

Prebiotic potential of enzymatically produced ulvan oligosaccharides using ulvan lyase of *Bacillus subtilis*, NIOA181, a macroalgae-associated bacteria

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Running title: Bioactive UOS production using ulvan lyase

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Abstract

Aims: To characterize the polysaccharide hydrolyzing potential of macroalgae-associated bacteria (MABs) for enzymatic production of oligosaccharides and determining their prebiotic potential.

Methods and Results: Approximately 400 MABs were qualitatively characterised for polysaccharide hydrolyzing activity. Only about 5 to 15% of the isolates were found to have the potential for producing porphyranase, alginate lyase and ulvan lyase enzymes which were quantified in specific substrate broths. One potential MAB, *Bacillus subtilis*, NIOA181, isolated from green macroalgae, showed the highest ulvan lyase activity. This enzyme was partially purified and used to hydrolyse ulvan into ulvan oligosaccharides. Structural characterization of ulvan oligosaccharides showed that they are predominantly composed of di-, tri-, and tetrasaccharide units. Results showed that the enzymatically produced ulvan oligosaccharides exhibited prebiotic activity by promoting the growth of probiotic bacteria and suppressing the enteric pathogens, which were higher than the ulvan polysaccharide and equivalent to commercial fructooligosaccharides.

Keywords: macroalgae, macroalgae-associated bacteria; ulvan lyase; ulvan oligosaccharide; prebiotic potential

Introduction

Macroalgae are a sustainable source of bioactive molecules, which have wide applications as functional foods in the nutraceutical and healthcare industry. Their global demand is progressively increasing and driving the expansion of its aquaculture industry. Cultivating macroalgae is also the need of the hour from the ecological perspective, as it facilitates CO₂ sequestration and is a widely accepted measure for reducing the carbon footprint (Duarte et al., 2017; Hurd et al., 2022). Macroalgae have the edge over other plants for their bioactive molecules, they are devoid of lignin and are composed of unique structural and storage polysaccharides, which are not commonly present in terrestrial plants (Li et al., 2020b; Jagtap et al., 2021). They grow predominantly in the coastal, rocky shore areas across all geographical locations, including the tropical, subtropical, temperate and polar regions (Selvarajan et al., 2019; Hung et al., 2021). The macroalgal polysaccharides have various bioactive potentials, but due to their high molecular weight and low solubility, they are primarily used only as hydrocolloids (Tanna and Mishra 2019). Studies have shown that these algal polysaccharides have wider applications when broken down enzymatically into low molecular weight oligosaccharides. These bioactive sugars of macroalgal origin are in great demand in the healthcare sector as oligosaccharides are accredited with “GRAS” (generally recognized as safe) status by the United States Food and Drug Administration (FDA 2016).

Enzymatic hydrolysis of complex, macroalgal polysaccharides requires specific enzymes for its breakdown into oligosaccharides. Macroalgae-associated bacteria (MABs) are the major source of these enzymes, such as agarase, carrageenase, porphyranase, alginate lyase, fucoidanase, laminarinase and ulvan lyase (Jagtap and Manohar 2021; Jagtap et al., 2022). Enzymes catalyzing algal polysaccharide breakdown are grouped into glycoside hydrolase (GH) and polysaccharide lyase (PL). The GH is the major group of enzymes that hydrolyse the breakdown of polysaccharides through the cleavage of β -(1→4)-glycosidic linkages and the PL enzymes acts through the β -elimination mechanism (Belik et al., 2020; Li et al., 2020a). Agarase, carrageenase, porphyranase, fucoidanase and laminarinase are the GH enzymes that cleave β -(1→4)-glycosidic linkages in agar, carrageenan, porphyran, fucoidan and laminarin, respectively (Przybylski et al., 2015; Zhang et al., 2019). Alginate lyase and ulvan lyase belong to the PL group, which can hydrolyse alginate and ulvan polysaccharides through the β -elimination into respective oligosaccharides. Alginate lyase is one of the well-studied carbohydrate modifying enzyme, catalyzing the hydrolysis of alginate into bioactive oligosaccharides mainly comprising of di-, tri- and tetrasaccharide units (Belik et al., 2020; Jagtap et al., 2022). However, porphyranase, fucoidanase, laminarinase and ulvan lyase are the least studied enzymes and there are very few reports on the bioactive potential of oligosaccharides produced using these enzymes

(Reisky et al., 2018; Qin et al., 2020). Though chemical processes are used to hydrolyse the algal polysaccharides into low molecular weight oligosaccharides, it involves treating polysaccharides with concentrated acid and heating at high temperatures for several hours, releasing monomers and undesirable toxic by-products such as furfural and 5-hydroxymethylfurfural into a final product, which reduces the purity of oligosaccharides (Lee and Lee 2016; Cheong et al., 2018). The oligosaccharides produced through chemical processes have limitations in functional food and nutraceutical applications as chemical treatment removes sulfation and methylation, which can reduce their bioactivity. Therefore, enzymatic hydrolysis of algal polysaccharides to produce stereo-specific bioactive oligosaccharides are gaining utmost importance because of the versatile nature of microbes producing these enzymes, which can be rapidly grown through batch or continuous cultivation technique in the laboratory with strong adaptability for commercial applications (Charoensiddhi et al., 2017; Gurpilhares et al., 2019).

Macroalgal diversity from India is well studied and documents approximately 5000 macroalgal species along the 7500 km coastal stretch. Extensive research on the diversity, aquaculture practices, production of value added products and extraction of bioactive molecules from different macroalgal groups are reported from the Indian sub-continent and its adjoining islands. Previous studies have reported the diversity and interactions of microbes in the macroalgal ecosystem from these regions (Singh and Reddy 2014; Naik et al., 2019). They have depicted that macroalgae provide a suitable substratum for the settlement of microorganisms and release various organic substances that promote bacterial growth and biofilm formation (Singh et al., 2011; Selvarajan et al., 2019). However, the biotechnological potential of the microbiome associated with macroalgae for its enzymes to produce algal oligosaccharides is not fully explored.

This study focuses on the characterization of bacteria associated with various macroalgae for their enzymatic potential to hydrolyse major algal polysaccharides such as porphyran, alginate and ulvan. One of the potential MAB, *Bacillus subtilis*, NIOA181, isolated from green macroalgae, showed the highest ulvan lyase activity, which was used to hydrolyse ulvan into ulvan oligosaccharides (UOS). Structural characterization of the UOS based on Fourier Transform Infrared (FTIR), High-Resolution Mass Spectrometry (HRMS) and Nuclear Magnetic Resonance (NMR) spectroscopy was carried out. The prebiotic activity of the enzymatically produced UOS was also determined to identify their potential to increase the growth of probiotic bacteria (*Lactobacillus acidophilus* and *Lactobacillus bulgaricus*) and suppress the growth of enteric *Escherichia coli* strains, which is known to elicit varied health benefits (Fernandez et al., 2011). This study will help to understand the diversity of bacteria

associated with macroalgae and their enzymatic potential to hydrolyse algal polysaccharides into low molecular weight bioactive oligosaccharides.

MATERIALS AND METHODS

Chemicals and bacterial strains

Analytical grade sodium alginate (Sigma Aldrich, USA), ulvan extracted from *Ulva lactuca* as described by (Lahaye et al., 1999) and porphyran extracted from *Porphyra* sp. as illustrated by (Zhang et al., 2019) were used for all experimental analysis. Zobell marine broth was procured from Himedia laboratories, India and Man-Rogosa-Sharp broth from Sisco Research Laboratories Pvt. Ltd. India of the microbiological grade. Fructooligosaccharides (FOS) from chicory, used as a model prebiotic compound, was purchased from Sigma Aldrich and other chemicals used were of analytical grade. The potential probiotics *Lactobacillus acidophilus* (NCIM 2285/ATCC4963) and *Lactobacillus bulgaricus* (NCIM 2056/ATCC8001) and an enteric culture of *Escherichia coli* strain DSM1576 (ATCC8739), were procured from National Collection of Industrial Microorganisms, CSIR-National Chemical Laboratory, Pune, India.

Isolation of MABs

Macroalgal samples were collected from major macroalgal beds along the Indian coast to isolate MABs. The sampling stations included Okha and Porbandar along the Gujarat coast, Malvan and Kunkeshwar along the Maharashtra coast, Vagator, Dona Paula, Palolem from the Goa coast and Mandapam along the Tamil Nadu coast (Figure 1). Representative macroalgae belonging to all three major groups of red, brown and green macroalgae were collected (Jagtap et al., 2021) and associated epiphytic MABs were isolated following the shake tube method (Singh et al., 2011). For this, macroalgal samples were rinsed with sterile seawater to remove the attached debris, sand particles and seashells. Approximately 1 g of the washed macroalgal sample (fresh weight) was re-suspended in 9 ml of sterile seawater to release all the epiphytic bacteria into suspension and serially diluted (10^{-3} - 10^{-4}) samples were spread plated on Zobell Marine agar microbiological plates under sterile conditions and incubated at 28°C for 24 - 48 h to obtain well-isolated colonies. At the end of the incubation period, morphologically distinct colonies were picked, streak purified and maintained on Zobell Marine agar plates for further analysis with designated isolate numbers NIOA1 to NIOA421 in sequence.

Qualitative analysis of MABs for enzymatic hydrolysis of algal polysaccharides

The purified MABs were grown on algal polysaccharide substrate plates to check their potential to hydrolyse porphyran, alginate and ulvan. In brief, the pure bacterial cultures were inoculated in a sterile Zobell Marine Broth (20 ml) and incubated in a shaker incubator for 24 h at 28°C. The culture

suspension (10 µl) with the absorbance of 0.8 to 0.9 at 600 nm was spot inoculated on specific substrate media plates supplemented with 0.5% of porphyran or alginate or ulvan in the basal salt solution, composed of 5% sodium chloride, 0.5% ammonium sulphate, 0.3% dipotassium hydrogen phosphate, 0.1% magnesium sulphate, 0.01% ferrous sulphate and 1.5% agar. The spot inoculated plates were incubated at 28°C for 48 h; after incubation, the plates were stained with substrate-specific staining solution; Lugol's iodine (0.5%) for porphyran, grams iodine (0.5%) for alginate and cetylpyridinium chloride (0.05%) for ulvan (Sawant et al., 2015). The zone of clearance around the bacterial colony was measured within 10 minutes of staining (Figure S1). The bacterial isolates were tested for their ability to catalyse the polysaccharide breakdown activity and grouped based on the zone of clearance. Those isolates with the highest zone of clearance (20-35 mm) were categorised as “potential isolates” with very good activity and with decreasing zone of clearance, the isolates were grouped as good (11-20 mm), minimum (1-10 mm) and no activity group. The potential MABs with good efficiency in breaking down representative algal polysaccharides were used for further analysis and identified using molecular techniques.

Phylogenetic characterization of potential polysaccharide hydrolyzing MABs

The potential MABs (19 isolates) with the highest polysaccharide hydrolyzing activity were identified based on the 16S rRNA gene sequence. Each purified potential bacterial isolate was grown in 10 ml Zobell Marine Broth at 28°C for 24 h. The bacterial cell pellets of the individually grown isolates with an absorbance of approximately 0.8 at 600 nm were collected through centrifugation (20000 g for 5 min) and used for genomic DNA extraction. The 16S rRNA gene fragment of the MABs was amplified using 27F and 1492R universal bacterial primers (Liao et al., 2011). The amplified fragment was purified using a PCR clean-up kit (Promega, USA) and sequenced bidirectionally on Genetic Analyzer 3130xl (ABI) based on the Big Dye v 3.1 chain terminator chemistry. 16S rRNA gene sequence obtained for individual isolates was compared with the NCBI database using the nucleotide BLAST search program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The partial 16S rRNA gene sequence of the 19 potential MABs are deposited in the NCBI GenBank database. Phylogenetic analysis of bacterial isolates was carried out on the MEGA X platform based on the sequence homology of the 16S rRNA gene to gene sequence from the NCBI database using the Maximum likelihood algorithm (Tamura et al., 2011) and the robustness was assessed with 1000 bootstrap replicates.

Quantitative estimation of enzyme activity in the potential MABs

The nineteen potential isolates were evaluated for their quantitative enzyme activity. For this, individual isolates were inoculated in basal salt solution amended with 0.5% polysaccharide substrates

(porphyran, alginate or ulvan) and grown at 28°C for 48 h. The enzyme activity of the potential isolates was assessed by measuring the release of reducing sugar following the dinitrosalicylic acid method (Miller, 1959) and galactose (20-100 µM) was used as a standard. Enzyme activity was expressed in U as the amount of enzyme required to liberate 1 µM of reducing sugar min⁻¹ mg⁻¹ of protein (Gao et al., 2019). The extracellular protein content in the broth was determined following the Bradford assay and varying concentrations of BSA (50-250 µg ml⁻¹) were used as a standard. The isolates NIOA181, NIOA284, NIOA379, NIOA323 and NIOA354, which showed very high enzyme activity, are deposited in the National Centre of Industrial Microorganisms, Culture collection at CSIR-National Chemical Laboratory, Pune, India. Of this NIOA181 (NCIM 5804), *Bacillus* sp., which showed very high ulvan lyase activity, was studied in detail and its ability to hydrolyse ulvan was characterised.

Biochemical characterisation of ulvan lyase (ULB1) from *Bacillus* sp. NIOA181

Ulvan lyase, ULB1 from *Bacillus* sp. NIOA181 was characterised for optimization of its potential to hydrolyse the algal polysaccharide ulvan. For this, *Bacillus* sp. NIOA181 was inoculated in 100 ml of basal salt solution, amended with 0.5% ulvan and allowed to grow at 28°C for 24 h. The cell-free supernatant containing the extracellular ulvan lyase was collected after centrifugation of the culture broth at 5000 g for 15 - 20 minutes. The supernatant was precipitated with saturated ammonium sulfate to collect the crude enzyme and the excess ammonium sulfate was removed by dialysis. The crude ulvan lyase was loaded on the DEAE sepharose column and eluted with a 0 - 1 M NaCl gradient and fractions were collected at regular intervals. Those fractions with enzyme activity were combined and dialysed against 50 mM Tris-HCL buffer (pH 8.0) and freeze-dried into powder form. The influence of different parameters such as the pH, temperature, varying NaCl concentrations, metals ions and chelating agents on the relative activity of the lyophilized enzyme was tested. The effect of pH (3 to 11) on ulvan lyase activity was determined at 50 mM of different buffers such as acetate buffer (pH 3.0 - 5.0), phosphate buffer pH 6.0 - 7.0), Tris-HCl buffer (pH 8.0 - 9.0) and carbonate-bicarbonate buffer (pH 10.0 - 11.0) and temperatures ranging from 20 - 60°C by incubating the lyophilized enzyme with 0.5% substrate for 30 minutes. Similarly the effect of NaCl concentrations (0.01 - 1 M), different metal ions (Li⁺, K⁺, Mg²⁺, Ca²⁺, Ba²⁺, Ni²⁺, Co²⁺, Cu²⁺ and Fe²⁺) at 10 mM concentration, 0.1% detergent or inhibitors (ethylene diamine tetraacetic acid (EDTA), dithiothreitol (DTT), urea, β-mercaptoethanol, sodium dodecyl sulfate (SDS), triton X-100, Tween 20, Tween 80 and cetyl trimethyl ammonium bromide (CTAB)) on ulvan lyase activity was determined at optimum conditions along with appropriate controls with no addition of metal ions or detergents.

Enzymatic production of UOS using ulvan lyase ULB1 from *Bacillus* sp. NIOA181

Enzymatic hydrolysis of ulvan was carried out to produce UOS using partially purified ulvan lyase, ULB1 from *Bacillus* sp. NIOA181 at the optimum conditions obtained from the characterisation studies (pH 8.0, temperature 40 °C, 0.01M NaCl, 10 mM Mg²⁺ and 0.1% of β-mercaptoethanol). For this, 1 ml (10 µg) of the enzyme was mixed with the 0.5% ulvan (100 ml) prepared in 50 mM Tris-HCl buffer at pH 8.0 and supplemented with 10 mM MgCl₂ and incubated at 40°C for 24 h and UOS production was determined using thin-layer chromatography. The UOS was purified on Sephadex® G-100 medium (Sigma-Aldrich, USA) and fractions (2 ml) were eluted with 50 mM Tris-HCL buffer (pH 8.0). The fractions were analysed for reducing sugar content using the dinitrosalicylic acid method (Miller, 1959). The fractions with the highest reducing sugar content were combined and freeze-dried and the white, crystalline ulvan oligosaccharides obtained were used for structural characterization and bioactivity analysis.

Structural characterization of enzymatically produced UOS

FTIR spectroscopy

The enzymatically produced UOS was analysed based on Fourier Transform Infrared Spectroscopy (Shimadzu-8201PC spectrometer, Japan) and evaluated for its functional groups (Hung et al., 2021). For this, lyophilized powder of UOS was individually mixed with dry potassium bromide and the FTIR spectrum was recorded using 50 scans in the spectral range of 4000–400 cm⁻¹.

HRMS spectrometry

HRMS analysis of UOS was performed using Bruker Impact HD Q-TOF spectrometer (Bruker Daltonics, Billerica, MA, USA). For this, UOS (100 µg ml⁻¹) prepared in acetonitrile/water (1:1 v/v) was injected through UHPLC (Dionex Ultimate 3000, Make-Thermo Scientific) to Electrospray Ionization (ESI)-Q-TOF system with a flow rate of 120 µl min⁻¹ and 90% acetonitrile in water containing 0.1% formic acid v/v was used as a mobile phase. The high-resolution mass spectrum was recorded for 10 minutes between 50–1500 m/z in positive ion polarity. The other MS parameters used were as follows: set capillary, 4000 V; set endplate offset, -500 V; set charging voltage, 2000 V; set nebulizer, 1.7 bar; set dry heater, 200°C; and set dry gas, 7.0 L min⁻¹. The spectra visualization and baseline correction were analysed using Bruker compass Data Analysis software 4.2 and the mass was confirmed by the m/z of the sample based on the available literature (Gao et al., 2019).

NMR spectroscopy

UOS at 20 mg ml⁻¹ concentration in deuterated chloroform was used for ¹³C NMR analysis on a BRUKER AVANCE III HD NMR 500 MHz a Liquid State 500MHz NMR spectrometer. The acquisition parameters were set as the number of scans, 4000; D1, 2.0 sec and Pulse program, zgpg30 for analysis.

Prebiotic activity of enzymatically produced UOS

The prebiotic activity of the enzymatically produced UOS was analysed based on their ability to promote the growth of two potential probiotics *L. acidophilus* and *L. bulgaricus* and suppress the growth of enteric culture, *E. coli*. The growth was determined by growing these isolates in a dextrose-free Man-Rogosa-Sharp medium supplemented with UOS (1%) as the sole carbon source by measuring the absorbance of the culture medium at 600 nm at 0 and 24 h. The prebiotic activity of UOS was compared with ulvan and commercial FOS from chicory, following the same methodology described above for UOS. To calculate the prebiotic score, 100 µl of starter and 24 h grown probiotic and enteric cultures were spread plated on basal Man-Rogosa-Sharp medium and incubated at 37°C for 24 h under anaerobic conditions in a RUSKINN CONCEPT 400M workstation. The growth of probiotic and enteric cultures was measured by enumerating the number of colony-forming units (CFUs).

The prebiotic activity score (PA) was calculated using the following equation.

$$PA = [(\log Px^{24} - \log Px^0) \div (\log Pg^{24} - \log Pg^0)] - [(\log Ex^{24} - \log Ex^0) \div (\log Eg^{24} - \log Eg^0)]$$

Where Px^{24} and Px^0 are the CFU measured of probiotic cultures from the 24 h grown and starter culture (at 0 h) respectively with 1% ulvan / UOS or FOS supplement; Pg^{24} and Pg^0 are the CFU measured from the 24 h grown and starter culture (at 0 h) respectively with 1% glucose supplement in the broth. Similarly, Ex^{24} and Ex^0 and Eg^{24} and Eg^0 is the CFU measure of enteric culture, *E.coli*. The CFU was enumerated by plating the broth on Man-Rogosa-Sharp plates (Hu et al., 2006).

Statistical analysis

All experimental studies were carried out in triplicates (n=3) and mean values are expressed along with standard deviations. Statistical significance was determined using one-way ANOVA and student's t-test and the significance level was set as $p \leq 0.05$.

Results

Isolation and screening of bacterial isolates

MABs were isolated from the most commonly grown macroalgae along the Gujarat, Maharashtra, Goa and Tamil Nadu coast. MABs associated with red macroalgae such as *Gracilaria*, *Halymenia*, *Porphyra* and *Hypnea*; brown algae such as *Padina*, *Spatoglossum*, *Sargassum*, *Stoechospermum* and *Sphacelaria* and *Chaetomorpha*, *Caulerpa* and *Ulva* of green macroalgae were isolated. Approximately 421 pure bacterial strains were isolated and about 152, 151 and 118 bacterial isolates were obtained from red, brown and green macroalgae, respectively (Table S1). These bacterial isolates were qualitatively analysed to check their potential to hydrolyse algal polysaccharides using representative algal polysaccharides porphyran, alginate and ulvan. The zone of clearance of all the isolates on substrate plates was measured to determine their efficiency. Of the 421 bacterial isolates tested, around 85 – 95% of the isolate did not show any clearance zone on polysaccharide substrate plates, which signifies that a large majority of the isolates are incapable of breaking down the complex polysaccharides. Of the 5 to 15% of the bacterial strains which showed enzyme activity, only 2 - 5% of the bacterial isolates exhibited very good activity with a zone of clearance in the range of 20 – 35 mm around the colony (Figure 2). Among the isolates tested, porphyranase activity was not seen in about 86% of bacterial isolates (360 isolates) and about 5% showed very good (19 isolates) activity. Similarly, 83% of the isolates showed no activity (351 isolates) for alginate lyase and only 4% showed very good (17 isolates) activity. The maximum number (96%) of isolates tested did not show ulvan lyase activity due to the heteropolysaccharide nature of ulvan and of the 4% of bacterial isolates which showed activity, only 2% (7 isolates) showed very good ulvan lyase activity (Figure 2). The nineteen isolates, which showed very good activity with a maximum clearance zone (> 20 mm) were characterised as potential bacterial isolates to hydrolyse porphyran, alginate and ulvan substrates (Table S2). Maximum porphyranase activity with a zone of clearance of 32 mm was observed in bacterial isolate NIOA284 and isolates NIOA112, NIOA174, NIOA284, NIOA319, NIOA321, NIOA327 and NIOA398, which were associated with red macroalgae showed good porphyranase activity. Similarly, bacteria isolated from brown macroalgae NIOA29, NIOA118, NIOA169, NIOA170, NIOA323, NIOA328, NIOA354 and NIOA388 exhibited good alginate lyase activity. Ulvan lyase activity was observed in five isolates, NIOA181, NIOA302, NIOA325, NIOA329 and NIOA379, associated with green macroalgae, which are shown to produce ulvan lyase for hydrolysis of ulvan and maximum ulvan lyase activity was recorded in strain NIOA181 with a zone of clearance of 25 mm (Table S2).

Phylogenetic characterization of potential polysaccharide hydrolyzing MABs

Phylogenetic analysis was carried out based on the 16S rRNA gene sequences of the 19 potential MABs and each of their closest bacterial taxa was identified using BLASTn sequence similarity (Table S3, Figure 3). The potential isolates were predominately grouped within Phylum Firmicutes and its diversity was represented by the genus *Bacillus* spp. (NIOA29, NIOA34, NIOA181, NIOA183, NIOA284, NIOA321, NIOA323, NIOA329, NIOA388), *Staphylococcus* spp. (NIOA118, NIOA174), *Exobacterium* spp. (NIOA302, NIOA96) and *Oceanobacillus* sp. (NIOA379). It was followed by representatives from Phylum Proteobacteria, which included *Acinetobacter* sp. (NIOA328), *Vibrio* sp. (NIOA169), *Pseudomonas* sp. (NIOA319), *Marinobacter* sp. (NIOA327) and genus *Micrococcus* sp. (NIOA354) belonging to Actinobacteria phylum (Figure 3). Maximum polysaccharide hydrolyzing bacteria (9 isolates) belonged to the phylum Firmicutes of the genus *Bacillus*.

Quantitative estimation of enzyme activity in the potential MABs

Based on qualitative analysis, a total of nineteen bacterial strains which showed very good activity were further characterised for quantitative estimation of specific enzyme activity. These bacterial isolates utilised the algal polysaccharides porphyran, alginate and ulvan as a carbon source by producing the specific enzyme to hydrolyse their glycosidic bonds. The enzyme activity in the potential MABs were estimated to quantify the specific enzymes porphyranase, alginate lyase and ulvan lyase produced by the individual isolates. The porphyranase activity recorded in the MABs was in the range of 61.9 - 208.6 U mg⁻¹ (Table 1). Of the 19 isolates, 8 potential MABs (NIOA181, NIOA284, NIOA323, NIOA327, NIOA328, NIOA354, NIOA379 and NIOA388) showed >130 U of porphyranase activity and the highest activity was shown by red macroalgae-associated bacterial strain NIOA284 with 208.6 U mg⁻¹ of enzyme activity. Similarly, bacterial isolates showed alginate lyase activity between 100.8 to 140.2 U mg⁻¹ and *Acinetobacter* sp., NIOA328, showed the highest activity (Table 1). Due to the complex structural composition of ulvan, the ulvan lyase enzyme is rarely studied; the bacterial isolates in this study showed ulvan lyase activity in the range of approximately 40 - 200 U mg⁻¹ (Table 1). The isolate, NIOA181, a green macroalgal epiphytic bacteria, showed the highest (211.5 mg⁻¹) ulvan lyase activity, identified as *Bacillus subtilis* and selected in this study for enzymatic production of UOS.

Biochemical characterization of ulvan lyase (ULB1) from *Bacillus* sp. NIOA181

The effect of different parameters such as pH (3 to 11), temperature (20 to 60 °C), NaCl concentrations (0.01 to 1 M), different metal ions (10 mM) and chelating agents (0.1%) on the ulvan lyase activity was determined. The maximum ulvan lyase activity was estimated to be at a mildly alkaline pH of 8,

with a decrease in activity at highly acidic (3 - 4) and alkaline pH (10 - 11) (Figure 4a). Optimum enzyme activity was in the temperature range from 30 to 45°C, with maximum activity at 40°C, reduction in activity was observed at low temperature (20°C) and at 50 and 60°C (Figure 4b). Maximum ulvan lyse activity was recorded at the lowest NaCl concentration tested at 0.01 M and a decrease in activity was seen from 0.05 to 1 M (Figure 4c). The effect of different metal ions Li⁺, K⁺, Mg²⁺, Ca²⁺, Ba²⁺, Ni²⁺, Co²⁺, Cu²⁺ and Fe²⁺ tested showed that the ULB1 activity was the highest in the presence of Mg²⁺ (10 mM) which was about 144% in comparison with the control which had no metal ion supplementation. About 120% of activity was recorded in the presence of K⁺, Ca²⁺ and Co²⁺ (Figure 4d). Metal ions such as Li⁺, Ba²⁺, Ni²⁺ and Zn²⁺ showed no impact on the ULB1 activity, whereas Cu²⁺ and Fe²⁺ had a negative effect. Of all the chelating agents tested for their influence on the ULB1 activity, β-mercaptoethanol and DTT had a positive impact, with the former showing maximum activity. Chelating agents Urea, SDS and Triton X-100 had no effect and the activity was almost similar to the control with no addition of chelating agents. However, EDTA, Tween 20, Tween 80 and CTAB negatively influenced the ULB1 activity (Figure 4e). Of the different parameters tested, optimum ULB1 activity was recorded at pH 8.0, temperature 40 °C, 0.01 M NaCl concentration and in the presence of metal ion Mg²⁺ and chelating agent β-mercaptoethanol, which was used for the enzymatic breakdown of ulvan into its UOS.

Structural characterization of enzymatically produced UOS

FTIR spectroscopy analysis

The structural characterization of the UOS was carried out based on FTIR spectroscopy to study their functional groups (Figure 5a). The spectrum shows characteristic peaks at 3280 cm⁻¹ in UOS, which may be attributed to the primary -OH group stretching vibrations. Similarly, the absorption band at 2930 cm⁻¹ can be associated with the -CH stretching vibration. The absorption band at 1417 cm⁻¹ and 1658 cm⁻¹ in the UOS represent symmetric and asymmetric carboxylate (-COO⁻) group stretching. The peaks at 1172 cm⁻¹ and 1080 cm⁻¹ are attributed to C-OH and C-O-C stretching in the pyranose ring of the rhamnose sugar, which is the major monomer of ulvan. The characteristic of uronic acid residues was also observed in the band at 810 cm⁻¹. All these results infer that the enzymatically produced UOS retains its sugar residues and functional groups, similar to the ulvan IR spectrum described in green macroalgae (Lahaye et al., 1999).

HRMS spectrometry analysis

Enzymatically produced UOS was analysed by HRMS to determine the degree of polymerisation, as the oligosaccharides are known to exist in dimer to 20 mers (Figure 5b). From the HRMS spectrum

studied, the highest significant peak at 269 m/z is attributed to Rha3S. The peaks at 295 m/z and 423 m/z are assigned to disaccharides of Rha-Xyl and Δ Rha3S. The prominent peak at 599 m/z and 665 m/z represents the presence of trisaccharides composed of Δ Rha3S-Xyl and Δ Rha3S-Xyl2S, respectively. The peaks at 699 m/z and 733 m/z are assigned to tetrasaccharides constituted by Δ Rha3S-Xyl-Rha and Δ Rha3S- Δ Xyl. The HRMS spectrum of the enzymatically produced UOS using ulvan lyase shows that di, tri and tetrasaccharides were produced from ulvan heteropolysaccharide.

NMR spectrum analysis

NMR spectrum based on ^{13}C was carried out for the enzymatically produced UOS to confirm the identity and configuration of the specific sugar moieties rhamnose, xylose and uronic acid (Figure 5c). The NMR spectrum confirmed the resonance at 96.7 and 95.9 ppm, which is attributed to the presence of α - and β -anomeric carbons in the reducing end of the rhamnose 3 sulfate residue, respectively. Methyl residue signals at 16.7, 16.8 and 16.9 ppm were observed, indicating the presence of L-rhamnose residue in UOS. The peak at 172.78 ppm represents C6 of 4-deoxy-L-threo-hex-4-enopyranosiduronic acid residue. The peak at 103 ppm implies the existence of C1 of β -D-xylose and the multiple peaks between 70-85 ppm represent C2-C5 of rhamnose, iduronic acid and glucuronic acid. These results of ^{13}C NMR analysis confirm the identity of the ulvan-specific sugars in UOS.

Prebiotic potential of enzymatically produced UOS

The prebiotic potential of UOS was compared with ulvan and commercial prebiotic FOS based on the growth profiling of probiotic bacteria, *L. acidophilus*, *L. bulgaricus* and enteric *E. coli*, along with their prebiotic activity score. The results show that the growth of probiotic bacteria, *L. acidophilus* and *L. bulgaricus* in the presence of ulvan, UOS and FOS were significantly different (Figure 6a). The ability of UOS (0.81) and FOS (0.81) to suppress the growth of *E. coli* was significantly equal and higher than ulvan (0.6). Such bioactive compounds, which show the ability to suppress the growth of enteric pathogenic strains of *E. coli* prove to be much efficient in their prebiotic activity. The prebiotic score was also calculated based on the CFU numbers obtained from the growth of probiotic and enteric bacteria in the presence of ulvan, UOS and FOS. The results show that UOS significantly promoted the growth of *L. bulgaricus* and *L. acidophilus* with a prebiotic score of 7.59 and 7.43, respectively, which was higher than ulvan and commercial prebiotic FOS; this was also supported by statistical analysis (Figure 6b).

Discussion

Macroalgae are known to harbor diverse microbial communities, contributing to their growth and defense. Many of these associated microbiomes are known to be the source of various extracellular

enzymes, including the carbohydrase enzymes, which are in huge demand, as they can catalyse the enzymatic breakdown of complex macroalgal polysaccharides into bioactive oligosaccharides (Selvarajan et al., 2019). These low molecular weight oligosaccharides are reported for various bioactivities such as antioxidant, immunomodulation, antiglycemic, anti-inflammatory, antitumor, prebiotic, antiobesity and antimicrobial (Jagtap and Manohar 2021). However, the major bottleneck for the production of these bioactive sugars is the identification of potential microorganisms capable of efficiently hydrolyzing macroalgal polysaccharides into oligosaccharides. Hence there is a continuous need to study the natural habitats for such microbes.

In this study, the MABs were isolated and characterised for their ability to utilize the model polysaccharides representing all three macroalgal groups. The results show that only about 10 – 15% of bacteria could hydrolyse the complex polysaccharides such as porphyran, alginate and ulvan. The bacteria that showed the potential to hydrolyse the unique and complex macroalgal polysaccharides are very scarce and known to contribute to the remineralization of macroalgae after the completion of a growth cycle (Florez et al., 2017; Naik et al., 2019). Studies in the water column of the seaweed ecosystem show the dominance of Firmicutes and Proteobacteria (Kizhakkekalam and Chakraborty 2019; Parvathi et al., 2020). Our study showed the diversity of potential MABs belonging to phylum Firmicutes (68%), followed by Proteobacteria (26%) and Actinobacteria (5%). Molecular tools based on Illumina sequencing and analysis were carried out to understand the diversity of microbiomes associated with macroalgae and described the presence of bacterial taxa belonging to Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Planctomycetes, Actinobacteria and Verrucomicrobia (Selvarajan et al., 2019; Comba González et al., 2021). The application of metagenomic tools for understanding functional diversity can provide more insights into the ecological and biotechnological potential of macroalgae-associated microbial communities (Lin et al., 2018).

Macroalgal polysaccharide hydrolysing activity has been reported from a large number of bacterial and a few fungal isolates, mostly from marine habitats (Jagtap et al., 2022). In our study, MABs, which had the highest porphyranase, alginate lyase and ulvan lyase activity, were reported by taxa belonging to Firmicutes and Proteobacteria. Among these isolates, NIOA181, *Bacillus* sp., and its enzyme ulvan lyase were studied in detail to produce UOS. Various studies have reported the polysaccharide hydrolyzing activity of *Bacillus* sp., which showed their ability to hydrolyse agar, carrageenan and alginate, but there are no reports on ulvan activity (Suzuki et al., 2003; Li et al., 2015, 2019; Chen et al., 2018). Very few studies have reported microbial ulvan lyase activity and enzymatic production of UOS (Table 2). Only one recent study has reported antioxidant and angiotensin I converting enzyme inhibition activity of UOS produced using non-specific enzymes such as amylase,

cellulase and xylanase (Hung et al., 2021). This is the first study, to the best of our knowledge, reporting ulvan lyase activity from a *Bacillus* sp., associated with green macroalgae. Green macroalgae of ulvaceae are mainly composed of sulfated heteropolysaccharide ulvan, accounting for 8-29% of the dry weight of *Ulva* biomass (Gurpilhares et al., 2016). The poor solubility of ulvan due to its high molecular and polydisperse nature, with its complex composition of rare sugars such as rhamnose, uronic acid and xylose, limits its applications in various food, biofuel and pharmaceutical industries (Kidgell et al., 2019). Identification of ulvan lyase producing microbes can be used for efficient utilization of macroalgal polysaccharides, which are not utilized to their full potential.

Structural characterization of the UOS produced using ulvan lyase, ULB1 from MAB isolate, NIOA181 was carried out to confirm its structural composition based on FTIR, HRMS and NMR. The FTIR characterization of enzymatically produced UOS shows functional group-specific to ulvan such as -OH stretching vibration, -CH stretching vibration, -COOH asymmetric and symmetric stretching vibrations, -S=O stretching vibration band, C-O vibration associated with a -C-O-SO₃ and bending vibration of -C-O-S, were detected in UOS at wavenumber 3280 cm⁻¹, 2930 cm⁻¹, 1658 and 1417 cm⁻¹, 1172 cm⁻¹, 1080 cm⁻¹ and 810 cm⁻¹, respectively. These peaks are similar to the earlier reports on UOS produced through enzymatic hydrolysis (Hung et al., 2021), which suggests that the enzymatic hydrolysis of ulvan could not remove the functional group-specific for parent polysaccharides. As ulvan is a polydisperse heteropolysaccharide constituted of repetitive disaccharide units of Rha3S-GlcA, Rha3S-IduA and Rha3S-Xyl (Lahaye et al., 1997). Enzymatic breakdown of ulvan can be achieved via hydrolysis or β -elimination mechanism using polysaccharide lyase (PL). Only a few PL families of ulvan lyase have been reported in the carbohydrate-active enzymes database (<http://www.cazy.org>), including PL24, PL25, PL28, PL37 and PL40 (Li et al., 2020a). PL24 and PL25 family ulvan lyase cleaves the glycosidic bond between GlcA-Rha3S and Rha3S-GlcA/IduA, respectively (Qin et al., 2018; Gao et al., 2019). Furthermore, PL28 family ulvan lyase cleaves the glycosidic bond next to both GlcA and IduA (Ulaganathan et al., 2018). The endolytic cleavage of ulvan through β -elimination using PL24 ulvan lyase from *Alteromonas* sp. (AsPL) forming disaccharides (Δ Rha3S) and tetrasaccharides (Δ Rha3S-Xyl-Rha) composed of unsaturated uronic acid at the non-reducing end (Lahaye et al., 1997; Qin et al., 2020). Similarly, ulvan hydrolysis using ulvan lyase of PL25 family from *Alteromonas* sp. A321 produced disaccharides of Δ GlcA-Rha3S and tetrasaccharides of Δ GlcA-Rha3S-IdoA-Rha3S (Gao et al., 2019). The ulvan depolymerization using ulvan lyase from *Formosa agariphila* KMM 3901T overexpressed in *Escherichia coli* results in the release of unsaturated disaccharides such as Δ Rha3S and a tetrasaccharide of Δ Rha3S-Xyl-Rha as the principal end products (Konasani et al., 2018). The enzymatically produced UOS was characterised to

confirm its structural configuration. FTIR spectroscopy confirmed its functional groups and the ¹³C NMR analysis was used to confirm the identity of the ulvan specific sugars in UOS. Further, mass spectrometry analysis was performed to estimate the sugar moiety based on the molecular weight. It indicated that the end products of enzymatic hydrolysis of ulvan using ulvan lyase ULB1 from NIOA181 were disaccharides of ΔRha3S, trisaccharides of ΔRha3S-Xyl, ΔRha3S-Xyl-2S and tetrasaccharides of ΔRha3S-Xyl-Rha, ΔRha3S-XylΔ with 4-deoxy-L-threo-hex-4-enopyranosiduronic acid at non-reducing end, which was similar to UOS produced using broad-spectrum ulvan lyase, from *Formosa agariphila* KMM 3901T (Lahaye et al., 1997; Konasani et al., 2018). The similarity in unsaturated (4-deoxy-L-threo-hex-4-enopyranosiduronic acid) end products and intermediate products without uronic acid composition confirm that the ulvan lyase ULB1 isolated from NIOA181 cleaves the 1, 4 linkages between Rha3S and GlcA or IdoA through endolytic cleavage.

The bioactive potential of the UOS was also studied based on the prebiotic activity, which was evaluated based on their capacity to increase the growth of probiotic bacteria. The enzymatically produced UOS showed one to two-fold higher activity than ulvan to proliferate the probiotic strains *L. acidophilus* and *L. bulgaricus*, which was equivalent to FOS. The prebiotic activity tested based on their ability to suppress the growth of enteric bacteria showed that UOS was equivalent to commercial prebiotic FOS and its prebiotic score UOS was recorded to be higher than ulvan and FOS. These enzymes are in global demand to hydrolyse the complex polysaccharides to produce bioactive oligosaccharides. The ulvan lyase of MAB, NIOA181, has the ability for enzymatic depolymerization of ulvan for the production of UOS with the prebiotic activity that could potentially be used in the healthcare sector for the efficient proliferation of gut microbiota. These prebiotic compounds produce small chain fatty acids, favoring the growth of probiotics and suppressing oxidative stress caused by enteric pathogens (Hamdy et al., 2018; Guarino et al., 2020). This provides protection against colon infections and inflammation, enhances mineral absorption and lipogenesis and prevents any other gastro-intestinal disorders, including colon cancer (Davani-Davari et al., 2019; Han et al., 2019). Identification and characterization of potential bacteria and its enzyme ulvan lyase in this work can be used efficiently for enzymatic hydrolysis and bioconversion of ulvan containing green macroalgae biomass to produce oligosaccharides with prebiotic activity for various healthcare applications.

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Conclusions: A potential MAB, NIOA181, producing ulvan lyase was isolated and used for the production of ulvan oligosaccharides with prebiotic activity.

Significance and Impact of Study: Rarely studied ulvan oligosaccharides with prebiotic activity can be widely used as an active pharmaceutical ingredient in nutraceutical and other healthcare applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Ashok S Jagtap (ORCID 0000-0003-4089-4718) made substantial contributions to the design of the work and wrote the first draft of the manuscript. Ashutosh S. Parab (0000-0002-5290-8454) performed the phylogenetic analysis and Nitin S. Kadam (0000-0003-1272-9862) characterized the samples and interpreted the results and Cathrine S Manohar (ORCID 0000-0003-0178-7784) contributed to the study conception and critically revised the work. All the authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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TABLE 1 Quantitative estimation of enzyme activity (U mg⁻¹ protein) in potential macroalgae-associated bacteria

Isolates	Porphyrinase	Alginate lyase	Ulvan lyase
NIOA29	61.9 ± 0.4	102.0 ± 5.4	62.7 ± 1.9
NIOA34	80.7 ± 4.8	110.7 ± 11.2	84.7 ± 1.1
NIOA96	67.6 ± 3.5	104.7 ± 9.7	37.7 ± 4.2
NIOA118	101.6 ± 11.0	105.0 ± 12.5	75.5 ± 2.7
NIOA169	77.6 ± 4.6	101.7 ± 11.3	75.4 ± 5.3
NIOA174	121.1 ± 1.0	100.8 ± 9.9	70.8 ± 1.2
NIOA181	174.7 ± 0.0	121.0 ± 1.6	211.5 ± 0.3
NIOA183	128.1 ± 4.3	102.7 ± 12.5	81.2 ± 0.6
NIOA284	208.6 ± 1.7	117.0 ± 3.5	140.1 ± 2.5
NIOA302	89.9 ± 4.3	115.6 ± 5.3	115.1 ± 3.3
NIOA319	81.7 ± 2.9	116.6 ± 12.5	69.3 ± 0.3
NIOA321	87.6 ± 13.4	117.9 ± 10.9	69.3 ± 1.1
NIOA323	190.1 ± 4.5	124.9 ± 3.6	164.3 ± 2.3
NIOA327	178.3 ± 0.7	138.6 ± 3.4	163.1 ± 3.7
NIOA328	170.2 ± 3.2	140.2 ± 2.5	144.4 ± 1.3
NIOA329	100.9 ± 0.3	123.2 ± 2.6	77.9 ± 4.1
NIOA354	182.9 ± 8.5	132.6 ± 2.63	116.7 ± 1.1
NIOA379	148.5 ± 3.8	122.1 ± 1.7	146.6 ± 3.8
NIOA388	132.6 ± 4.9	124.6 ± 8.6	78.8 ± 2.9

TABLE 2 Studies on microbial ulvan lyase from marine sources and characterization of the enzymatically hydrolysed product

Source or enzyme	Degree of polymerization	Bioactivity	Reference
<i>Persicivirga ulvanivorans</i>	DP2 - DP4	-	Collen et al. 2011
<i>Alteromonas</i> sp.	NA	-	Coste et al. 2015
<i>Alteromonas</i> sp.	DP2, DP4, DP6	-	He et al. 2017
<i>Alteromonas</i> sp. LOR	DP2, DP4	-	Foran et al. 2017
<i>Pseudoalteromonas</i> sp.	Di-, Tetra-	-	Qin et al. 2018 Reisky et al. 2018;
<i>Formosa agariphila</i>	Di-, Tetra-	-	Konasani et al. 2018
<i>Alteromonas</i> sp. A321	Di-, Tetra-	-	Gao et al. 2019
<i>Alteromonas</i> sp.	Di-, Tetra-	-	Qin et al. 2020
<i>Glaciecola</i> KUL10	DP2 - DP8	-	Mondal and Ohnishi 2020
<i>Pseudomonas vesicularis</i> and <i>Aeromonas salmonicida</i>	NA	Antioxidant and ACE inhibition activity	Hung et al. 2021
<i>Bacillus</i> sp. NIOA181	Di, Tri and Tetra	Prebiotic activity	This study

FIGURE LEGENDS

FIGURE 1 Sampling locations at the major macroalgal beds of the Indian coast along Gujarat, Maharashtra, Goa and Tamil Nadu coast

FIGURE 2 Percentage frequency of macroalgae associated bacterial isolates with no activity (white), minimum (grey), good (dotted) and very good (black) for hydrolysis of algal polysaccharides (a) porphyranase, (b) alginate lyase and (c) ulvan lyase based on qualitative plate assay

FIGURE 3 Phylogenetic tree showing the position of macroalgae associated bacteria with potential to hydrolyse algal polysaccharides based on the maximum-likelihood analysis of the 16S rRNA gene sequences. Values at nodes indicate bootstrap support from 1000 replicates and scale represents the number of substitution per amino acid

FIGURE 4 Influence of different parameters (a) pH (white bars) (b) temperature (gray bars) (c) NaCl concentration (dotted bars) (d) metal ions (grid bars) e) chelating agents (black bars) on the relative activity (%) of ulvan lyase

FIGURE 5 Structural characterization of enzymatically produced ulvan oligosaccharide from ulvan based on (a) FTIR (b) HRMS and (c) NMR spectrum

FIGURE 6 Prebiotic potential of ulvan oligosaccharides based on the (a) growth profiling of probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and enteric *Escherichia coli* and (b) prebiotic activity score of probiotic bacteria in the presence of ulvan (white bars), ulvan oligosaccharides (grey bars) and fructooligosaccharides (dotted bars); a, b, c represents mean values within a bar with unlike superscript letters shows significant difference ($p \leq 0.05$).

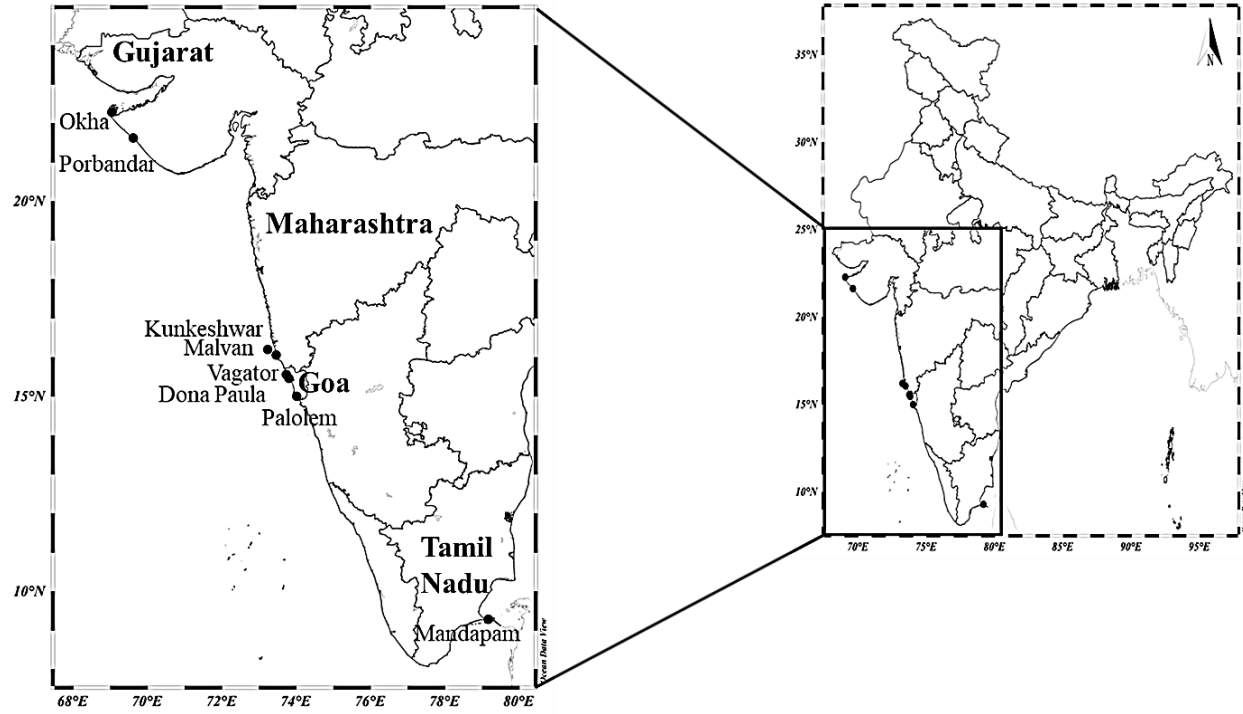


FIGURE 1

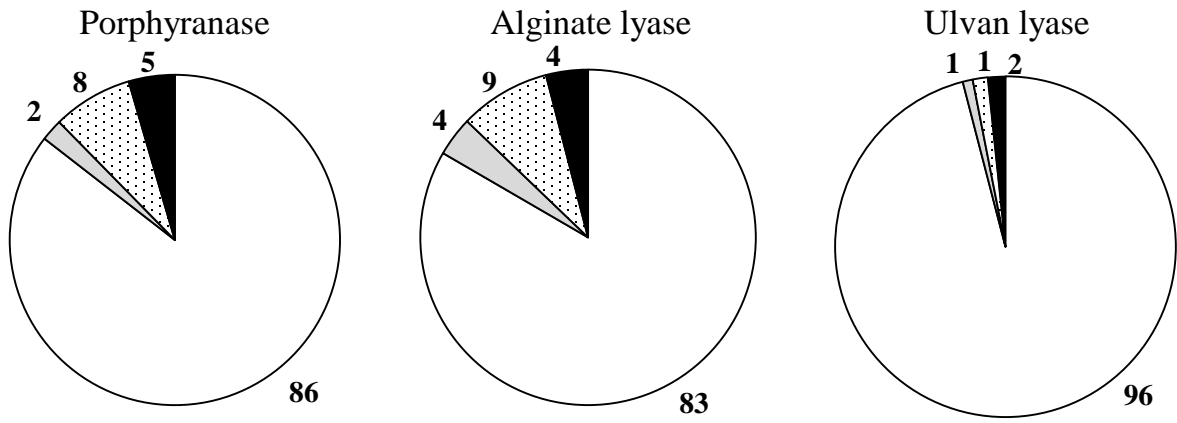


FIGURE 2

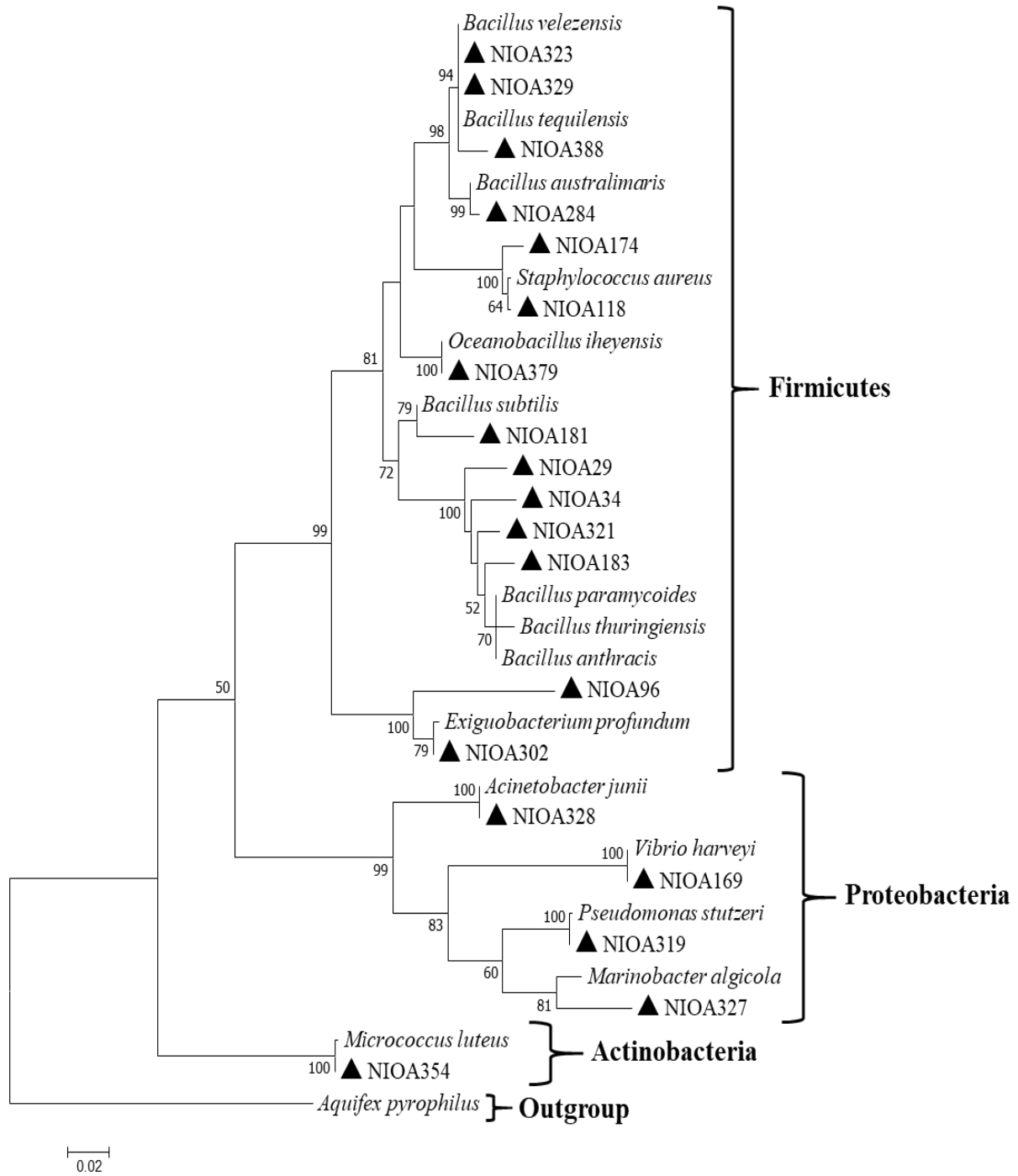


FIGURE 3

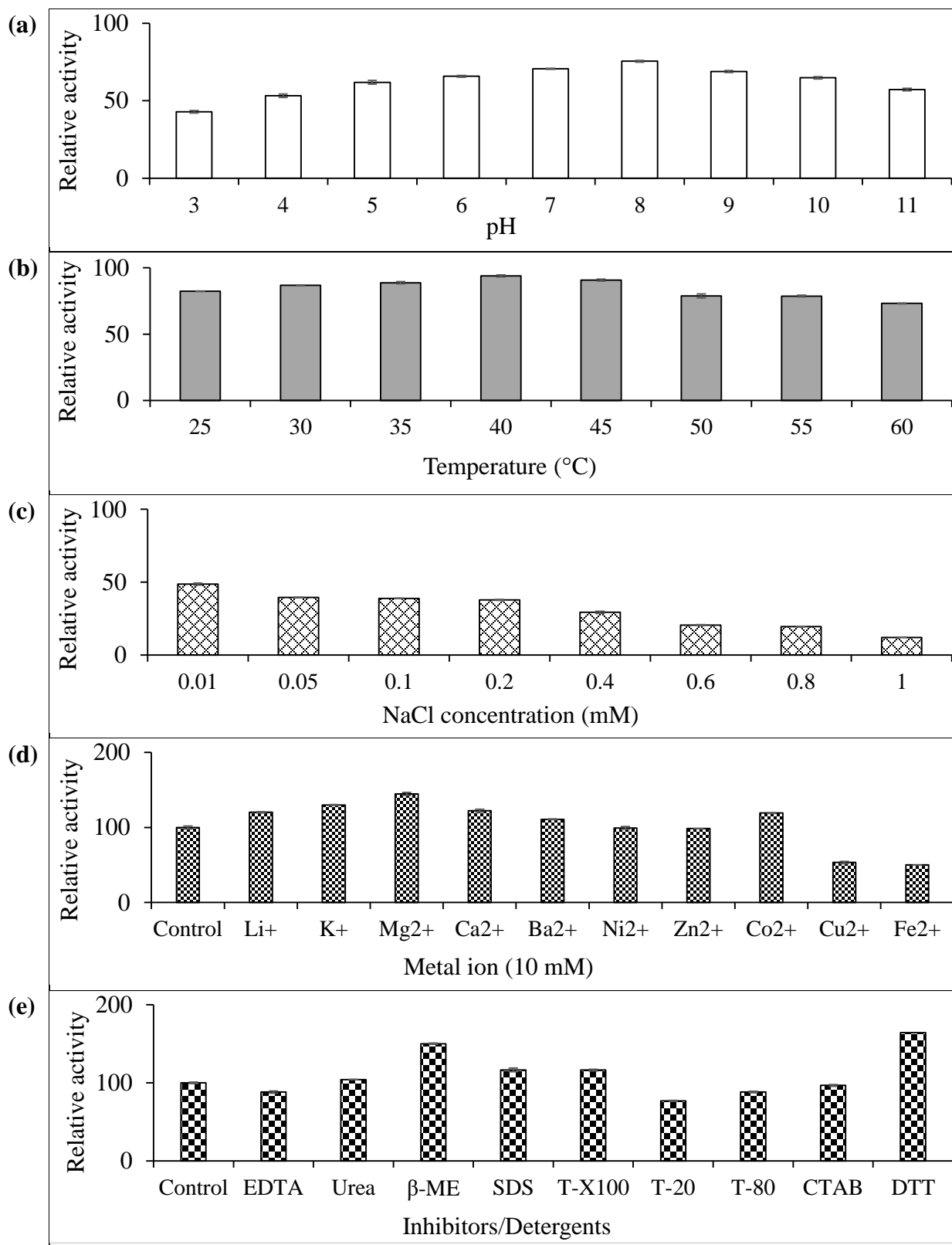
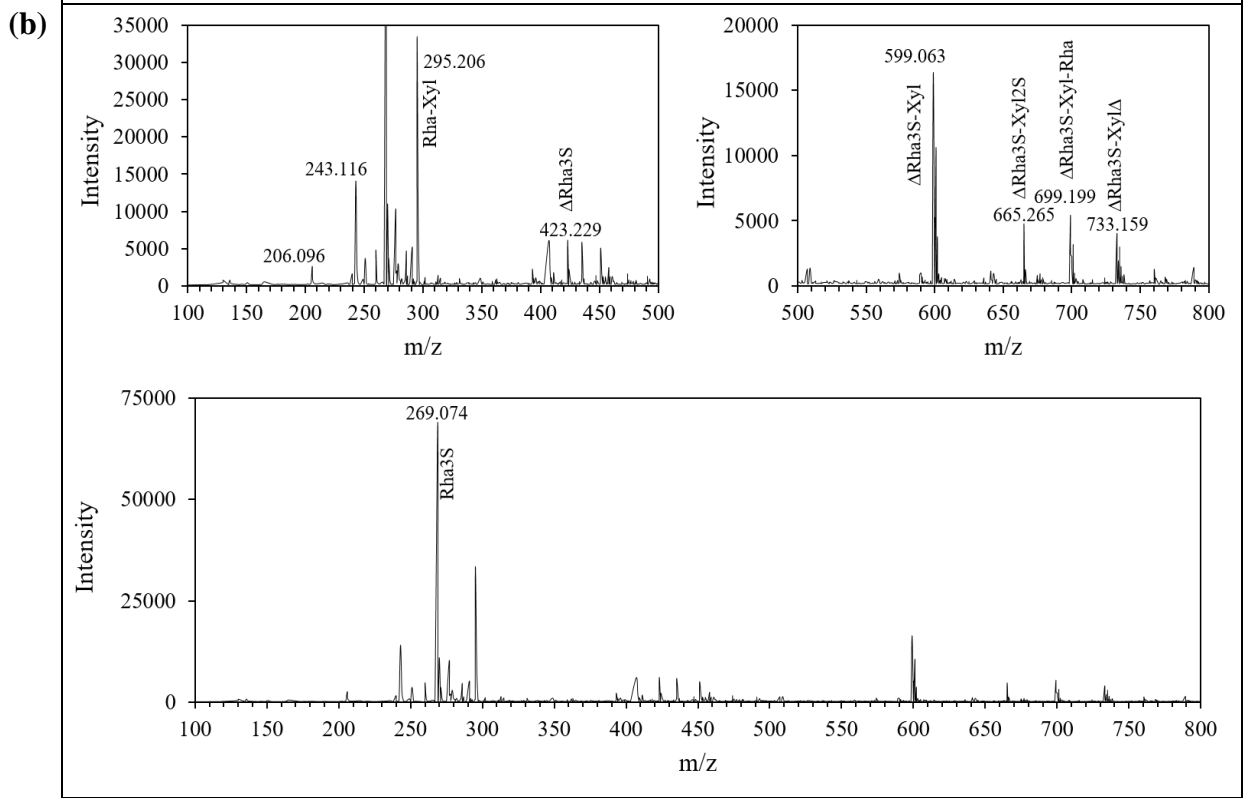
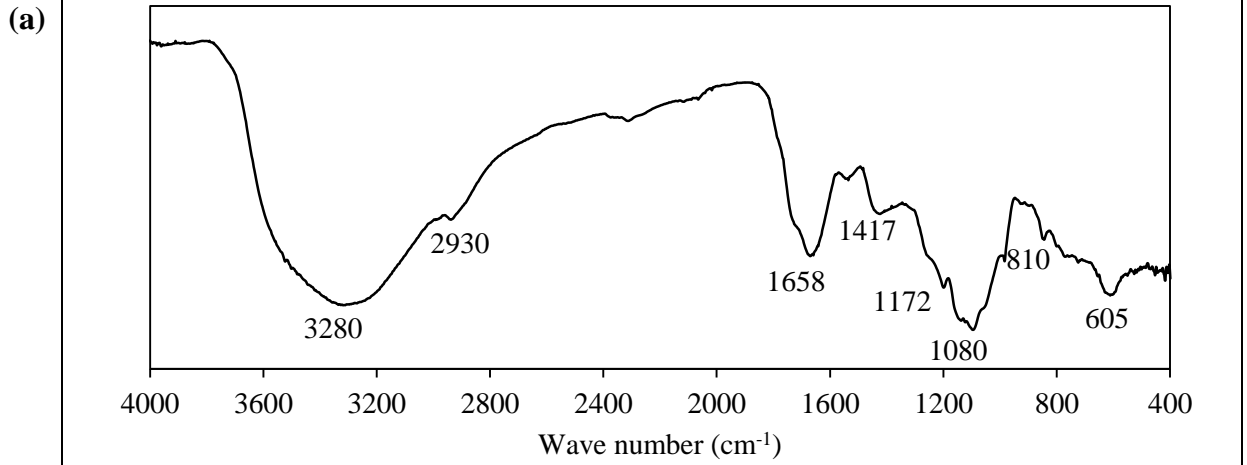


FIGURE 4



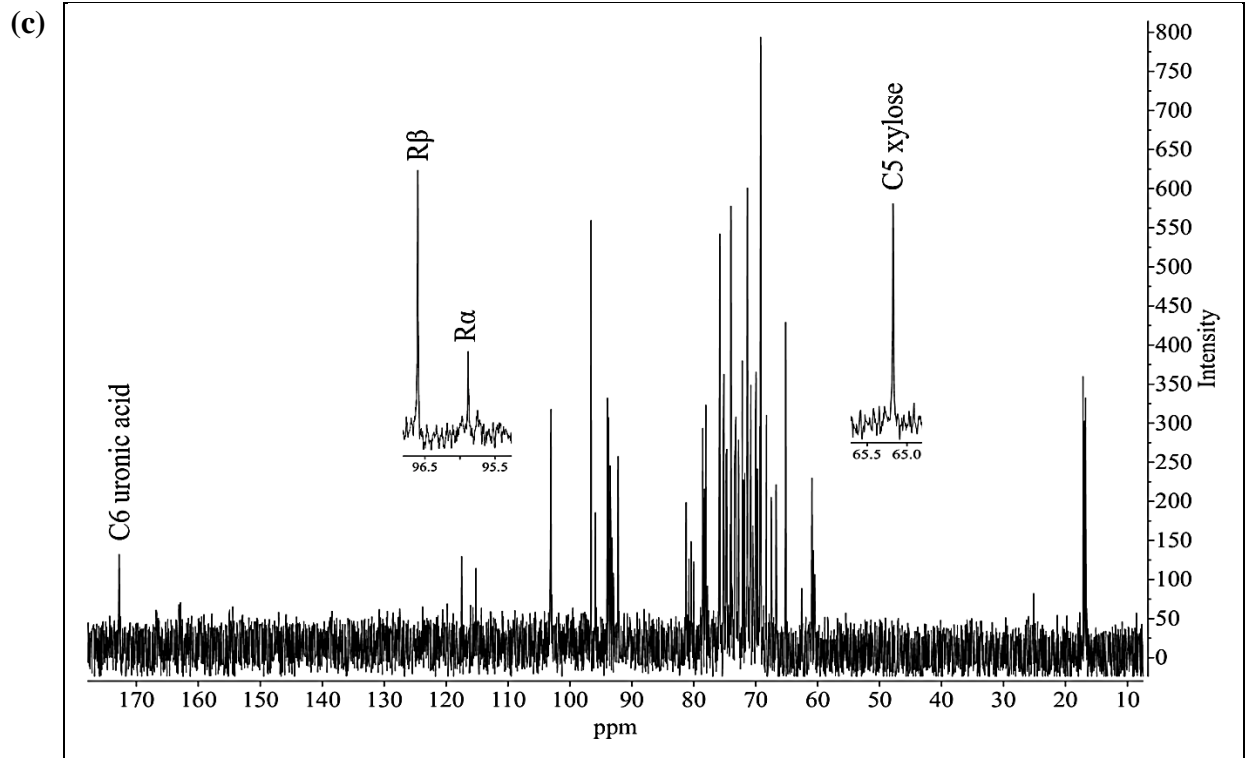


FIGURE 5

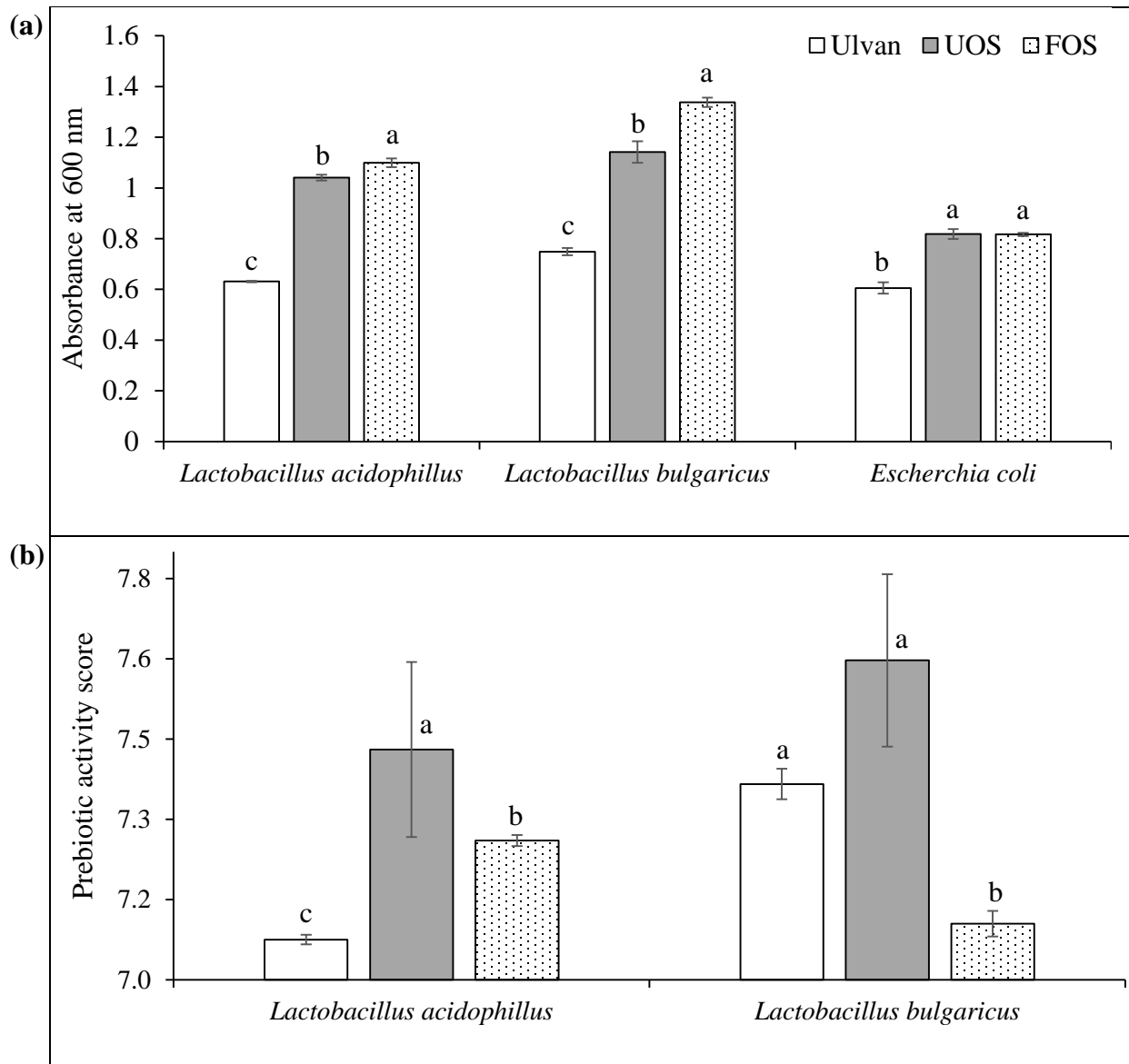


FIGURE 6