebiotic activity score

According to Hueber *et al*. (2007), the prebiotic activity score was calculated using the following equation:

Prebiotic activity score = {(probiotic log CFU/mL on the prebiotic at 24 hrs – probiotic log CFU/mL on the prebiotic at 0 hr) / (probiotic log CFU/mL on glucose at 24 hrs – probiotic log CFU/mL on glucose at 0 hr)} - {(enteric log CFU/mL on the prebiotic at 24 hrs – enteric log CFU/mL on the prebiotic at 0 hr) / (enteric log CFU/mL on glucose at 24 hrs – enteric log CFU/mL on glucose at 0 hr)} .

By definition, substrates with a high prebiotic activity score support good growth of the probiotic bacteria, with viable cell (CFU/mL) comparable with that when grown on glucose.

2.7 Statistical analysis

Descriptive results are presented as mean plus or minus standard deviation. ANOVA was used to assess the significance of descriptive data. *P* values <0.05 were considered significant. Dunnett's was used to identify the group that were similar with respect to the mean.

3. Results and discussion

3.1 The amount of sugar of freshwater macroalgae and saltwater macroalgae

The concentration of total sugar and reducing sugar were 0.764 and 0.197 mg/g in *Spirogyra* sp., 0.367 and 0.082 mg/g in *Cladophora* sp., 0.484 and 0.055 mg/g in *C. lentillifera*, and 0.253 and 0.037 mg/g in *C. corynephora*, respectively. DP of sample extracts were 3.8, 4.5, 8.8 and 6.8, respectively (Table 1). DP of 2-9 or sometimes 8-20 monomers are classified as oligosaccharides that correspond to the prebiotic definition. Moreover, prebiotics is thought to be non-digestible by the human or animal digestive enzyme (Saad *et al*., 2013). These oligosaccharides seem easier for fermenting by gut microbiota (de Jesus Raposo *et al*., 2016).

Table 1. Total sugar, reducing sugar concentration and DP of two freshwater macroalgae and two saltwater macroalgae.

Aqueous extracts of macroalgae	Total sugar (mg/g)	Reducing sugar (mg/g)	DP
Spirogyra sp. (Tao)	0.764	0.197	3.8
Cladophora sp. (Kai)	0.367	0.082	4.5
Caulerpa lentillifera (Sea Grape)	0.484	0.055	8.8
Caulerpa corynephora (Konnok)	0.253	0.037	6.8

3.2 Growth of Lactobacillus plantarum and Escherichia coli on macroalgae extracts

Crude *Spirogyra* sp., *Cladophora* sp., *C. lentillifera* and *C. corynephora* extracts were selected as carbon source for in vitro fermentation study. In this experiment, *L. plantarum* TISTR862 and *E. coli* TISTR073 were represented as lactic acid bacteria and enteric strain. The populations of tested strain in basal medium added with different carbon sources from macroalgae were shown in Tables 2-3. Basal medium without carbon source and with glucose served as blank and control group, respectively. Results demonstrated that the population of *L. plantarum* and *E. coli* were higher in sample extracts from freshwater than saltwater macroalgae. In contrast, the population of *E. coli* was not promoted in basal medium added with crude extracts from saltwater macroalgae. Growth of enteric strain (*E. coli*) on *C. corynephora* extracts and *C. lentillifera* extracts were lower than growth on *Cladophora* sp. extracts, *Spirogyra* sp. and glucose as carbon sources in basal medium. It might indicate that crude extracts from saltwater macroalgae were utilized less well by enteric strain than

Table 2. Growth of single tested strains of *L. plantarum* in the basal medium added with different carbon source when incubating for 60 hrs.

Carbon sources	*L. plantarum* (log CFU/mL)					
	0 hrs	12 hrs	24 hrs	36 hrs	48 hrs	60 hrs
Non-carbon	5.63±0.07	5.78±0.06	6.27±0.06	5.88±0.01	5.70±0.05	5.51±0.03
Glucose	5.76±0.02	6.13±0.01	7.12±0.03	7.17±0.05	7.12±0.03	6.70±0.21
Spirogyra sp. or Tao	5.82±0.01	7.30±0.08	7.45±0.10	7.38±0.08	7.16±0.67	7.07±0.27
Cladophora sp. or Kai	6.04±0.03	7.42±0.21	7.72±0.21	7.38±0.21	7.37±0.06	6.58±0.06
Caulerpa lentillifera or Sea Grape	5.90±0.03	5.97±0.01	7.14±0.03	6.96±0.01	6.77±0.05	6.66±0.07
Caulerpa corynephora or Konnok	5.70±0.07	5.83±0.12	7.25±0.07	7.32±0.01	7.05±0.05	6.89±0.07

Table 3. Growth of single tested strains of *E. coli* in the basal medium added with different carbon source when incubating for 60 hrs.

Carbon sources	*E. coli* (log CFU/mL)					
	0 hrs	12 hrs	24 hrs	36 hrs	48 hrs	60 hrs
Non-carbon	4.77±0.21	6.90±0.15	6.17±0.01	6.16±0.08	6.01±0.10	5.96±0.08
Glucose	4.90±0.02	7.04±0.01	6.99±0.05	6.92±0.07	6.80±0.03	6.63±0.05
Spirogyra sp. or Tao	4.77±0.03	7.28±0.21	7.12±0.21	6.97±0.20	6.96±0.15	6.77±0.14
Cladophora sp. or Kai	5.04±0.07	7.43±0.01	7.27±0.05	7.19±0.07	7.16±0.15	7.11±0.14
Caulerpa lentillifera or Sea Grape	4.77±0.10	5.20±0.08	5.07±0.06	4.84±0.27	4.69±0.05	4.47±0.03
Caulerpa corynephora or Konnok	4.69±0.06	5.04±0.27	4.77±0.29	4.60±0.01	4.60±0.06	4.47±0.27

crude extracts from freshwater macroalgae. Lower cell counts at 24 hrs, may be the consequence of biochemical processes during later stages of growth and death of the bacteria (Shalini *et al*., 2017).

The prebiotic activity score was calculated based on the change of growth in probiotic and pathogen after 24 hrs of incubation time. Using the prebiotic activity score equation, the prebiotic activity score of a particular oligosaccharide can be determined relative to tested strain (Huebner *et al*., 2007). With the resulting score of activity, it was possible to compare prebiotic substances. From this research (Figure 2), the highest score was obtained from *C. corynephora* extracts (1.10) follow by *C. lentillifera* extracts (0.77), *Cladophora* sp. extracts (0.173) and *Spirogyra* sp. extracts (0.07). The higher the prebiotic activity score derived from the higher the relative growth of probiotic and lower the relative growth of pathogen (Rubel *et al*., 2014)

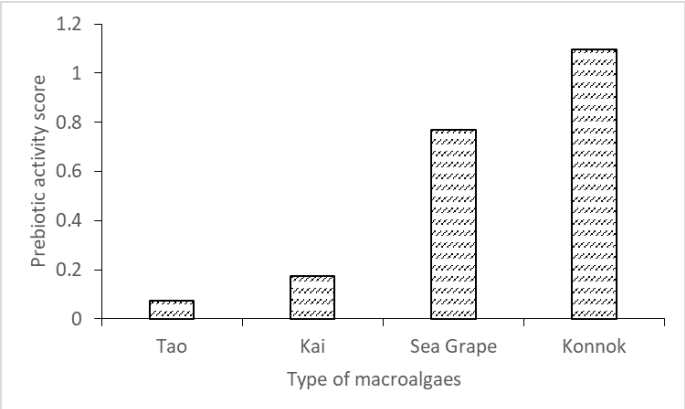

Figure 2. Prebiotic activity score of each macroalga

Saltwater macroalgae extracts could stimulate *L. plantarum* but could not stimulate *E. coli*. These were considered as a primary property of prebiotic substance. Saltwater macroalgae are rich in carbohydrates. The major component in green saltwater macroalgae are polysaccharide (25 - 75% of the dry weight (DW)): of total dietary content (29-67%) and soluble dietary fibre (17-24%) (Charoensiddhi *et al*., 2017). The structural polysaccharides of saltwater macroalgae are sulphated polysaccharides, such as ulvans and sulphated galactans, xylans, mannan, while the main storage polysaccharide is starch (Charoensiddhi *et al*., 2017). According to Fithriani (2015), reported about *Caulerpa* sp. that richness in sulfated polysaccharides. The most polysaccharides from saltwater macroalgae can be considered as dietary fiber that is resistant to digestion by enzymes present in the human gastrointestinal tract, and selectively stimulate to the growth of beneficial gut bacteria (de Jesus Raposo *et al*., 2016). These effects may provide health benefits to humans through a prebiotic effect (O'Sullivan *et al*., 2010).

3.3 Effect of Caulerpa lentillifera extracts on commercial culture

C. lentillifera extracts were selected for the next study with commercial culture due to the high prebiotic activity score. The preliminary prebiotic effect of the selected crude extracts has been evaluated by comparison with FOS. The dosage used in the growth assay was determined by inoculating commercial culture and *E. coli* supplemented with varying concentration of FOS and *C. lentillifera* extracts. The tested prebiotic sample were analyzed at 1.5%, 2.5% and 3.5% (w/v). Regarding the commercial cultures (*L. casei, L. lactis, S. thermophiles* and *B. animalis*), a stimulatory effect on the growth was observed for 24 hrs using *C. lentillifera* extracts and FOS as carbon source. Viable cell numbers obtained were compared (Figure 3). The viable cell numbers of *S. thermophiles* reached a value of 4.5 log CFU/mL when used FOS as carbon source in basal medium. The increasing growth of *L. casei, L. lactis, S. thermophiles* and *B. animalis* in media added with FOS were higher than media added with *C. lentillifera* extracts. Prebiotic activity score of *C. lentillifera* extracts and FOS are represented in Figure 4. The prebiotic activity score of the pure prebiotic provides a reference level of prebiotic activity score. In the present study, the prebiotic activity score is indicative of selective use of carbon sources by 4 different probiotic strains in comparison with *E. coli*. The prebiotic activity score of 3.5% (w/v) *C. lentillifera* extracts were 1.19, 0.55, 0.72 for *Lactobacillus, S. thermophiles* and *B. animalis*, respectively. FOS supported the growth of all the four probiotics strains suggesting its great prebiotic efficacy. *C. lentillifera* extracts had a lower score for all probiotic strains compared to FOS. The prebiotic activity score of 3.5% (w/v) FOS for these cultures were 2.05, 0.69 and 0.87, respectively. It might indicate that algae belonging

to Division Chlorophyta, like *Caulerpa*, are rich in glucomannans, mannans, xylans, sulfated polysaccharides and pectins. Sulfated polysaccharides found in a wide range of algae exhibit their prebiotic effect by inhibiting the adhesion of pathogens (Kho *et al*., 2017). The results in the present study provide evidence for each microbial strain dependence on different carbon sources.

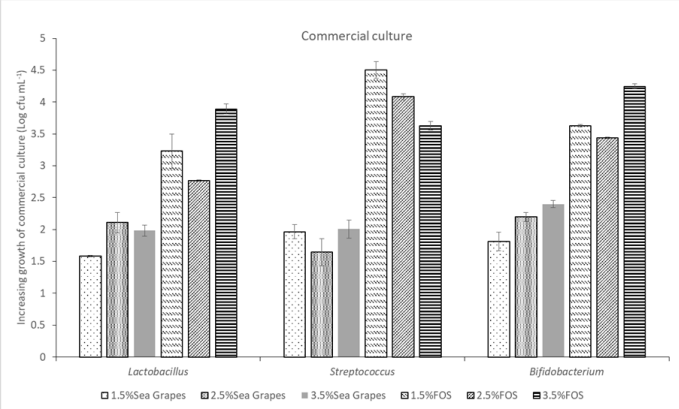

Figure 3. Increasing growth of commercial culture in basal medium added with different concentration of carbon sources.

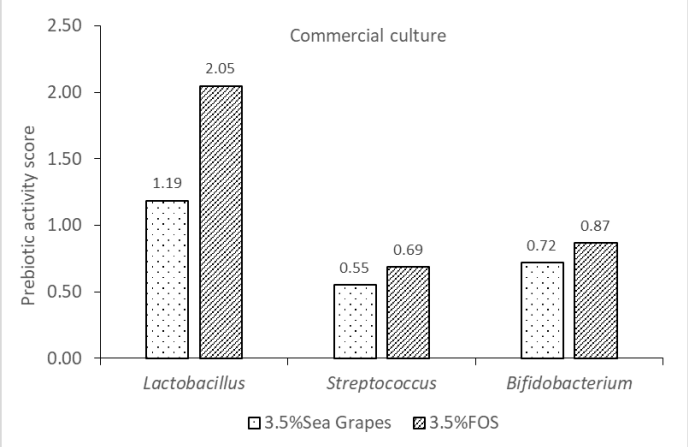

Figure 4. Prebiotic activity score of commercial cultures grown on basal medium supplemented with 3.5% (w/v) *C. lentillifera* (sea grapes) extracts and 3.5% (w/v) FOS.

4. Conclusion

The effectiveness of prebiotic substances depends on its ability to be selectively fermented by and to support the growth of specific targeted organisms (Huebner *et al*., 2007). But, this work was the preliminary study that shows the effect of crude macroalgae extracts on the growth of lactic acid bacteria. The results obtained here showed that crude saltwater macroalgae extracts could be considered to growth promotion for lactic acid bacteria. Therefore, the results of this work could support that the consumption of saltwater macroalgae could improve the health of consumers. However, additional research is required to establish the in vivo prebiotic capacity of saltwater macroalgae aiming its inclusion in functional food development.

Conflict of interest

The authors declare that they have no conflict of interest.

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