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Identification of The Potential of Degrading Carrageenan in Red Algae *Kappaphycus alvarezii* Symbiotic Bacteria

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Abstract— *Kappahycus alvarezii is a red alga contained large amount of bioactive material, such as carrageenan. Carrageenan is useful as a raw material for several industries and can be degraded by marine bacteria through breaking the linkages in polysaccharide carrageenan into oligosaccharide carrageenan. The aim of this study is identification of degrading carrageenan in K. alvarezii symbiotic bacteria. The results showed there was 14 isolate bacteria, and all of the isolates have clear zone on congo red staining activity. The isolate bacteria were 7 genera as K. alvarezii symbiotic bacteria, such as Labrenzia sp., Alteromonas sp., Vibrio sp., Celeribacter sp., Pseudoalteromonas sp., Phaeobacter sp. and Cobetia sp. Labrenzia sp., Alteromonas sp., Vibrio sp., Pseudoalteromonas sp., Phaeobacter sp. were recognized to have strong interactions with carrageenan in red algae, while the other Celeribacter sp. and Cobetia sp. have strong interactions with alginate in brown algae.*

Keywords— ***Kappahycus alvarezii, carrageenan, degrading bacteria.***

I. INTRODUCTION

Kappahycus alvarezii is red algae that generally has cylindric thallus, smooth surface, cartilaginous and consists of several types based on the color, such as green, yellowish green, gray, brown and red (Parenrengi *et al*., 2010). *K. alvarezii* was lived in tidal habitats, coral reef flats and attached to hard substrates (Erlania, 2013). *K. alvarezii* was contained large amount of carrageenan and used as stabilizer and gelling agent in processed meat, ice cream, chocolate, pudding, pet food, shampoo, toothpaste and cleaning products industrial (Hotchkiss *et al*., 2016; Barret, 2018).

In the ecosystem, bacteria were played an important role because of its ability to degrade organic matters to inorganic matters (Ginting *et al.*, 2019). The existence of symbiont bacteria was to protect their host and produce secondary metabolites (Funty, 2015). The use of secondary metabolites in algae, for example as bioactive materials (Nurhaedar, 2008). Carrageenan as a bioactive material in algae can be degraded by marine bacteria, especially gram-negative bacteria and produced enzymes to degrade carrageenans and it was useful for several industries (Chauhan & Saxena, 2016). Carrageenans degradation was a process to break the linkages in polysaccharide carrageenans to be oligosaccharide carrageenan with low molecular weight (Ghanbarzadeh *et al*., 2018). Several studies showed that *Pseudoalteromonas* sp. (Li *et al.*, 2013); *Tamlana* sp. was isolated from red algae *Hyalosiphonia caespitosa* (Sun *et al*., 2010) and *Cythopaga* sp. was isolated from red algae *Eucheuma gelutinue* (Mou *et al.*, 2004) have degrading carrageenan ability. Based on previous several studies, there is no *K. alvarezii* symbiotic bacteria in degrading carrageenan. Therefore, it is necessary to conduct research on the identification of degrading carrageenans in *K. alvarezii* symbiotic bacteria.

II. MATERIAL AND METHODS

Kapphycus alvarezii AND CARRAGEENAN USED IN THIS STUDY

Algae *K. alvarezii* were collected from USA Marine Biological Institute, Kochi University, Japan and floated in Uranouchi Bay, Tosa, Kochi Prefecture, Japan for one week to collect the bacteria. While several commercial carrageenan was used in this study, κ-carrageenan and λ-carrageenan purchased from Wako.

ISOLATION OF *K. alvarezii* SYMBIOTIC-BACTERIA

Arificial water agar medium was made with adding 0.5% carrageenan and incubated with red algae *K. alvarezii* for 3 days. After 3 days, the artificial seawater bacteria were dropped around 0.1 ml in marine broth agar. Moreover, bacteria were incubated at 25°C for 2 days to get the pure bacteria cultured.

DEGRADING CARRAGEENAN SCREENING BY CONGO RED STAINING

Bacteria in marine broth agar medium was inoculated to marine broth medium and incubated at 25°C for 2 days. Congo red agar medium was made from 4 gr gar medium, 25 ml 0.5% carrageenan and 1 ml congo red. Bacteria colonies from marine broth medium was dropped into congo red agar medium for 0.1 ml. The bacteria were incubated at 25°C for 2 days to identify the clear zone. Formed clear zone was an indicator of the carrageenan degrading activity existence. Bacteria with clear zone was inoculated and analyzed by 16S rDNA.

16S rDNA ANALYSIS OF *K. alvarezii* SYMBIOTIC-BACTERIA

A colony of 14 isolate bacteria were used as a template for PCR. Isolate bacteria were amplified by using universal primer pr0R2 (5'-AGAGTTTGATCMTGGCTCAG-3') dan 534R (5'-ATTACCGCGGCTGCTGG-3'). PCR products were applied to agarose gel electrophoresis and purified using Wizard® SV Gel and PCR Clean-Up System (Promega). The purified DNA were sequenced by ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems Japan) using BigDye® Terminator v3.1 and analyzed with BLAST on NCBI.

III. RESULT AND DISCUSSION

Bacteria isolation found 14 isolate bacteria with big size colonies. Congo red staining activity showed that there was clear zone in 14 isolate bacteria (Fig 1). Congo red was used for degrading carrageenans activity because congo red have strong interaction with polysaccharide which contained cellulose linked by β-1,4-glycosidic linkages (Teather & Wood, 1982). FAO (2003) was explained that red algae contain carrageenan and cellulose which insoluble in water and alkali. The clear zone was formed because of the reaction between *congo red* and β-1,4-glycosidic linkages in cellulose polymer (Missa *et al*., 2016).

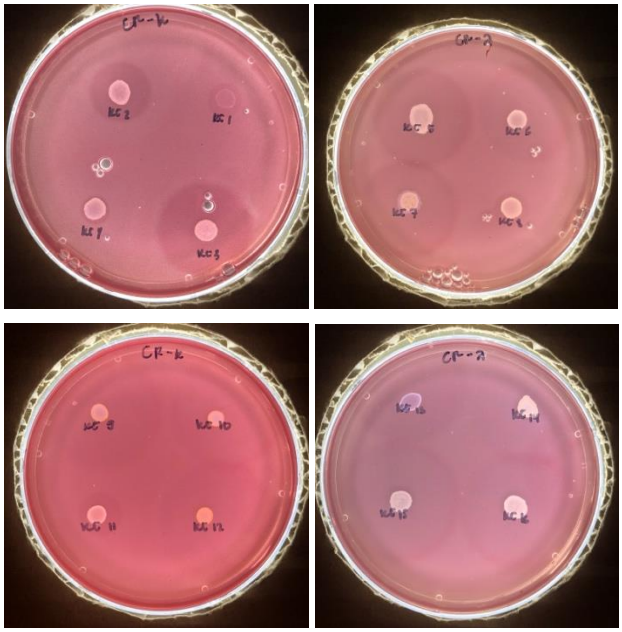

Fig 1: Congo red staining activity on 14 isolate bacteria

Electrophoresis showed that the measurement of bacteria DNA fragment was about 500 bp and it was compared to the marker 1 kbp DNA (Fig 2). The purification of DNA was measured by absorbance 260 nm and 280 nm. It showed that the absorbance of purified DNA was about 1.73-2.07 (Table 1). Thermo Fisher Scientific (2010) explained that the ratio of absorbance 260 nm and 280 nm was about 1.8 and it was "pure" for DNA purification.

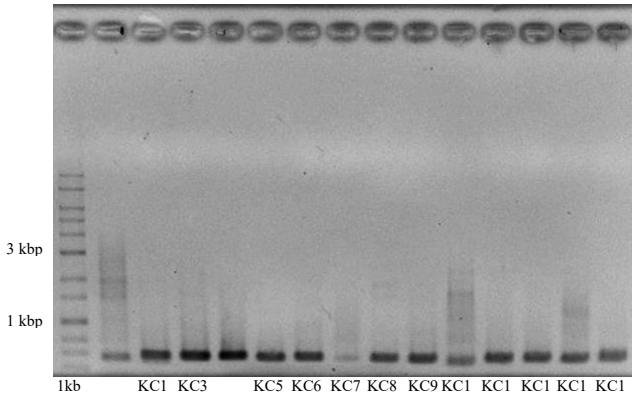

Fig 2. DNA fragment of K. alvarezii symbiotic bacteria

Table 1. Absorbance of K. alvarezii symbiotic bacteria DNA purification

Bacteria	$A_{260/280}$

Isolate 1	1.91
Isolate 2	1.95
Isolate 3	1.91
Isolate 4	2
Isolate 5	2.04
Isolate 6	1.9
Isolate 7	1.73
Isolate 8	1.93
Isolate 9	1.89
Isolate 10	1.76
Isolate 11	2.07
Isolate 12	1.97
Isolate 13	1.93
Isolate 14	1.82

16S rDNA showed that there were 7 genera of symbiotic bacteria in *K. alvarezii*, such as *Labrenzia* sp., *Alteromonas* sp., *Vibrio* sp., *Celeribacter* sp., *Pseudoalteromonas* sp., *Phaeobacter* sp. and *Cobetia* sp. (Table 2). Azizi *et al.*, (2018) found some bacteria was associated with 4 types of *K. alvarezii* and classified by 11 genera, such as *Alteromonas* sp., *Aestuariibacter* sp., *Idiomarina* sp., *Jejuia* sp., *Halomonas* sp., *Primoskyibacter* sp., *Pseudoalteromonas* sp., *Ruegeria* sp., *Terasakiella* sp., *Thalassospira* sp. and *Vibrio* sp. All of bacteria in this study was gram-negative bacteria and have carrageenan degrading ability by congo red staining activity. Chauhan & Saxena (2016) explained that carrageenase enzyme was only produced extracellularly by gram-negative bacteria.

Table 2. 16S rDNA of K. alvarezii symbiotic bacteria

***K. alvarezii* symbiotic bacteria**		***Identity***	**Reference**
Isolate 1	*Labrenzia* sp.	99.77%	*Labrenzia* sp. THAF35, Accession No. CP045380
Isolate 2	*Alteromonas* sp.	98.75%	*Alteromonas tagae*, Accession No. NR_043977
Isolate 3	*Alteromonas* sp.	99.58%	*Alteromonas tagae*, Accession No. NR_043977
Isolate 4	*Vibrio* sp.	99.60%	*Vibrio campbellii* MMRF1060, Accession No. MT307282
Isolate 5	*Alteromonas* sp.	100%	*Alteromonas macleodii* ROA033, Accession No. MT515801
Isolate 6	*Vibrio* sp.	99.80%	*Vibrio rotiferanus* AM7, Accession No. AP019798
Isolate 7	*Celeribacter* sp.	100%	*Celereibacter naphthalenivorans* EMB201, Accession No. NR_137260
Isolate 8	*Pseudoalteromonas* sp.	100%	*Pseudoalteromonas* sp. L10, Accession No. MN889153
Isolate 9	*Pseudoalteromonas* sp.	100%	*Pseudoalteromonas* sp. S4498, Accession No. MT514367
Isolate 10	*Phaeobacter* sp.	99.33%	Uncultured bacterium 5M23, Accession No. JF272132
Isolate 11	*Pseudoalteromonas* sp.	100%	*Pseudoalteromonas* sp. Md236, Accession No. AY461673
Isolate 12	*Vibrio* sp.	100%	*Vibrio campbellii* 1511126, Accession No. CP025953
Isolate 13	*Cobetia* sp.	100%	*Cobetia pacifica* GPM2, Accession No. CP047970
Isolate 14	*Alteromonas* sp.	100%	*Alteromonas macleodii* ROA033, Accession No. MT515801

The first *Labrenzia* sp. in red algae was *Labrenzia polysiphoniae* in red algae *Polysiphonia* sp. (Romanenko *et al*., 2019). *Alteromonas* sp. was found as *Kappahycus alvarezii* symbiotic bacteria and showed a pathogenetic. *Alteromonas* sp. was able to be pathogen agent that caused *ice-ice* symptoms (Syafitri *et al*., 2017). On the other hand,

Alteromonas sp. showed a potential to degrade some polysaccharide, such as alginate (Neumann *et al.*, 2015), ulvan (Koch *et al.*, 2019); agar (Wang *et al*., 2005); ι-karagenan (Barbeyron *et al.*, 2019) and κ-karagenan (Barbeyron *et al*., 1994). Araki *et al*., (1999) and Zhu & Ning (2016) found high activity of κ-carrageenase enzyme through *Vibrio* sp. purification. Moreover, *Pseudoalteromonas* sp. had an ability to utilize κ-carrageenan and ι-carrageenan for their energy source (Hettle *et al*., 2019). *Pseudoalteromonas* sp. was also degraded κ-carrageenan (Liu *et al*., 2011) and λ-carrageenan (Guibet *et al*., 2007). Furthermore, *Phaeobacter inhibens* was found in red algae *Tichocarpus crinitus* to degrade the carrageenan (Kalitnik *et al*., 2017). Based on the several studies, *Labrenzia* sp., *Alteromonas* sp., *Vibrio* sp., *Pseudoalteromonas* sp. and *Phaeobacter* sp. were recognized to have strong interactions with red algae through the utilization of red algae carrageenan. Whereas *Celeribacter* sp. and *Cobetia* sp. was found in brown algae through the utilization of brown algae alginate. Ihua *et al*. (2020) showed that *Celeribacter* sp. was found on brown algae thallus *Laminaria digitata* and Yagi *et al*. (2016) explained that *Cobetia* sp. was isolated from brown algae *Padina arborescens* with alginate degrading enzyme.

IV. CONCLUSION

In this study, we found 7 genera of *Kappahycus alvarezii* symbiotic bacteria, such as *Labrenzia* sp., *Alteromonas* sp., *Vibrio* sp., *Celeribacter* sp., *Pseudoalteromonas* sp., *Phaeobacter* sp. and *Cobetia* sp. All of the bacteria showed an activity on congo red staining based on the formed clear zone. The clear zone was indicated the carrageenan degrading activity.

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