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Isolation, Characterization and Enzymatic Potential of Epiphytic Bacteria Associated with the Red Seaweed *Kappaphycus alvarezii*

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KEYWORDS

Seaweed-associated bacteria, marine microbiota, enzyme activity ,Agarase, Carrageenase.

ABSTRACT:

Epiphytic bacteria on seaweed plays an mportant role in nutrient cycling and are a natural source of carohydrases. In this study the epiphytic bacteria associated with red seaweed Kappaphycus alvarezii have been isolated and characterised by standard culture-based methods for the determination of their biochemical diversity and potential enzyme function. Their biochemical profile indicated the presence of metabolically versatile bacteria typical of the genera commonly found in marine macroalgae (Vibrio, Pseudomonas, Bacillus and related taxa). The results suggest that polysaccharide degrading enzymes such as agarases, alginate lyases and cyto-carrageenases are produced, which are relevant for breaking down the components of the algal cell wall (agar and carrageenan). In summary, the results demonstrate the functional relevance of epiphytic bacteria for the degradation of structurally complex polysaccharides and the potential of Kappaphycus alvarezii as a source of enzymes for bio-engineering and environmental applications.

1.Introduction

Marine life is recognised as a vast reservoir of organisms with a wide range of bioactivities. Seaweed is one of the most important marine communities with a diverse microbial population. Exploring the marine environment has allowed the algae to produce different secondary metabolites to survive the stresses of the environment. These metabolites are known to have valuable biological activities(Cadar et al., 2025; El-Beltagi et al., 2022).Marine macroalgae and related microorganisms have coevolved since early evolution and have formed functionally integrated symbionts. Although seaweed is extensively studied for its environmental and industrial value, the structure and function of its microbial communities are still poorly understood. Recent 16S rRNA gene-based studies indicate that the epiphytic bacterial communities are strongly influenced by host taxonomy, with more similarities within the same phylum of algae than between different phyla, indicating host specificity and possible coevolution. Despite the taxonomic variation, the functional redundancy of these microbial communities indicates resilience to environmental changes. Microbes associated with seaweed play a key role in the growth, defence and adaptation of the host, which makes them key drivers of ecological function. In this context, the enzymatic activity of seaweed-associated microorganisms is an important functional link between microbial diversity and bio-technological potential. (Egan et al., 2013; Farhan et al., 2025).

Cells in all living things contain enzymes, chemicals that act as biocatalysts, catalyzing or accelerating biological reactions. Enzymes have superior selectivity, variable activity and higher catalytic efficiency than chemical or synthetic catalysts. These advantages have led to the increased use of enzymes in a wide range of sectors, such as the chemical, food and pharmaceutical industries. This has increased the demand for high quality enzymes produced using reasonably priced commercial methods. (Farhan et al., 2025; Robinson, 2015). Marine macroalgae are a diverse and specialised community of epiphytic bacteria that are important for nutrient cycling, surface colonisation and ecological stability in coastal ecosystems. These microorganisms

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have evolved to use structurally complex polysaccharides such as agar, carrageenan, alginate, cellulose, laminar and various sulfate galactones (Egan et al., 2013) in the cell walls of seaweed. Consequently, seaweed-associated bacteria are well known producers of commercially important hydrolytic enzymes, including amylases, proteases, cellulases, agarolytics, alginate lyases and carrageenases. These enzymes have a high specificity of substrates and a catalytic activity, characteristics which make them valuable for a number of commercial processes in the pharmaceutical, food, agro-industrial, textiles and bioremediation industries. Compared to synthetic chemical catalysts, microbial enzymes offer better biocompatibility, less reaction-related energy requirements and a wider range of substrate handling capabilities, which have stimulated worldwide interest in their sustainable production (Trincone, 2011).

Red algae, including *Kappaphycus alvarezii*, are particularly well known for their rich polysaccharide composition, dominated by carrageenan which provide a suitable environment for bacteria that can produce agarases and carrageenases. These bacteria play a key ecological role by helping to break down macroalgae biomass and recycle organic matter in the marine environment (Adalsteinsson et al., 2025) (Satheeja Santhi et al., 2014). Previous studies have reported agarolytic and carrageenergic genera such as *Vibrio, Zobellia, Flammeovirga* and *Pseudomonas* many of which are natural epiphytes of commercially grown red algae. Understanding the diversity and enzymatic capabilities of these bacteria is not only important for the environmental protection but also for identifying strains with biotechnology applications in the development of functional foods, the production of bioactive oligosaccharides and the valorisation of seaweed biomass(Saravanan et al., 2024)(Veerakumar & Manian, 2022).

In recent years, there has been increased interest in the exploration of marine microbial resources in view of the growing demand for sustainable bio-based industries and the global expansion of the marine plant-based industry. Cultivated red algae such as *Kappaphycus alvarezii* play a major role in the production of carrageenan and other hydrocolloids, but their microbiological composition is still less well characterised. Many previous studies focused on alginate- or cellulose-degrading bacteria from brown algae, while relatively few studies looked at the diversity of bacteria able to degrade red algae polysaccharides. Furthermore, although enzymes for the degradation of marine polysaccharides have been reported, the relationship between specific seaweed hosts and their enzymatic communities of bacteria is not yet sufficiently understood (Saravanan et al., 2024). Addressing this gap is crucial to discovering new enzyme sources, improving seaweed treatment technologies and promoting the development of functional compounds derived from marine organisms. By characterising the bacterial community associated with *Kappaphycus alvarezii* and by screening for key hydrolytic enzymes, the study advances current knowledge of the functional diversity of the red algae and provides a basis for future applications in marine biotechnology.

The purpose of the study was to isolate and preliminarily characterise bacteria related to *Kappaphycus alvarezii* and to identify isolates that are able to degrade seaweed polysaccharides such as agar, alginate and carrageenan. This integrated approach provides basic information on the functional diversity of the seaweed microbial community and supports the future exploration of marine bacteria as promising bio-technological applications.

1. Materials and Methods

2.1 Sample Collection

Kappaphycus alvarezii was collected from Mandapam , Ramanathapuram district of Tamil Nadu, India.The samples were transported to the laboratory in plastic bags containing seawater collected at the point of collection. The collected seaweed sample was washed with sterile seawater until it was free of unwanted impurities and adhering particles of sand.

2.2 Isolation of Bacteria

The samples were washed three times with autoclaved seawater to remove loose epiphytes and sand particles from the samples. After rinsing, the surface of the thallus was swabbed using sterile cotton swabs and swabbed onto the Zobelle Marine Agar (HiMedia) plates. The inoculated plates were incubated for 5 days at 30°C according to Lemos et al. (1985) After incubation, morphologically different colonies were selected and repeatedly restreaked to obtain separate, individual colonies(Karthick & Mohanraju, n.d.).

2.3 Amylase Activity

The amylase activity was assessed on nutrient agar plates supplemented with 2% (w/v) soluble starch and agar of 2% (w/v). Each purified isolate was streaked on the medium and incubated for 24 hours at 30°C. After incubation, the plates were flooded with iodine solution to visualize the degradation of the starch. Isolates that produce extracellular amylase will show a clear decolorised zone along the line of stripes, in contrast to the blue-black colouration of the starch. (Veerakumar & Manian, 2022)

2.4 Agarolytic Activity

The agarolytic activity was tested using the screening medium containing 0.05 % (w/v) of yeast extract, 0.25 % (w/v) of peptone and 2% (w/v) of agar as sole carbon source, pH adjusted to 7.8. Each isolate was streaked on the° medium and incubated for 3 days at 30°C. The plates were examined for agarolytic activity, as indicated by agar liquefaction or by shallow troughs along the line. Morphologically distinct colonies have been restreakedhos to ensure purity. For confirmation, Lugol's iodine (2 g potassium iodide and 1 g iodine in 200 ml distilled water) was poured over cultures from three days old cultured on the same medium. Isolates producing clear zones after staining were identified as bacteria that degrade agar. (Khambhaty et al., 2008).

2.5 Alginate lyase Activity

The activity of alginate lyase on bacterial isolates was evaluated on agar plates with 0.5% of sodium alginate, 0.5% of potassium sulphate, 0.2% of dipotassium phosphate, and 2% of magnesium sulphate, adjusted for pH 7.2 to 7.4. Each purified isolate was then rubbed on the surface of ALG plates and incubated at 30°C for 2 to 4 days until a visible growth was apparent. After incubation, the plates were immersed in the *Gram's iodine* solution. Isolates produced alginate lyase showed a clear zone along the line of stripes against a bleached background. Only isolates with clear bands were selected for further analysis (Wang et al., 2017).

2.6 Carrageenase Activity

κ-Carrageenase activity was assessed using κ-carrageenan medium composed of 2% (w/v) κ-carrageenan, 0.1% (w/v) yeast extract, and 0.5% (w/v) peptone. Each purified isolate was dotted onto the solid medium and incubated at room temperature (27°C) for 4 days. Following incubation, the plates were flooded with Lugol's iodine solution. The formation of a clear halo surrounding the colony indicated the hydrolysis of κ-carrageenan and confirmed κ-carrageenase activity in the isolate (Wijaya et al., 2021).

2.7 Lipase Activity

The activity of lipase in isolates has been investigated by means of a modified phenol red-tween agar plate test. Nutrient agar with seawater was supplemented with phenol red (0.01% w/v) and Tween (1% w/v) as lipid substrates. Before sterilisation, the pH of the medium was adjusted to approximately 7.3. The isolates were placed on plates and incubated at 30 °C for 48 to 72 hours, showing the apparent colour change of the medium from red to orange or yellow following the release of fatty acids and the subsequent acidification of the medium by lipid hydrolysis.(Ramnath et al., 2017).

3. Morphological and Biochemical Characterization

3.1 Morphological Characterization

Gram-staining has been performed by the standard Gram reaction method (Hucker & Conn, 1923). The 24-hour cultures were stained, air-dried and examined under the influence of oil immersion (100x) to determine cell morphology and Gram-positive reactions.

3.2 Biochemical Characterization

Preliminary biochemical assays on isolates have been performed according to standard microbiological protocols (Cappuccino and Sherman, 2014). Tests included catalase, oxidase, indole synthesis, methyl red (MR), Voges-Prostauer (VP) and citrate. All media and reagents were sourced from HiMedia Laboratories (India) and the tests were carried out according to the manufacturer's instructions. The results of biochemical profiling were used to support the provisional identification of the isolates.

3.3 Molecular Characterization

The genomic DNA was extracted from an overnight culture of KA2 as it showed the highest activity out of all four isolates, using a commercial bacterial genomic

DNA kit (HiMedia, India) according to the manufacturer's instructions. Following the PCR conditions described by Weisburg et al., the 16S rRNA gene was amplified by the 27F (5′-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5′-TACGGCTTGATCAG-3) universal primers. (1991) The amplicons were examined on 1% *agar* gel, cleaned and subjected to Sanger sequencing. The resulting sequences were compared with the NCBI GenBank database using the BLASTn tool to identify the species-by-species level. The phylogenetic sequencing of selected isolates was performed by MEGA7 using a *neighbor-joining* approach and a thousand bootstrap replicates (Kumar et al., 2016).

4. Results

4.1 Isolation of Epiphytic Bacteria

After swabbing the thallus and culturing on Zobelle marine agar, a total of 4 bacterial isolates were successfully obtained from the surface of *Kappaphycus alvarezii*. Different colonies with different pigmentation, morphology and elevations were selected for analysis.

Figure 1: Isolated bacteria from seaweed**.**

4.2 Amylase Activity

Extracellular amylase production has been confirmed by the presence of distinct clearance around bacterial colonies on starch agar following iodine flooding. These zones were due to the hydrolysis of starch in the medium and showed that the isolates were actively secreted amylase under the culture conditions tested.

Figure 2: Amylase activity of the four isolates KA1,KA2, KA3 and KA4.

4.3 Agarolytic Activity

All isolates tested on agarolytic media showed a visible agar liquefaction or shallow trough around the line of streak after 72 hours of incubation. After flooding the plate with Lugol's iodine solution, the agar-degrading isolates formed clear halos, which confirmed the positive agarolytic activity. This suggests the presence of bacteria that can use agar as a source of carbon, which is consistent with the typical red seaweed-associated microbial community.

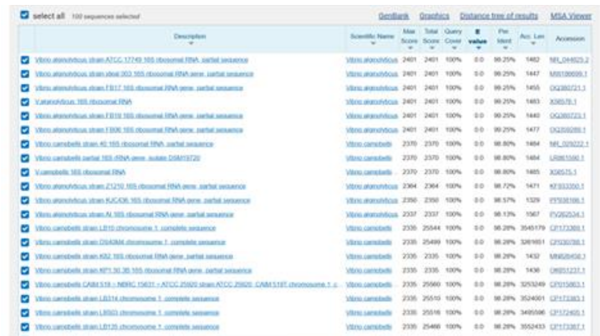

Figure 3: Agarolytic activity of the four isolates KA1,KA2, KA3 and KA4.

4.4 Alginate Lyase Activity

Screening on ALG plates revealed that several isolates produced clear zones after Gram- iodine treatment, which confirmed the activity of alginate lyase. These isolates were able to hydrolyze sodium alginate, which indicates metabolic flexibility although alginate is not a major component of the cell wall of *Kappaphycus alvarezii*.

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Figure 4*:* Alginate lyase activity of the four isolates KA1,KA2, KA3 and KA4.

4.5 Carrageenase Activity

The plates with the KA-carrageenase showed well defined halos around the inoculated colonies after iodine staining, indicating positive activity of KA-carrageenase. These isolates were able to degrade the major sulphate galactane present in red seaweed, carrageenan. Bacteria producing carrageenase are considered environmentally important for the turnover of carrageenan in marine environments.

Figure 5: Carrageenase activity of the four isolates KA1,KA2, KA3 and KA4.

4.6 Lipase Activity

Methyl red-associated changes in colour were observed in Tween 80 agar. Lipase activity has therefore been detected in the isolates.

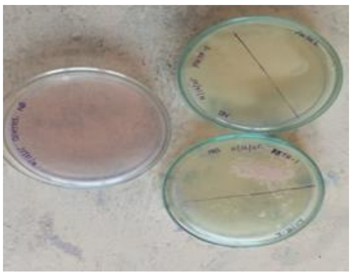

Figure 6: Lipase activity of the four isolates KA1,KA2, KA3 and KA4.

4.7 Biochemical Characterization

All four isolates exhibited a similar biochemical profile, being oxidase positive, catalase positive, MR positive, VP and citrate negative. Three isolates were indole positive, while KA4 was indole negative. The similarity in biochemical characteristics suggests that the isolates are closely related and may belong to the same genus, possibly representing different strains of a Vibrio-like bacterium.

Biochemical tests	**KA1**	**KA2**	**KA3**	**KA4**
Colony morphology	Circular	Circular	Circular	Circular to irregular
Indole test	+	+	+	_
Methyl Red	+	+	+	+
VP	_	_	_	_
Citrate	_	_	_	_
Catalase	+	+	+	+
Oxidase	+	+	+	+

Table 1: Biochemical characterization of the four isolates

4.8 Molecular Characterization

The 16S rRNA gene sequence obtained from the selected bacterial isolate was analysed by BLASTN against the GenBank database of NCBI. BLAST results showed that the isolate had the highest degree of sequence similarity with *Vibrio alginolyticus*. The top hits had a 100 % coverage of the query with a maximum sequence identity of 99.25 % and an E value of 0.0, indicating a high-level match. The closest matches were found in several strains of *V. alginolyticus*, including strain ATCC 17749. A comparably lower sequence similarity (98.1-98.8) has been observed in closely related species such as *Vibrio campbellii*. Based on high degree of identity, full coverage of the query and zero E value, the isolate was

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classified as *Vibrio alginolyticus*.The sequence has been submitted at NCBI with accession number PZ006106.

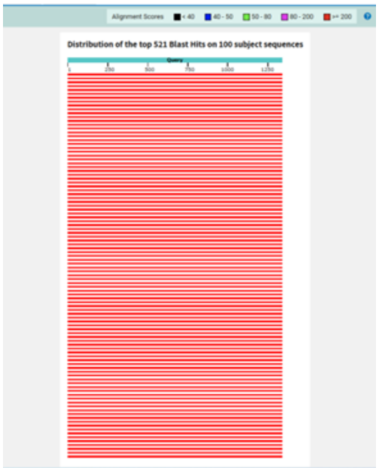

Figure 7: Molecular Characterization of the isolate KA2

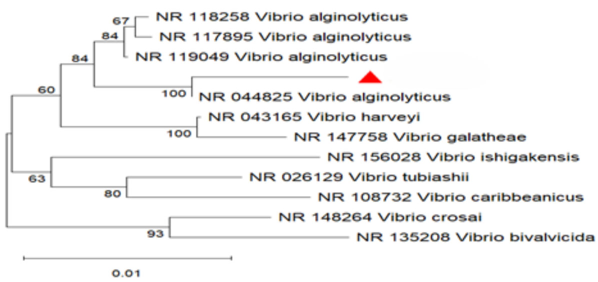

Figure 8: Phylogenetic Tree of KA2

5. Discussion

This study shows that the epiphytic bacterial community associated with *Kappaphycus alvarezii* has a high capacity for the degradation of polysaccharides, as demonstrated by isolates positive for amylase, agarolytic, alginate lyase and κ-carrageenase-positive isolates. The variety of enzyme activities observed suggest that seaweed-associated bacteria play an active ecological role in the transformation of marine polysaccharides that are structurally complex. This enzyme potential is consistent with previous reports suggesting that macroalgae contain specialized microbes that are able to degrade polymers of the algal cell wall (Goecke et al., n.d.).

The predominance of agarolytic and carrageenan-rich strains is consistent with the biochemical composition of red algae cell walls rich in agar, agaropectin and carrageenan (Duckworth & Yaphe, 1971).Red algae such as Kappaphycus and Gracilaria are known for hosting agar- and carrageenan-degrading bacteria, especially *Vibrio, Pseudomonas, Flammeovirga* and *Zobellia.* (M et al., 2017)(Sun et al., 2021). Agarases (EC 3.2.1.81) produced by marine bacteria are particularly common in epiphytic micro-organisms and contribute to nutrient cycling and the dynamics of the algal film.

The ability of the isolates to hydrolyze multiple complex substrates indicates the presence of various carbohydrate-active enzymes (CAZymes), in particular glycoside hydrolases and lyases, which are involved in the degradation of sulphated galactane and uronic acid rich polymers. These multifunctional polysaccharides are of great industrial interest, particularly for applications in biotechnology, bioremediation and the production of bioactive oligosaccharides(Ch et al., n.d.). Overall, the predominance of agarolytic-, alginate lyase-, amylase- , lipase- and carrageenase- positive isolates is consistent with the patterns observed in seaweed-associated microbiomes but also underlines the enzymatic diversity of the epiphytic community of *Kappaphycus*. This work adds to growing evidence that tropical red algae host bacteria that can degrade a broad range of marine polysaccharides and may be a valuable reservoir for the discovery of new marine enzymes.

6. Conclusion

This study successfully isolated and characterised epiphytic bacteria associated with *Kappaphycus alvarezii*, showing diversity in morphology, gram-staining and biochemical properties. These preliminary observations highlight that red algae contain a diverse microbial community adapted to the polysaccharide-rich surface environment of the host. This diversity is an important marker of functional specialisation of marine epiphytic bacteria. Enzymatic testing showed that several isolates have significant extracellular hydrolytic potential, in particular agarolytic, alginate lyase and carrageenase-like activity. These enzymes are central in the breakdown of structurally complex red algae polysaccharides, indicating that the bacteria involved are actively involved in nutrient cycling and turnover of organic matter in marine ecosystems. The detection of several positive activities also highlights the potential industrial importance of these isolates, particularly for applications in bioconversion, functional synthesis of oligosaccharides and marine biotechnology.

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Conflict of Interest

There is no conflict of interest.

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