ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF MARINE BACTERIA CONSORTIUM FOR CARRAGEENAN BIOMASS DEGRADATION

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ABSTRACT-For isolating carrageenan degrading bacteria, carrageenophytes, sediments and seawater collected from various localities along the coasts of Tamil Nadu were grown in mineral medium with carrageenan as sole carbon source. Bacterial isolates with carrageenolytic property were identified on the basis of their ability to form soft pits on the surface with clear haloes around its colonies, gradually turning the solid medium to liquid. Out of 38 isolates exhibiting carrageenolytic activity 3 each from marine sediments and decomposed algal samples and 13 from fresh seaweeds with strong carrageenolytic lesions were identified. Bacteria consortium *Brevibacterium iodinum*ACa01 **KJ754134** and *Pseudomonas aeruginosa*ACa02 **KJ754135** found effective for degrading carrageenan polymer as well as carrageenan biomass (*Hypnea musciformis*). Saccharification was more effective on spent biomass than on fresh biomass of *Hypnea musciformis* by mild acid pre-treatment followed by bacterial consortium than those biomasses directly subjected to bacterial consortium. In this study, *Brevibacterium iodinum*ACa01 **KJ754134** and *Pseudomonas aeruginosa*ACa02 **KJ754135** as consortium capable of degrading carrageenan rich seaweed biomass reported for the first time.

Keywords: **Carrageenolytic; Saccharification; bacterial consortium; *Brucella iodinum*; *Pseudomonas aeruginosa***

Introduction

Carrageenan is a common name of polysaccharide found in certain species of red seaweeds (Rhodophyceae) belonging to the family *Solieriaceae, Rhabdoniaceae, Hypneaceae, Phyllophoraceae, Gigartinaceae, Furcellariaceae* and *Rhodophyllidaceae* (Schneider and Wynne 2007). They are water soluble polymers with high molecular weight (Necas and Bartosikova 2013) and were recorded up to 75 % of the dry weight in some species of *Kappaphycus* (*K. striatum*-75.6% and K. *alvarezii*- 71.0%) containing 15% to 40 % of ester-sulphate (Jamal and Ikasari 2014). Since this sulphated polymer exhibit unique rheological (Beltagy 2012) and physiochemical properties, carrageenan has been used in various industries as texturing and gelling agents (Necas and Bartosikova 2013), pelletizing agents and emancipating modernizers of medications in pharmaceutical industries (Ma et al. 2013). This carrageenan structurally categorized into various types based on the number and position of sulphate group(s) and polymer assembly as alternating 3-linked α-D-galactopyranose and 4-linked β-D galactopyranose (Ma et al. 2013). At least 15 different types of carrageenan are known (Lahaye 2001; Cunha and Grenha 2016) however, the most industrially relevant carrageenans are kappa (κ), iota (ι) and lambda (λ) forms (Cardoso 2014).

The primary differences which influence the properties of κ, ι and λ carrageenan are the number and position of ester sulphate groups as well as the content of 3,6-anhydrogalactose (3,6-AG) (Ziayoddin et al. 2010). Higher levels of ester sulphate reduce the solubilizing temperature and gel strength; Kappa type carrageenan has an ester sulphate content of about 25 to 30% and a 3,6-AG content of about 28 to 35%. Iota type carrageenan has an ester sulphate content of about 28 to 30% and a 3,6-AG content of about 25 to 30%. Lambda type carrageenan has an ester sulphate content of about 32 to 39% without 3, 6-AG (Stanley 1987; Knudsen et al. 2015). The α (1, 4) linked galactose units in carrageenan are in the D configuration while in agars they are in L configurations (Rees 1969). Carrageenan composition mainly depends upon the source of seaweeds, life stage and the method of extraction which suggest the physico-chemical properties of a variety of food industry appliances (Knudsen et al. 2015; Craigie 1990; Usov 1998).

As early as 1943, Mori extracted carrageenase from marine mollusk (Zhang and Kim 2010) for carrageenan degradation. Recently attention on degraded carrageenans gaining interest because of this sulfated oligosaccharides have diverse biological and physiological activities which include anticoagulation, anti-inflammation, anti-thrombosis, antitumor, viral inactivation etc., Biological activities of these degraded carrageenan lie on structural parameters like molecular mass, degree of sulfate esterification, linking position of sulphate groups etc., (Ziayoddin et al. 2010). Recently carrageenan seaweeds considered as potential feet stock of marine biomass for energy production (Jiang et al. 2016). Therefore the carrageenan polymers as well as its biomass are the potential source for various applications upon degradation. But the complexities in the structure of carrageenan pose challenge to degrade because of structure specificity and linkages that require enzymes with broad spectrum of activation and/or specific methods for degradation (Lang et al. 2014). As marine source, carrageenan degradation by bacteria of marine origin considered advantageous than eukaryotic marine fungi which would exhaust the degraded products (Faturrahman et al. 2011; Chauhan and Saxena 2016). Marine bacteria such as *Alteromonas carrageenovora*, *Cytophaga drobachiensis*, *Vibro* sp.,

Zobellia galactanovorans and *Pseudomonas elongata* capable of degrading carrageenan polymer are reported (Vijayaraghavan et al. 2012). But bacteria capable of degrading carrageenan polymers in biomass are advantageous for carrageenan based biomass saccarification for energy production because compounds and pigments in the biomass interferes the degradation efficiency of those bacteria identified as suitable for degrading carrageenan polymer (Sarwar et al. 1985; Williams et al. 2013). In this study, marine bacterial consortium capable of degrading carrageenan polymer as well as biomass characterized using 16S rRNA gene sequencing is presented.

Materials and Methods

Sample collection

For isolating carrageenan degradation bacteria from the carrageenophytes, healthy thalli collected using sterile forceps were immediately transferred to aseptic vials containing sterilized seawater and transported to the laboratory. The respective algae in decaying condition, sediments and seawater were also sampled at the same site (Table 1). Samplings were done at four seasons (pre-monsoon July 28, monsoon Nov 15, post-monsoon Mar 8 and summer May 5) along the coast of Pamban, Gulf of Mannar (Palk Bay, India) in the year 2014. Spent samples at the disposal site of carrageenan industries (Madurai, Tamil Nadu) were collected aseptically and brought to the laboratory and sundried stored in the desiccators until further study.

Isolation of carrageenan degrading bacteria (Youssef et al. 2012)

Sample of fresh and decaying seaweed thallii weighing 1.0 g were homogenized under aseptic conditions using 10 ml of sterile distilled water. Sediment weighing 1.0 g was extracted with sterile sea water using orbital shaker for 30 minutes and the volume was made up to 10 ml. From each sample, 1 ml was transferred aseptically to 250 ml conical flasks containing 100 ml of mineral medium (g l^{-1}, $(NH_4)_2SO_4$, 5; NH_4Cl, 0.7; NaCl, 20; $CaCl_2 \cdot 2H_2O$, 0.2; K_2HPO_4, 3; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.3; $FeSO_4 \cdot 7H_2O$, traces; agar-agar, 15. pH 7.5) containing 0.1 % carrageenan as the sole carbon source and incubated for 3 days at 37ºC at 180 rpm. After incubation, serial dilutions of 10^{-5} to 10^{-9} were prepared. From each dilution, 100 µl was spread on Petri plates containing ca. 20 ml of mineral medium with 1.5 % carrageenan. The plates were incubated at 30ºC up to 7 days and results were recorded. Isolates showing carrageenolytic activity activity by developing lesion were picked and subsequently re-streaked for getting pure culture. Based on the morphology, Gram's staining and biochemical characteristics carried out as described in the Bergey's manual (Ziayoddin et al. 2010; Garrity et al. 2005; Khambhaty et al. 2007), 19 isolates identified as carrageenan degrading were designated with unique codes and stored in glycerol suspension (glycerol/bacterial broth of 1:1 v/v) in Eppendorf tubes at -80ºC for further study.

Screening the potential isolates

To identify the potential isolates among the 19 carrageenan degrading bacteria, exponential culture broth of 100 µl of each isolate was inoculated into 100 ml of mineral medium containing 0.1 % carrageenan at 37ºC under 180 rpm for 5 days. For carrageenan degradation, Bacterial growth as OD at 660 nm as well as total sugar (Dubois et al. 1956), reducing sugar (Miller 1959) and protein (Lowry et al. 1951) levels in the medium after inoculation were recorded everyday using culture supernatant obtained by centrifugation at 7000 rpm for 15 minutes at 4ºC.

Bacterial consortia for carrageenan degradation

The bacterial isolates (ACa 01, ACa 02, ACa 04 and ACa 07) found as potential were combined as mentioned in the Fig. 3. The exponential broth of 100 µl containing equal volume of each bacterial broth in the consortium was inoculated into 100 ml of mineral medium containing 0.1 % carrageenan incubated at 37ºC in 180 rpm. The carrageenan degradation was assessed as described in the section 2.3.

Carageenase activity in bacteria consortium (Vijayaraghavan et al. 2012)

The exponential 100 µl culture of ACa 01, ACa 02 and ACa 01 + ACa 02 was inoculated separately into 100 ml of mineral medium containing 0.1 % carrageenan and incubated at 37ºC in 180 rpm. For carrageenase assay, 1.0 ml of each culture supernatant (centrifugation at 7000 rpm for 15 minutes at 4ºC) as crude carrageenase was incubated with 2.0 ml substrate (0.25 % carrageenan in 20 mM Tris-HCl buffer, pH 7.5) at 30 ± 2°C for 2 h. The reaction was stopped by heating in a boiling water bath for 15 min (Khambhaty et al. 2007; Thompson et al. 1994). Boiled enzyme was used as blank. The reducing sugar generated was determined by the Somogyi-Nelson method (Nelson 1944) using D-galactose standard.

Saccharification of carrageenan biomass by bacterial consortia ACa 01+ACa 02

The red seaweed, *Hypnea musciformis* weighing 5.0 Kg collected along the coast of Thondi (Balk Pay, India) was washed thoroughly in tap water and shade dried for 5 days. Shade dried specimen was chopped and pulverized into fine powder of 70 mm mesh size and the powdered sample was treated as fresh biomass. A portion of fresh biomass powder of 500 g was subjected to carrageenan extraction (Craigie and Leigh 1978) and the residue was treated as spent biomass. Total sugar (Dubois 1956) and reducing sugar (Miller 1959) of fresh and spent biomass were recorded.

For saccharification, 100 µl exponential culture of equal volume of ACa 01 and ACa 02 was inoculated into 250 ml conical flask containing 100 ml mineral medium with 0.1 % seaweed biomass (fresh and spent separately) pre-treated with and without 1 % H_2SO_4 for 30 minutes at 121ºC and incubated at 37ºC in 180 rpm. Carrageenan biomass degradation as saccharification by reducing sugar yield (Miller 1959) in the medium was assessed through the total sugar and reducing sugar level everyday for 4 days in the culture supernatant (centrifugation at 7000 rpm for 15 min at 4ºC). Experiments were carried out in triplicates and mean values are expressed.

Characterization of bacterial consortium

Morphological and biochemical characteristics of bacteria consortium (ACa 01 and ACa 02) were carried out based on the procedure of Bergey's manual (Ziayoddin et al. 2010; Garrity et al. 2005; Khambhaty et al. 2007). The molecular characterization by 16S rRNA gene sequencing through PCR amplification was carried out (Nithya and Pandian 2010; Amutha and Kokila 2012; Lokhande and Pethe 2016) and the nucleotide sequences were deposited in GenBank.

Results and discussion

Degraded products of carrageenan have potential values in food, medicine and fuel productions. Oligosaccharides released by acidic or enzymatic hydrolysis of carrageenan polysaccharide have been recognized as potential bioactive compounds in medicine and prebiotics in food. Carrageenan polymer could be degraded into monomers which would be used as a source for producing biogas or bioethanol through fermentations (Jiang et al. 2016). Various methods like as mild acid treatment, temperature treatment and microbial inoculation (bacteria and fungi) are employed for carrageenan degradation (Vera et al. 1998). The method chosen for hydrolysis not only depends on the broken products aimed but also depends on the nature of substrate (carrageenan polymer or biomass) subjected for degradation. In this study, a method is developed for degrading the carrageenan polymer as well as biomass through mild acid treatment followed by marine bacterial consortium isolated.

Isolation of carrageenan degrading bacteria

Samples collected from the various substrata along the various coasts were grown in mineral medium with carrageenan as sole carbon source for 7 days (Table 1). Those bacterial isolates utilizing carrageenan as its carbon source produced lesions on the medium and were picked up and sub-cultured in regular interval of 7 days until confirmed as single isolate based on the morphology and Gram's staining as described in the Bergy's Mannual (Ziayoddin et al. 2010; Garrity et al. 2005; Khambhaty et al. 2007). The diameter of the single, isolated lesion formed on the medium due to carrageenan degradation (observed as wells) was measured in mm diameter (Table 1). The carrageenolytic wells were appeared as lytic diluted lesion on the mineral medium (Fig. 1). In the screening, 38 bacterial isolates showing carrageenan degradation was recorded (Table 1). Previously, several bacteria capable of degrading carrageenan (Chauhan, and Saxena 2016) were isolated from various substrata like marine sediments (Vashist et al. 2013; Hu et al. 2009), seawater (Ma et al. 2007), seaweeds (Vera 1998; Tayco et al. 2013) and non-marine environments such as river (Agbo and Moss 1979), soil (Suzuki et al. 2003) and plant root (Hosoda et al. 2003). In the present study, bacteria showing carrageenan degradation were isolated from various marine substrata among the 38 isolates exhibit carrageenolytic lesion observed on the plates, 19 chosen as potential. In the 19 isolates, 3 are (ACa 02, 18, 19) from the marine sediment of Pudumadam coast, 3 (ACa 14, 15, 16) are from mixed samples of *Hypnea musciformis* and *Hypnea valentiae* of Thiruchendur coast and the remaining 13 are from other carrageenophyte samples (Table 1). As reported in the earlier studies (Chauhan and Saxena 2016; Vijayaraghavan et al. 2012; Khambhaty et al. 2007; Sarwar et al. 1983a; Potin et al. 1991; Mou et al. 2003; Araki et al. 1999; Ostgaard et al. 1993; Michel et al.2000; Michel et al. 2001; Shangyong 2013; Yao et al. 2013; Ziayoddin et al. 2014; Li et al. 2015; Zhu and Ning 2016) bacteria isolates showing good carrageenan degradation were isolated from marine sediments and seaweeds in this study for carrageenan degradation.

Screening the potential isolates

The results present in the Fig. 2 are the carrageenan degradation potential of 19 bacterial isolates evaluated on the basis of growth and level of total sugar, reducing sugar and protein in the mineral medium which contained carrageenan as sole carbon source. Although all the isolates reached the stationary phase of growth on 4th day after inoculation, isolates ACa 07 and ACa14 showed maximum growth. Bacterial growth result a decrease in the total sugar in the medium that cause phycocolloid degradation which showed increase in the level of reducing sugar and proteins in the medium (Chiura and Tsukamoto 2000). Extracellular enzymes including those responsible for carrageenolysis estimated in the total protein are synthesized and released in the medium by bacteria for degrading carrageenan polymer while utilizing for carbon source during growth (Schroeder et al. 2003; Shin et al. 2010). In the medium, decrease in total sugar and increase in reducing sugar were at high in the medium grown by isolates ACa01, 02, 03 and 07 whereas total protein was high as increasing order by isolates ACa02, ACa13, ACa11 and ACa10 on 4th day after inoculation. This observation indicates as those isolates (ACa10, ACa11 and ACa13) synthesis high protein and less reducing sugar inadequately synthesizing enzyme protein of carrageenolysis whereas the isolate ACa02 recorded high protein and reducing sugar support degradation. High reducing sugar recorded in the medium as result of growing isolates such as ACa 01, ACa03 and ACa07 would also favored the synthesis of carrageenolytic protein as like ACa02 than other isolates and these are selected as the potential for carrageenan degradation.

Bacterial consortium for carrageenan degradation

There are various types of carrageenan (kappa κ, iota ι and lambda λ) and each one is a complex polymer with different linkages which require complex enzymes for degradation or one or more bacteria or a bacterium capable of producing complex enzymes for effective or complete degradation (Vera et al. 1998). Studies reported that the strains of *Alteromonas* which readily released carrageenase into the medium yield monosaccharides as major end-products. The combined action of three strains of *Cytophaga* sp. is responsible for the degradation of polysaccharide of red alga *Rhodella reticulate* (Panova et al. 2002). In the present study, potential bacterial isolates ACa01, ACa02, ACa03 and ACa07 were combined for carrageenan polymer degradation (Fig.3) and results show more growth and carrageenan degradation when bacteria inoculated in-combination. Exponential growth phase of bacteria in-combinations was reached on 2nd day itself (Fig.3) compared to the individual bacteria reached on 4th day (Fig.2). The total sugar level was declined rapidly from 1st day and exhausted on 4th day indicate speedy carrageenan degradation by bacteria in-combinations and this reflect in the maximum increase in the reducing sugar level in the medium. Among the bacteria combinations, maximum reducing sugar level was recorded on 4th day after inoculation of ACa01 + ACa02 (Fig.3). The total protein which also includes carrageenolytic enzymes recorded maximum on 3rd day in-combinations of ACa01+07 and on 4th day of all other bacterial combinations. Thus high level of total protein support the carrageenan hydrolysis reflect more reducing sugar in the medium was grown by bacterial combination ACa01 + ACa02 on 4th day but recorded lower amount of total protein over ACa02 + ACa07 combination. This observation shows that maximum total extracellular protein in the medium by bacterial combination of ACa02 + ACa07 would less influence on carrageenolysis on the other hand which indicated by decreased reducing sugar level in the medium by bacterial combination of ACa01 + ACa02 support the synthesis of carrageenolytic protein for degradation. Thus consortium of ACa01 + ACa02 induces the synthesis of carrageenolytic protein

underlay quorum sensing phenomenon (Sun et al. 2012). The combined growth of ACa01 + ACa02 at different pH and temperatures show optimum carrageenase enzyme activity at pH 7.5 in 37ºC (Fig.4).

Saccharification of seaweed biomass by bacteria consortium

Thermal and acids (HCl/H_2SO_4) are commonly employed for treating the various types of seaweed biomass for hydrolysis (Jiang et al. 2016). There are commercial enzymes obtained from microbial source used for hydrolysing of seaweeds biomass (Yazdani et al. 2014). In some studies, bacteria are used for saccharification of carrageenan polymers as well as biomass to release monosugars which subjected to fermentation (Yanagiawa et al. 2013). The saccharification of seaweed biomass generally contingent upon the temperature applied, acid concentration, properties of enzymes, microbes etc., (Tian et al. 2014). Thus choice of identification and optimization of suitable saccharification methods are vital for maximum reducing sugar production (Jiang et al. 2016; Pasanda et al. 2016). The method suitable for saccharifying the carrageenan polymer may not be efficient for carrageenan biomass because biomass constitutes non-polysaccharide compounds which affect the saccharification process. In this study, the bacterial consortium identified for degrading the carrageenan polymer was employed for carrageenan biomass hydrolysis. In this study total sugar and reducing sugar estimated from the various samples of carrageenophyte *Hypnea musciformis* after treating with different sacharification methods are presented in the Table 2. The saccharified carrageenan spent samples were recorded less amount of total sugar compared to fresh biomass. The fresh and spent biomasses were saccharified by two methods after hot stream digestion at 121ºC for 30 minutes. In one method, biomass was subjected to bacterial consortium and the second method by mild acid (1 % H_2SO_4) treatment followed bacterial consortium. Insignificant difference in the level of the total sugar and reducing sugar content was recorded between the samples of acid pretreated and un-treated one among the same type of biomass. Whereas inoculation of bacterial consortium significantly improved the reducing sugar yield in both type of biomass. This support that bacterial consortium identified in this study can be used not only for carrageenan polymer hydrolysis but also for biomass. High conversion of reducing sugar recorded in the spent samples when subjected to mild acid treatment followed by bacterial consortium.

Characterization of ACa01 + ACa02 through 16S rRNA gene sequencing

The morphological and biochemical characteristics were the bases for the identification of bacteria. Both the isolates were Gram-negative rod, non-motile and positive for oxidase, urease, Voges-\Proskauer, nitrate reduction and citrate utilization tests and negative for catalase and indole production tests. They grew from 30 to 40ºC.

The 16S rRNA sequence analyses results showed that the isolate ACa01 had 99% sequence similarity to *Brevibacterium iodinum* and deposited in NCBI KJ754134 (Fig.5) and isolate ACa02 show 100% similar to *Pseudomonas aeruginosa* deposited in NCBI KJ754135 (Fig.6).

Conclusion

It is concluded that among the bacteria capable of degrading carrageenan isolated from the carrageenan yielding red seaweeds, seawater and marine sediments, bacteria consortium *Brevibacterium iodinum*ACa01 **KJ754134** and *Pseudomonas aeruginosa*ACa02 **KJ754135** found effective for degrading carrageenan polymer as well as biomass (*Hypnea musciformis*). Saccharification was more effective on spent biomass than on fresh biomass of *Hypnea musciformis* by mild acid pre-treatment followed by bacterial consortium than those biomasses directly subjected to bacterial consortium. In this study, *Brevibacterium iodinum*ACa01 (**KJ754134)** and *Pseudomonas aeruginosa*ACa02 (**KJ754135**) consortium capable of degrading carrageenan rich seaweed biomass was reported for the first time.

Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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