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Alteromonas macleodii KS62 (MTCC 12606): A Novel κ-Carrageenase Producing Microorganism

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Abstract: A κ-carrageenan degrading bacterium was isolated from decaying red seaweeds. Based on biochemical characterization and 16S rRNA sequence the isolate was identified as *Alteromonas macleodii* KS62. To the best of our knowledge, this is the first study reporting κ-carrageenan degrading bacterium of species *Alteromonas macleodii*. κ-carrageenase enzyme production of 1.6U/ml (12.5U/mg protein) was obtained when *Alteromonas macleodii* KS62 was grown in a medium containing κ-carrageenan as carbon source. The κ-carrageenase production further increased to 2.9U/ml (14.1U/mg protein) when *Alteromonas macleodii* KS62 was grown in a medium supplemented with *Kappaphycus* red seaweed powder. This the first report on κ-carrageenase production using red seaweed biomass as substrate. Efficient κ-carrageenase production establishes the potential of red seaweed biomass to serve as a low cost substrate for economic production of κ-carrageenase enzymes. Enzymatic hydrolysis of κ-carrageenan was studied. κ-carrageenan tetrasaccharide and hexasaccharide were found to be the major end products of enzymatic hydrolysis.

Keywords: *Alteromonas macleodii*, K-carrageenan, K-carrageenase, Enzyme, Red Seaweeds, *Kappaphycus*

1. Introduction

Carrageenans are cell wall polysaccharides of certain red seaweeds. The cell wall makes up to 65% of dry weight of red seaweed biomass and thus, carrageenans are a major constituent of red seaweeds. They are made of D-galactose and 3,6-anhydro-D-galactose units joined by alternating α-1,3 and β-1,4 bonds. Carrageenans are highly sulphated and are classified based on the positions and number of sulphate groups per disaccharide unit. κ-carrageenan contains one sulphate group per disaccharide unit. It is made of repeating units of 3,6-anhydro-D-galactose and D-galactose-4-sulphate. κ-carrageenases are microbial enzymes which degrade κ-carrageenan. These enzymes are useful to obtain red algal protoplasts for extracting valuable biomolecules like fatty acids, vitamins, carotenoids, proteins [1]. These enzymes can also be used to convert the abundant and high carbohydrate content biomass of red seaweeds to sugars which can be then fermented to obtain bioethanol, an emerging biofuel. They can serve as tools for structural analysis of cell walls of red seaweeds. They are useful to obtain low molecular weight carrageenan oligosaccharides which have anti-viral [2], anti-tumor [3] and immune-regulatory [3, 4] activities.

Only a few κ-carrageenan degrading microorganisms are known. Ability to degrade κ-carrageenan was for the first time reported in *Pseudomonas carrageenovora* (now reclassified as *Pseudoalteromonas carrageenovora*) [5]. Since then, κ-carrageenan degrading ability has been reported in the following genera: *Cytophaga* [6], *Pseudomonas* [7], *Tamlana* [8], *Pseudoalteromonas* [9, 10], *Zobellia* [11], *Cellulosimicrobium* [12], *Cellulophaga* [13], *Pedobacter* [14]. To the best of our knowledge, there are no reports on κ-carrageenases from *Alteromonas macleodii*. We, therefore, report for the first time κ-carrageenan degrading activity by newly isolated *Alteromonas macleodii* strain KS62.

The objective of present study was also to utilize red seaweed biomass as substrate for κ-carrageenase production. As red seaweeds are abundant and cheaply available their utilization can bring down the cost of commercial production

of κ-carrageenase. Enzymatic hydrolysis of κ-carrageenan was also studied.

2. Materials and Methods

2.1. Isolation and Characterization of κ-Carrageenan Degrading Microorganism

Decaying red seaweeds were collected from Kovalam beach near Chennai, India. For isolation, the decaying red seaweeds were enriched for five days in isolation/enrichment medium containing κ-carrageenan as the sole source of carbon (in g/l): κ-carrageenan 1, $NaNO_3$ 2, NaCl 20, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5, $Fe_3(PO_4)_2$ 0.01, $CaCl_2$ 0.1. Single isolated colonies were obtained by repeated sub-streaking on plates containing the above medium supplemented with 1% κ-carrageenan. Here, κ-carrageenan served as solidifying agent as well as the sole carbon source. Some colonies showed visible pits around them on κ-carrageenan plates. These colonies were screened for extracellular κ-carrageenase activity by incubating 50µl of culture supernatant in wells made in the centre of petri plates containing 1% κ-carrageenan gel. The plates were incubated at 30°C for 24 hours and then stained with Gram's iodine for 15 minutes to look for degradation zones. The isolate KS62 showed the best activity. Thus, isolate KS62 was selected for further studies.

Biochemical characterization of isolate KS62 was carried out by methods described elsewhere [15]. The biochemical tests performed included indole production, methyl red-Voges Praskeur (MR-VP), citrate utilization and, production of the enzymes catalase, oxidase, urease and arginine dihydrolase. Gram's staining was performed for morphological characterization of the isolate. 16S rRNA sequencing was carried out for phylogenetic analysis.

2.2. κ-Carrageenase Production by the Isolate When Grown on κ-Carrageenan

Isolate KS62 was grown in medium containing (in g/l): κ-carrageenan 1, yeast extract 0.6, NaCl 8.33, K_2HPO_4 1.6, $MgSO_4 \cdot 7H_2O$ 0.5, $Fe_3(PO_4)_2$ 0.01, $CaCl_2$ 0.47. 3% of culture grown in initial isolation/enrichment medium (composition given in previous section) for 24 hours was added as inoculum. Submerged fermentation was carried out for 18 hours at 30°C, 150 rpm. At the end of 18 hours, the culture was harvested and extracellular κ-carrageenase production was determined.

2.3. κ-Carrageenase Production by the Isolate When Grown on Red Seaweeds

Kappaphycus red seaweed was obtained from seaweed farmers in Tuticorin, Tamil Nadu, India. It was washed thoroughly with tap water followed by distilled water and dried in a hot air oven at 60°C overnight. The dried seaweed was then ground to obtain a coarse powder. Different concentration (0.5, 1, 2, 5, 7.5, 10 and 15 g/l) of this *Kappaphycus* seaweed powder was added to inorganic salts mixture containing (in g/l): NaCl 8.33, K_2HPO_4 1.6, $MgSO_4 \cdot 7H_2O$ 0.5, $Fe_3(PO_4)_2$ 0.01, $CaCl_2$ 0.47. Red seaweed powder served as carbon and nitrogen source. The medium was autoclaved and inoculated with 3% of 24-hour culture grown in initial isolation/enrichment medium. Submerged fermentation was carried out for 18 hours at 30°C, 150rpm. At the end of 18 hours, the culture was harvested and extracellular κ-carrageenase production was determined.

2.4. Enzymatic Hydrolysis of κ-Carrageenan

5ml of crude enzyme, produced in the κ-carrageenan medium, was incubated with 15ml of 0.75% κ-carrageenan in 25mM Tris-HCl (pH 8.0) buffer at 35°C. 2ml of samples were removed at different time points and enzyme reaction was stopped by boiling for 15 min. The amount of reducing sugars released were determined by 3,5-dinitrosalicylic acid (DNS) reagent [16].

To determine the molecular mass distribution of the hydrolysis products, the 12 hours hydrolysate was analysed in negative ion mode by electrospray ionisation mass spectrometry (maXis impact Bruker Daltonics ESI-Q TOF-MS).

2.5. κ-Carrageenase Enzyme Assay

At the end of 18 hours of growth, cells were removed by centrifuging at 8000rpm for 10min at 4°C. The cell-free culture supernatant obtained was taken as crude enzyme extract. Enzyme was incubated with 0.75% κ-carrageenan in 25mM Tris-HCl buffer (pH 8.0) at 35°C for 30 minutes. The reaction was stopped by boiling the assay mixture for 15 minutes. An enzyme blank assay mixture containing enzyme denatured by pre-boiling for 15 minutes was included as control. The reducing sugars formed were measured by 3,5-dinitrosalicylic acid (DNS) reagent [16], using D-galactose as standard. One unit of κ-carrageenase activity was defined as the amount of enzyme required to release one µmole of D-galactose equivalents per minute under the above mentioned conditions. Protein concentration of crude enzyme extract was measured by Folin-Lowry assay [17], bovine serum albumin (BSA) served as standard.

3. Results and Discussion

3.1. Isolation and Characterization of Isolate KS62

67 single colonies were obtained by streaking on κ-carrageenan plates. 8 of these colonies showed visible pits around them, indicating κ-carrageenan degradation. These colonies were screened for extracellular κ-carrageenase production by the semi-quantitative plate assay described in Materials and Methods section. Isolate KS62 exhibited best κ-carrageenase activity. The results of biochemical characterization of the isolate can be found in Table 1. Isolate KS62 formed white round colonies with smooth margins. Cells were observed as Gram negative rods under microscope.

Table 1. *Biochemical characterization of isolate KS62.*

Sr. no.	Biochemical Test	Result
1	Catalase	Positive
2	Oxidase	Positive
3	Indole Production	Negative
4	MR	Negative
5	VP	Negative
6	Citrate Utilization	Negative
7	Nitrate Reduction	Positive
8	Urease	Positive
9	Arginine dihydrolase	Negative

3.2. Phylogenetic Analysis and Identification of Isolate KS62

The 16S rRNA sequence of isolate KS62 was found to have up to 99.93% homology with *Alteromonas macleodii*. Thus, isolate KS62 was identified as belonging to species *Alteromonas macleodii*. *Alteromonas macleodii* KS62 has been deposited at Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India; with accession number MTCC 12606. The 16SrRNA sequence of *Alteromonas macleodii* KS62 has been submitted to GenBank and has been assigned accession number KY608081. To the best of our knowledge, this is the first report on κ-carrageenan degrading bacterium from species *Alteromonas macleodii*.

3.3. κ-Carrageenase Production on κ-Carrageenan and Red Seaweeds

Alteromonas macleodii KS62 exhibited κ-carrageenase production of 1.6U/ml (12.5U/mg protein) when grown in medium containing κ-carrageenan. Next, κ-carrageenase was produced using red seaweed biomass as substrate.

India, with its long coastline, can produce a million tonnes of seaweeds annually and the production can be year round. Unprocessed seaweeds, thus, represent a cheap and easily available source of biomass. Red seaweeds are rich in carbohydrates, mainly glucans and galactans [18, 19]. *Kappaphycus* seaweeds accumulate large quantities of κ-carrageenan as their cell wall component. These seaweeds, rich in κ-carrageenan, can therefore serve as an excellent substrate for production of κ-carrageenase enzyme. In present study, for the first time, *Kappaphycus* red seaweed was used as substrate for κ-carrageenase production. The media contained a mixture of inorganic salts; dried *Kappaphycus* seaweed powder was added to serve as carbon and nitrogen source. Figure 1 shows the effect of *Kappaphycus* seaweed powder concentration on κ-carrageenase production. Maximum κ-carrageenase production of 2.9U/ml (14.1U/mg protein) was obtained when 5g/l of *Kappaphycus* seaweed powder was added to the medium. The increased production in the seaweed supplemented medium can be attributed to the fact that this submerged fermentation resembles the natural habitat of the isolate, isolated from decaying red seaweeds in the first place.

Previous studies have reported κ-carrageenase production comparable to that obtained in present study. Extracellular κ-carrageenase production of 1.54U/ml was reported when free cells of *Cellulosimicrobium cellulans* were cultured in an optimized medium, the production increased to 2.22U/ml when immobilized cells were used [12]. A *Cellulophaga lytica* strain isolated from sediments of a carrageenan production base in China has been reported to secrete 1.648U/ml of κ-carrageenase in culture supernatant [13]. Similarly, isolated *Pedobacter hainanensis* strain released 1.84U/ml of κ-carrageenase in extracellular medium [14].

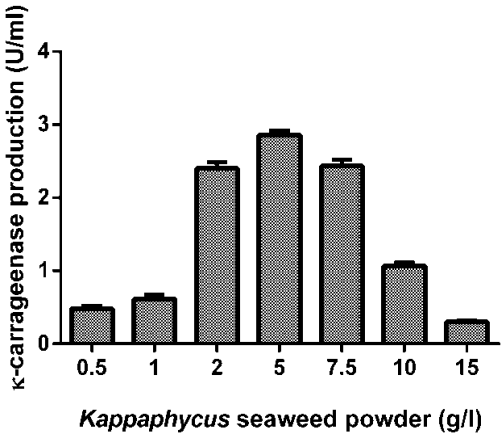

Figure 1. *Effect of Kappaphycus seaweed powder concentration on κ-carrageenase production.*

3.4. Enzymatic Hydrolysis of κ-Carrageenan

Time course of enzymatic hydrolysis of κ-carrageenan was studied by incubating crude enzyme with the substrate for different incubation times. As can be seen in Figure 2, the reducing sugars increased till 1 hour and then the rate of increase slowed down. However, when the hydrolysis products were analysed by thin layer chromatography (TLC), it was seen that the size of hydrolysis products continued decreasing after 1 hour (Figure 3). The depletion of undigested κ-carrageenan with increasing incubation time could be clearly seen in TLC. Also, the size of hydrolysis products decreased with increasing incubation time. At the end of 12 hours of enzymatic hydrolysis, 2 major hydrolysis products could be seen in TLC.

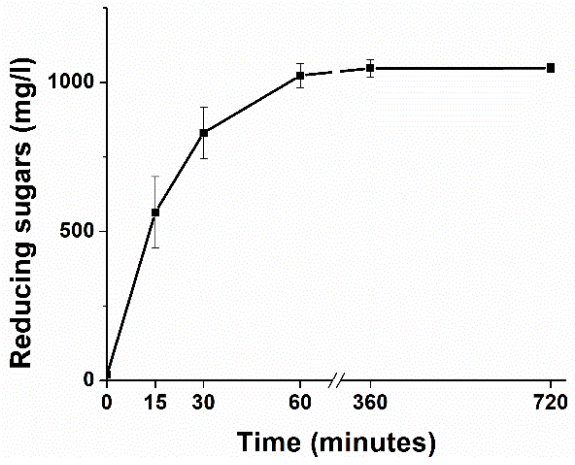

Figure 2. *Time course of enzymatic hydrolysis of κ-carrageenan.*

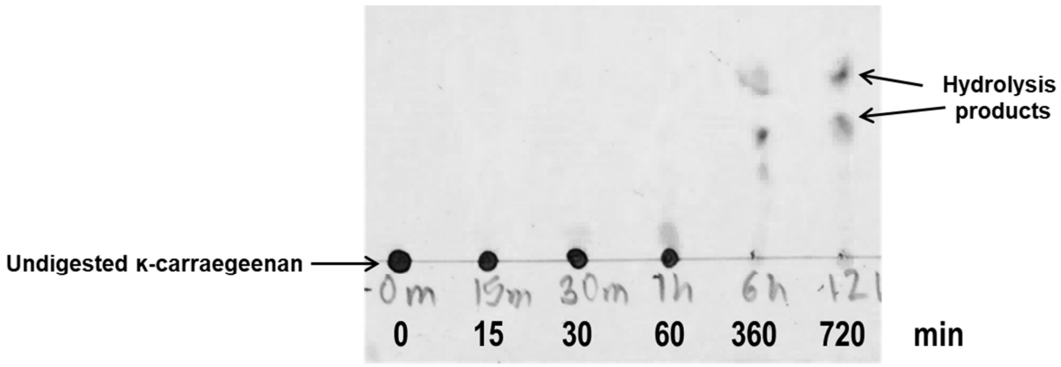

Figure 3. *TLC of κ-carrageenan enzymatic hydrolysis products.*

The 12 hours enzymatic hydrolysate of κ-carrageenan was analysed by negative ion mode ESI-MS. The oligosaccharide products identified based on their m/z values are listed in Table 2. Here, the repeating disaccharide κ-neocarrabiose unit is denoted as (A-G4S), where A = 3,6-anhydro-D-galactose and G4S = D-galactose-4-sulphate. Tetrasaccharide ion ($[(A\text{-}G4S)_2]^{2-}$ m/z = 394), tetrasaccharide ion with one sodium ion ($[(A\text{-}G4S)_2Na]^-$ m/z = 811) and tetrasaccharide ion with a potassium ion ($[(A\text{-}G4S)_2K]^-$ m/z = 827) were detected in the mass spectrum. Also, hexasaccharide ion with a potassium ion was detected ($[(A\text{-}G4S)_3K]^{2-}$ m/z = 606). Thus, enzymatic hydrolysis of κ-carrageenan yielded tetrasachharide and hexasaccharide as end products.

Table 2. *Oligosaccharide products detected in ESI-MS of 12 hours enzymatic hydrolysate of κ-carrageenan.*

Observed m/z	Composition assignment
394	$[(A\text{-}G4S)_2]^2$-
811	$[(A\text{-}G4S)_2Na]$-
827	$[(A\text{-}G4S)_2K]$-
606	$[(A\text{-}G4S)_3K]^2$-

A = 3,6-anhydrogalactose;
G4S = D-galactose-4-sulphate;
Na = sodium ion;
K = potassium ion.

4. Conclusions

In present study, a κ-carrageenan degrading microorganism was isolated and characterized. The isolate was found to have Gram-negative rod-shaped cells when seen under a microscope. Based on biochemical characterization and 16S rRNA sequence, the isolate was identified as *Alteromonas macleodii* KS62. To the best of our knowledge, this is the first report on κ-carrageenase producing microorganism belonging to species *Alteromonas macleodii*.

The isolate exhibited κ-carrageenase production of 1.6U/ml (12.5U/mg protein) when grown in a medium supplemented with κ-carrageenan as carbon source and yeast extract as nitrogen source. The production further increased to 2.9U/ml (14.1U/mg protein) in a medium supplemented with red seaweed (*Kappaphycus*) biomass as carbon and nitrogen source. κ-carrageenase production obtained is comparable to or slightly more than that reported in other studies [12-14]. It is of interest that this strain *Alteromonas macleodii* KS62 secretes these κ-carrageenase enzymes along with a range of other proteins and enzymes in the extracellular milieu via membrane vesicles as we reported earlier [20].

This is the first study successfully utilizing red seaweed whole biomass powder as substrate for κ-carrageenase enzyme production. Using the cheap red seaweed biomass as substrate for κ-carrageenase production will help achieve economically sustainable large scale production of κ-carrageenase enzymes.

Enzymatic hydrolysis of κ-carrageenan revealed two major end products of hydrolysis identified as tetrasaccharide and hexasaccharide of κ-carrageenan by ESI-MS analysis. These low molecular weight κ-carrageenan oligosaccharides can find pharmaceutical applications as bioactive molecules.

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