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A comprehensive approach to evaluate microplastic biodegradation potential of mangrove rhizobacteria

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Abstract

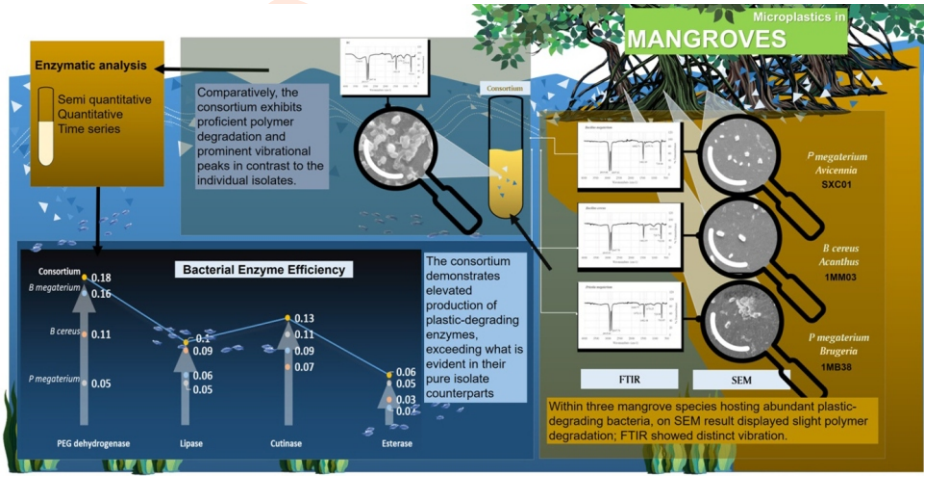
Aim: The study investigates the microplastic degradation potential of mangrove rhizobacteria and their efficiency as a consortium.

Methodology: Rhizosphere sediments were collected from three common mangrove species, *Avicennia* sp., *Acanthus* sp. and *Bruguiera* sp. in Kerala, India. Rhizobacteria were isolated, characterized and a consortium was formulated, which were analyzed for the production of plastic-degrading enzymes. Structural changes in PVC microplastic films treated with individual cultures and consortium were determined by FTIR spectroscopy and Scanning Electron Microscopy.

Results: The most abundant bacteria from each of the three mangrove species were identified as *Priestia megaterium* (SXC01) from *Avicennia* sp., *Bacillus cereus* (1MM03) from *Acanthus* sp. and *Priestia megaterium* (1MB38) from *Bruguiera* sp. Semi-quantitative and quantitative analysis showed that *Priestia megaterium* (SXC01) was efficient in the production of PEG dehydrogenase, *Bacillus cereus* (1MM03) in lipase production and *Priestia megaterium* (1MB38) in esterase and cutinase synthesis. Interestingly, the bacterial consortium showed higher production of enzymes and also exhibited greater stability in their attachment to the PVC surface compared to individual bacterial isolates. Further, FTIR spectral studies revealed pronounced vibrations in the hydroxyl (OH) regions, C-H regions and C-Cl stretching regions, providing evidence of bacterial PVC degradation.

Interpretation: This study highlights the potential of consortium in the effective degradation of microplastics, surpassing individual isolates in enzymatic activity. To mitigate microplastic contamination, mangrove rhizobacteria are important players with potential uses in the restoration and maintenance of mangrove ecosystems.

Key words: Bacterial consortium, Mangrove ecosystems, Microplastic degradation, Rhizobacterial strains



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Introduction

Microplastics are plastic particles less than 5 mm in length that easily contaminate the environment due to their minute size. The microscopic size coupled with ubiquity and slow degradation rates has aggravated the persistence of these microplastics in the environment (Thompson *et al.,* 2004; Chamas *et al.,* 2020). In addition, the interaction of microplastics with other pollutants has resulted in a combinatorial adverse effect on the environment, thus making microplastic contamination the most significant issue presently confronting the globe (Wang *et al.,* 2017; Frias *et al.,* 2010; Mammo *et al.,* 2020; Zhang *et al.,* 2022). The microplastics in the environment include those manufactured for a variety of uses such as microbeads or naturally weathered particles from larger plastic objects which subsequently reach the aquatic bodies (Caruso, 2019; Loganathan and Kizhakedathil, 2022). The primary sources may be runoff from landfills, road-making paints, tyre debris, sewage, agriculture practices, synthetic cloth manufacturing and fragmentation of plastics (Bao *et al.,* 2023). They get heavily accumulated in water bodies, amounting to 150 million tonnes in the marine environment. Additionally, 10 to 20 million tonnes of plastic reportedly enter the oceans annually (Jambeck *et al.,* 2015; Mazhandu *et al.,* 2020; Tursi *et al.,* 2022).

Due to the dispersion of water waves, flow rate, as well as the complexity of the mangrove roots, microplastics aggregate to a significant amount on the ridges of mangroves (Van *et al.,* 2021). Such accumulation further disseminates into the environment by entering the mangrove food web, considerably influencing its functioning (Talukdar *et al.,* 2023). Mangrove ecosystems reside in the coastal zones and are frequently exposed to microplastics due to their structural complexity (Deng *et al.,* 2021). According to the latest report, the current global mangrove distribution is 145,068 km^2, with major contributions from Asia, South America and Australia (Jia *et al.,* 2023). Microplastic pollution has been reported in various mangrove compartments like mangrove surface seawater, sediments and biota (Rochman *et al.,* 2016; Goswami *et al.,* 2020).The presence of microplastics has been reported in the mangrove ecosystems of Jamaica, South Africa, Malaysia, India, Brazil, Iran, Mexico, Indonesia and Singapore (Govendar *et al.,* 2020; Deng *et al.,* 2021; Siahaan and Patria, 2021; Mendes *et al.,* 2023; Cordova *et al.,* 2023).

In India, microplastic pollution has been reported in the Indus, Ganges and Brahmaputra rivers in Northern and Central India; Tamil Nadu and Kerala in South India; marine environment, river ecosystems and Saltpans in Western India; and in East and North-East India, river ecosystems like the Brahmaputra, Hooghly and beaches of Odisha (Chinglenthoiba *et al.,* 2022). According to Gupta *et al.* (2021) and Kumkar *et al.* (2021), microplastic abundance in Indian estuaries ranged from 0.107 particles m^{-3} and 0.41 (± 0.05) to 0.23 (± 0.02) particles l^{-1}. Each year, the Bay of Bengal receives roughly 4 million tonnes of plastic garbage from all the river systems not only from India but also from Bangladesh (Adyel and Macreadie, 2021). Microplastics have also been detected in three different environmental compartments, including soil (933±564 particles kg^{-1}), sediment (1275±532 particles kg^{-1}) and water (101.6 24 particles l^{-1}) from the Mangalavanam Bird Sanctuary, a protected mangrove forest in Cochin, Kerala (Kannankai *et al.,* 2022).

Such reports have also been encountered in the other less protected parts of Cochin mangroves and southwestern India (Shylaja *et al.,* 2021; Valsan *et al.,* 2024). To mitigate microplastics from water, different technologies like physical, chemical and biological methods have been developed (Liu *et al.,* 2023). Although such techniques are available, the complete removal of microplastics from water resources has been inefficient. The use of physical methods is associated with increased implementation costs, while adsorption and chemical treatments cause secondary pollution. In contrast, biological approaches do not cause any environmental issues but have low degradation efficiency (Gao *et al.,* 2022). However, microbial biodegradation, especially using fungi, actinobacteria and bacteria is an effective way to remove microplastics from soil (Avinash *et al.,* 2023; Namasivayam and Avinash, 2023; Namasivayam *et al.,* 2023). Zhai *et al.* (2023) proposed primary processes involved in the microbial decomposition of microplastics, which included, biodeterioration: enzymatic destruction of microplastics, bio-fragmentation: the process of integrating the microplastics into microbial cell walls and bioassimilation: using microbes as a substrate for creating biofilms. Thus, microbes are beneficial in microplastic degradation due to their affordability, versatility and inherent biodegradability (Cai *et al.,* 2023).

These ecosystems are inhabited by a milieu of bacterial communities that hitherto would possess special features to thrive in such environments and could even degrade microplastics. Therefore, utilizing the innate microbial potential offers hope for creating long-term solutions to lessen the negative effects of plastic pollution on the ecosystem. Only a few microorganisms belonging to phyla Proteobacteria, Bacteroidetes and Actinobacteria (Yan *et al.,* 2023; Auta *et al.,* 2022) with the ability to degrade microplastic have been reported in mangrove sediments. Major types of plastic-degrading enzymes that bacteria produce for breaking down plastics are Polyethylene glycol dehydrogenase (PEG), esterase, lipase and cutinase with respective degradation pathways (Ali *et al.,* 2023). In view of the above, this study was conducted to determine the microplastic degradation potential of abundant bacterial isolates associated with the rhizosphere of common mangroves like *Avicennia* sp., *Acanthus* sp., and *Bruguiera* sp., located in Cochin Backwaters, Kerala. The study also aimed to understand the degradation efficiency of consortia of these isolates with enzymatic profile and chemical bond transformation.

Materials and Methods

Sample collection: Sediment samples were collected from the rhizospheres of three abundant mangrove species viz., *Avicennia*

sp., *Acanthus* sp., and *Bruguiera* sp., located in Panambukad island of Cochin, Kerala, India. The samples were collected in a sterile container with a sterile spatula and brought to the Microbiology Laboratory for further analysis (Gottlein, 2006).

Isolation of bacteria: The sediment samples were weighed up to 10 g, suspended in 90 ml of autoclaved ambient water, serially diluted to 10^{-5},100 µl of samples from dilutions were plated onto nutrient agar plates and incubated for 48 hr at 25°C (Clark, 1965). Following incubation, the most abundant bacterial cultures associated with each of the three mangrove species were selected as they would hitherto have a profound role in degrading the pollutants encountered, especially microplastics.

Biochemical characterization: The isolates were subjected to culture-based biochemical identification procedures, including Gram staining and motility, IMViC, TSI agar test, determination of urease, nitrate reduction, mannitol motility, catalase, oxidase, and sugar fermentation using glucose, lactose, sucrose, and maltose (Gerhardt *et al.,* 1981). Based on the results, ABIS software was used to (Sorescu and Stoica, 2021) determine the genera of the organisms.

Molecular characterization: Genomic DNA was extracted from each isolate and purified using the standard phenol-chloroform method (Sambrook and Russel, 2001). The genomic DNA samples were subjected to Polymerase Chain Reaction using the universal 16S rRNA primers (forward: 5'AGATTT GATCMTGGCTCAG3, reverse: 5'CGGTTACCTTGTTAC GACTT3'). The PCR conditions used were: denaturation (95 °C - 2 min, 95°C- 40 sec), annealing (55°C - 40 sec), and polymerization (72°C-1 min 50 sec, 72°C-10 min) for 30 cycles. The amplicons were then electrophoresed in 1% agarose gel and observed under a transilluminator. The PCR products were sequenced using Sanger's technique (Enfys Life Sciences Pvt Ltd., India). NCBI BLAST (Basic Local Alignment Search Tool) was used to carry out a homology comparison of 16S rRNA sequences of three distinct bacterial strains and was identified up to the species level. Enzymatic studies were carried out for all isolates individually and as a consortium.

Pre-consortium analysis and Consortium formulation: The antagonistic activity test was used to check the interaction exhibited between the bacterial isolates in the consortium. A 24-hr-old culture of a selected isolate was swabbed on nutrient agar plates with diffusion wells. About 0.1 ml of another bacterial culture was dispensed into diffusion wells and incubated at 25°C for 24 hr. The presence of a clear zone around the wells indicated inhibition. The above procedure was repeated for different combinations of three isolates. The growth of each bacterial isolate was evaluated in the consortium. About 1 ml of 24-hr-old culture of each bacterial isolate was inoculated into a conical flask containing 20 ml nutrient broth and incubated at room temperature (25°C) for 24 hr. The sample was serially diluted and 100 µl was spread over nutrient agar plates. Based on the colony morphology, the characteristics of colony-forming units of each isolate were recorded. Following the successful pre-analysis of isolates for consortium formulation, the consortium was prepared in 1:1:1 ratio by mixing 1 ml of each of the three 24 -hr- old ($6x10^{-5}$ cfu ml^{-1}) isolates into 5 ml fresh nutrient broth and incubated at 25°C for 24 hr (Irkitova and Grebenshchikova, 2018).

Semi-quantitative analysis of plastic degrading enzymes: The isolated bacteria were subjected to semi-quantitative analysis of plastic degrading enzymes such as PEG dehydrogenase, esterase, lipase and cutinase with their respective special media as follows: For PEG dehydrogenase: 2% PEG was added to a mineral salt medium with Coomassie Blue as an indicator and 1% agar (Rosario and Baburaj, 2017), esterase: media containing 1% Tween 80 (polyoxyethylene-sorbitan monooleate) as a substrate with Phenol Red as an indicator and 1% agar (Plou *et al.,* 1988), lipase: tributyrin agar containing 5 gl^{-1} peptone, 3 gl^{-1} yeast extract, 1.5 g agar with a final pH of 7.5 and Phenol Red as an indicator (Bharathi *et al.,* 2019), cutinase: Nutrient Broth Yeast extract (NBY) agar (0.8% Nutrient broth, 0.2% yeast extract, 1.5% agar and 0.4% tomato cutin) (Gururaj *et al.,* 2021). Each of the three bacterial isolates was spot inoculated on the above media, incubated at 25°C for 48 hr, and the zone of clearance was measured. Similarly, the above analysis was carried out for the consortium as well, and the diameter of the clearance zone was recorded.

Quantitative analysis of plastic degrading enzymes: Quantitative analysis of each enzyme production by the isolates was conducted by culturing in a conical flask containing 50 ml of respective culture media and incubating for 48 hr in a rotary shaker. Further, the samples were centrifuged at 8000 rpm for 15 min and the optical density of the supernatants was estimated for PEG dehydrogenase (λ max =580 nm) (Sedmak and Grossberg, 1977), esterase, lipase, and cutinase (λ max =610 nm) (Gaur *et al.,* 2018) using UV-Visible Spectrophotometer (Systronics, India). The production of enzymes was analyzed for the bacterial consortium also and optical density(OD) was determined.

Time series analysis of enzyme production and growth: Approximately, 1 ml (25×10^{-5} cfu ml^{-1}) of bacterial culture was inoculated into a sterile conical flask containing 25 ml of respective culture media amended with four enzyme substrates under study and incubated at 25°C for 11 days. Subsequently, 4 ml of the culture suspension was centrifuged at 8000 rpm for 5 min at an interval of every 24 hr. The optical density of the supernatant was measured using a spectrophotometer (Systronics, India) to determine the enzyme production. PEG dehydrogenase was estimated at 580 nm wavelength, while esterase, lipase and cutinase were estimated at a wavelength of 610 nm. Further, the pellet was dissolved in fresh nutrient broth, mixed and optical density was read at λ max =600 nm to measure cell density. Thus, enzyme production and cell growth of the individual isolates and bacterial consortium were also performed by repeating the above procedure for eleven days (Gomes *et al*., 2013).

Microplastic film degradation analysis: PVC plastic films were cut into 2x2 mm fragments and transferred to a fresh solution of 70 ml Tween 80, 10 ml sodium hypochlorite and 983 ml distilled water for 60 min (El-Shafei *et al.,* 1998). The strips were washed with distilled water for 1 hr and then placed aseptically in 70% ethanol for 30 min. The pre-treated PVC strips were then placed in five conical flasks with 50 ml of sterile mineral salt media which were further inoculated with 1 ml of consortium, 1 ml of each of the three individual isolates and uninoculated which formed the control. The experimental and control flasks were incubated at 25°C for 30 days and thereafter the plastic films were analyzed using Fourier Transform Infra Red spectrometer Mid IR (Nicolet™ iS50 FTIR Spectrometer, United States) in the spectral range 400–4000 cm^{-1} to observe the structural modifications in the PVC films and Scanning Electron Microscopy (JEOL - Model JSM-6390 Scanning Electron Microscope, Japan) to examine the polymer structure (Akarsu *et al.,* 2023).

Statistical Analyses: One Way ANOVA followed by Bonferroni post-hoc analysis was performed to detect if there was any significant variation in enzyme production between the bacterial isolates and bacterial consortium using JASP software (The JASP Team, 2024). Data generated from the semi-quantitative and quantitative enzyme production as well as the 11-day enzyme degradation studies were subjected to the above statistical analyses.

Results and Discussion

Characterization of bacterial isolates using biochemical tests and 16S rRNA sequencing revealed that the most abundant bacteria from each of the three mangrove species were identified as *Priestia megaterium* SXC01 (GenBank accession number: OR807429) from *Avicennia* sp., *Bacillus cereus* 1MM03 (GenBank accession number: OQ439614) from *Acanthus* sp., and *Priestia megaterium* 1MB38 (GenBank accession number: OQ439612) from *Bruguiera* sp. (Table 1). The above three abundant bacterial isolates were used for the formulation of a bacterial consortium. Various studies have been conducted to develop synthetic bacterial consortiums to evaluate their potential capabilities as consortia (Singh *et al.,* 2023). In a consortium, the activities of the bacterial isolates could be enhanced or reduced depending on the interactions among them due to oscillating environmental conditions. However, the bacterial consortium designed artificially in the laboratories held a chance of developing antagonistic relationships with each other among the constituent bacteria (Hibbing *et al.,* 2010). Thus, pre-consortium studies are required to assess their stability as consortium. There were no inhibitory activities observed among the constituent strains in the present study, and this is corroborated by the absence of clear zones in the agar diffusion experiment. The preconsrotium study on the analysis of growth in the three isolates showed that a stable growth was observed in all the isolates over a period of 6 days (Fig. 1.). The exponential phase of bacterial isolates varied among the isolates under study. The figure unequivocally demonstrates that the growth of all three isolates progressed steadily even in consortium, with *Priestia megaterium* 1MB38 reaching the exponential phase on the second day, *Priestia megaterium* SXC01 on the third day and *Bacillus cereus* 1MM03 on the fourth day.

The enzymatic analysis confirmed that the strains *Bacillus cereus* 1MM03, *Priestia megaterium* 1MB38 and *Priestia megaterium* SXC01 from mangrove rhizosphere sediments were able to produce plastic-degrading enzymes like PEG dehydrogenase, esterase, lipase and cutinase as described earlier from bacteria associated with polluted soil, mangrove sediments and even animal gut (Auta *et al*., 2017; Meng *et al.*, 2021; Wróbel *et al.,* 2023). In addition, semi-quantitative and

Table 1: Characterization of bacterial isolates from mangrove species

Parameters	*Priestia megaterium* (SX01)	*Bacillus cereus* (1MM03)	*Priestia megaterium* (1MB38)
Gram staining	Gram positive rods	Gram positive rods	Gram positive rods
Motility	+	+	+
Indole	-	-	-
MR	+	-	+
VP	-	+	-
Citrate	+	+	+
TSI	+	-	+
Mannitol	+	-	+
Nitrate	+/-	+	+/-
Urease	+	+/-	+
Glucose	+	+	+
Lactose	+	-	+
Sucrose	+	+/-	+
Maltose	+	+	+
Catalase	+	+	+
Oxidase	-	+	-

Table 2: FTIR peaks wavenumber (cm^{-1}) of control sample and biodegraded PVC plastic films

FTIR Peaks wavenumber (cm^{-1})					
Control	**SXC01**	**1MM03**	**1MB38**	**BC**	**Group and Assignmnet**
3375.82	-	-	-	3296.81	O-H stretching - vOH
2915.27	2915.6	2195.39	2915.44	2915.17	C-H Asymmetric stretch - vasCH2
2847.39	2847.61	2847.79	2847.74	2847.61	C-H symmetric stretch - vsCH2
1640.76	1652.71	-	1649.77	1642.66	C=Cbending- vC=C
-	-	-	1472.11	-	C-H bending-δasCH3
1462.66	1462.49	1462.65	1462.48	1462.65	C-H bending -δasCH3
1377.76	1377.71	-	1378.25	1378.41	C-H bending - δsCH3
-	-	1031.9	-	1079.5	vC- C
-	-	729.74	729.98	-	C-Cl stretching - vC- Cl
718.96	719.06	718.9	718.97	719.04	C-Cl stretching - vC- Cl

quantitative analysis of enzyme production by the isolates in the current investigation revealed that all bacterial isolates produced lipase and cutinase most profoundly, nevertheless, strain *Priestia megaterium* 1MB38 was less successful in producing esterase (Fig. 2). *Bacillus* sp. has proved to be a promising microbe in the degradation of microplastics (Auta *et al.,* 2017). In addition, *Rhodococcus* sp. (Auta *et al.,* 2018), *Pseudomonas* sp., *Pandoraea* sp., *Dyella* sp. (Ren and Ni, 2023), *Gordonia* sp., and *Novosphingobium* sp., (Liu *et al.,* 2023) are also known to degrade plastics. With their incredible enzymatic efficiency, bacteria as well as bacterial consortia show different levels of plastic polymer degradation (Lokesh *et al.,* 2023). Generally, plastics are found with either, C-C backbone (polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC)) or heteroatomic backbone (polyethylene terephthalate (PET) and polyurethane (PUR) (Wei and Zimmermann, 2017). In the semi-quantitative examination, the largest zones of clearance were observed for PEG dehydrogenase produced by SXC01 (diameter - 0.4 cm), 2 cm each for lipase and cutinase by all isolates and 1 cm for esterase produced by *Priestia megaterium*. The biofilm-producing bacteria *B. megaterium* has been reported as a potent isolate in the breakdown of polymer membrane on polyether sulfone (PES) (Bhuvaneswari *et al*., 2016). Similarly, *B. cereus* was found to degrade high-density polyethylene and *P. megaterium* polyester and polypropylene (Wróbel *et al.,* 2023). The current study is the first report of *Bacillus* sp. isolated from mangrove rhizosphere sediments showing microplastic degradation potential. The highest optical densities, however, in quantitative analysis were 1.00 for PEG dehydrogenase by *Priestia megaterium* SXC01 and *Bacillus cereus* 1MM03, 0.16 for lipase by *Bacillus cereus* 1MM03, 0.56 for cutinase by *Bacillus cereus* 1MM03, and 0.56 for esterase by Bacillus cereus 1MM03. The results proved that the isolates *Priestia megaterium* SXC01 and *Bacillus cereus* 1MM03 were potent enough to produce PEG which is an important enzyme in microplastic degradation. (Hajighasemi *et al.,* 2019; Kawai, 2005).

According to earlier reports (Lima *et al.,* 2004; Sekhon *et al.,* 2006), *B. megaterium* showed the potential of producing lipase enzymes that were highly stable at high temperatures and in the presence of organic solvents. Similarly, *B. cereus* producing lipase which was stable at varying temperatures, pH and salinity has been described (Mohammed *et al.,* 2022) along with lipase-producing *P. megaterium* (Balasubramanian *et al.,* 2023). *B. megaterium* strains that produced esterase were previously found in soil samples from South Korea (Jung *et al.,* 2003) and *B. cereus* strain was discovered in a hot spring in Bakreshwar, West Bengal, India (Ghati and Paul, 2015). *Salinimicrobium* sp., *Rhizobium* sp., (Kawai and Enokibara, 1996), *Pseudomonas* sp., (Toyama *et al.,* 1995), *Flavobacterium* sp. (Yamanaka and Kawai, 1989), *Sphingomonas* sp., and *Stenotrophomonas* sp., (Hu *et al.,* 2007) have been found to produce PEG dehydrogenase. However, studies on the ability of *Bacillus* sp., and *Priestia* sp., in the production of cutinase are meagre. Interestingly, bacterial consortium surpasses all other values in both quantitative and semi-quantitative analyses compared to the individual strains, as illustrated in Fig. 2.

The 11-day time series analysis of enzyme production and cell growth of three isolates is depicted in Fig. 3. *P. megaterium* (SXC01) was found to generate PEG dehydrogenase with an average OD value of 0.12±0.05, followed by *B. cereus* (1MM03) with an OD value of 0.06±0.04. *P. megaterium* (1MB38) had a high cell density of 0.32, which indicated significant growth in the culture medium. However, the growth of the potent strains *Priestia megaterium* SXC01 was modest, with an OD of 0.16. Strains, *Bacillus cereus* 1MM03 and *Priestia megaterium* SXC01 had similar lipase production with an average OD value of 0.05±0.04 and 0.05±0.02 respectively and a high cell density with an OD of 2.44 in the culture medium. Comparatively, *Priestia megaterium* 1MB38 produced lipase enzymes less efficiently than other isolates. However, esterase and cutinase production by the isolate *Priestia megaterium*

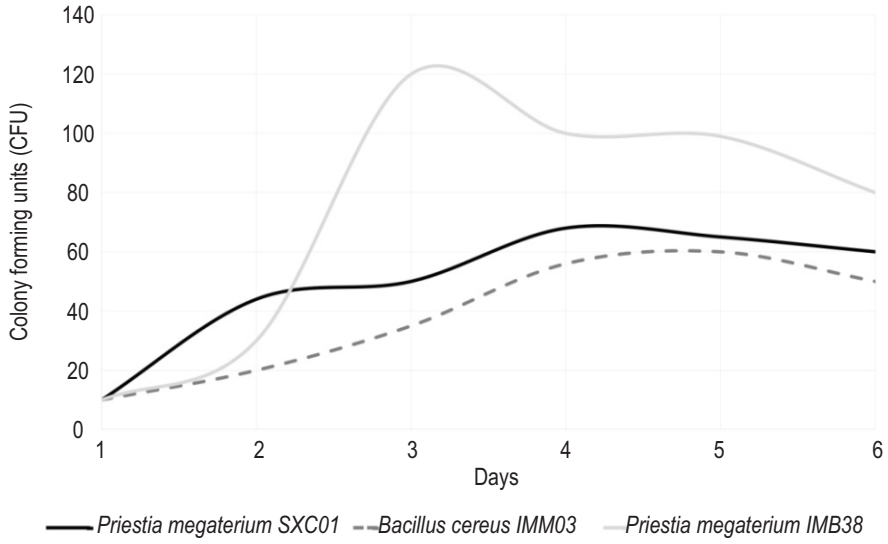

Fig. 1: Growth curve of *P. megaterium, B. cereus* and *P. megaterium* in bacterial consortium.

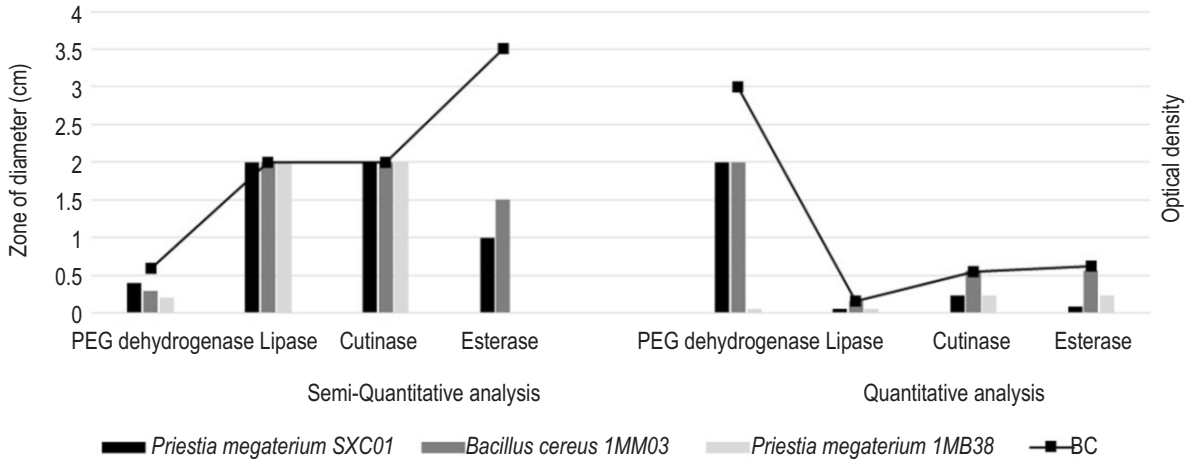

Fig. 2: Semi-quantitative and quantitative analysis of individual strains and bacterial consortium (BC) for plastic degrading enzymes.

1MB38 was higher with an average OD of 0.04±0.02 and 0.09±0.03, respectively. A similar result was found in the case of cell density analysis with OD 3.8 and 0.45, respectively, for the above two enzymes.

Thus in the present study, *Priestia megaterium* SXC01 was effective in the production of PEG dehydrogenase, *Bacillus cereus* 1MM03 in lipase production, and *Priestia megaterium* 1MB38 in esterase and cutinase production which highlight the microplastic biodegradation potential of these bacteria and the role they play in the detoxification of plastics thus supporting the mangrove plants in their survival. Previously, *Bacillus* sp. and *Priestia* sp. have been reported to be plant growth promoting bacteria producing an array of enzymes to help adapt the plant counterpart tolerate challenging ambient conditions (Mohanan *et al.,* 2020, Guzman *et al*., 2022). The time series analysis of enzyme production and growth of bacterial consortium showed that it efficiently produced plastic degrading enzymes in the order, PEG dehydrogenase (OD 0.18)>cutinase (OD 0.13)>lipase (OD 0.1)>esterase (OD 0.06) (Fig. 4). Similarly, the cell densities in

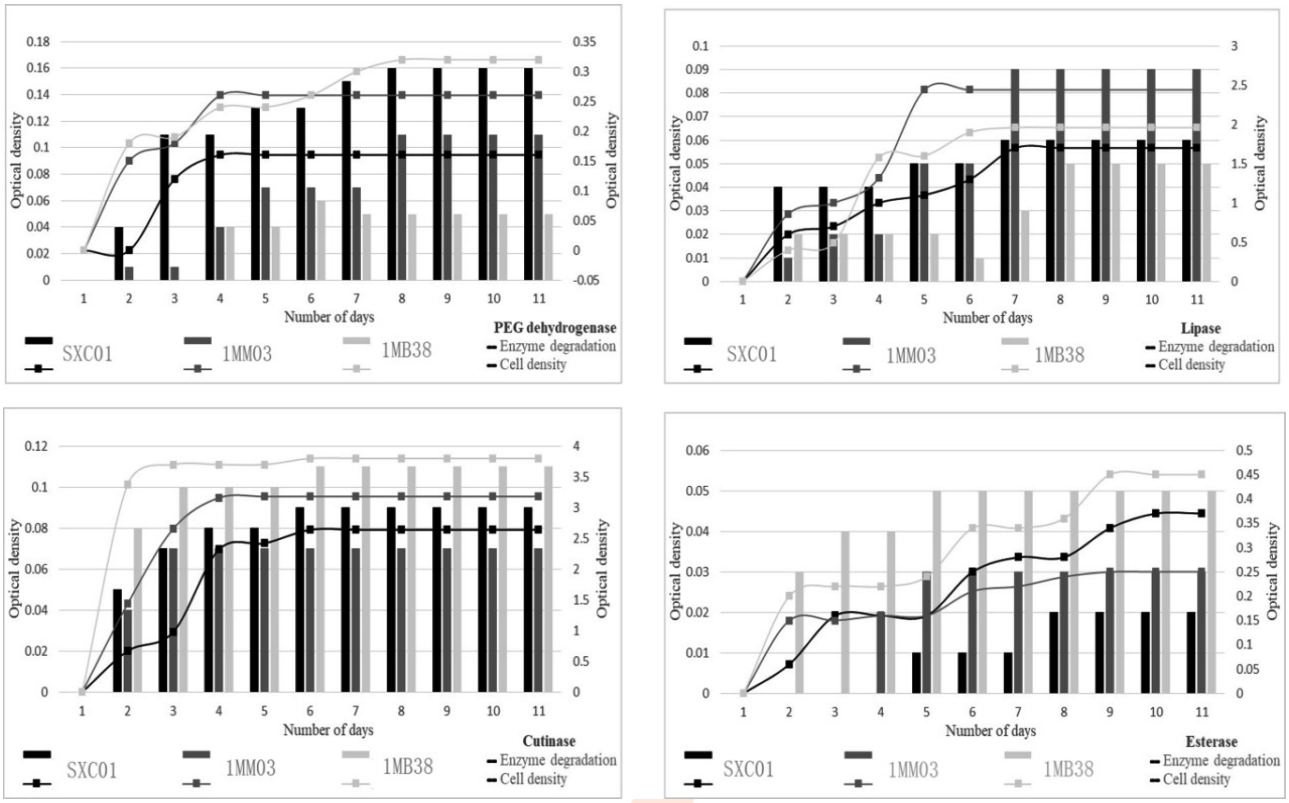

Fig. 3: Enzymatic production (bars) and Growth curve (lines) analysis test result of PEG dehydrogenase, esterase, lipase, and cutinase by *P. megaterium SXC01, B. cereus 1MM03 and P. megaterium1MB38.*

Table 3: ANOVA conducted for semi-quantitative, quantitative and time series enzyme production between individual isolates and bacterial consortium

Analysis	Cases	Sum of squares	df	Mean square	F	p
Semi-	E	21.141	3	4.047	$4.832 \times 10^{+15}$	<0.001
Quantitative	I	5.991	3	1.997	$2.402 \times 10^{+13}$	<0.001
	E x I	13.772	9	1.53	$1.049 \times 10^{+15}$	<0.001
	E	20.099	3	6.7	$5.74 \times 10^{+14}$	<0.001
Quantitative	I	5.774	3	1.925	$1.155 \times 10^{+15}$	<0.001
	E x I	8.914	9	0.99	$8.490 \times 10^{+13}$	<0.001
Time Series	E	0.063	3	0.021	$3.265 \times 10^{+31}$	<0.001
Enzyme	I	0.025	3	0.008	$3.764 \times 10^{+30}$	<0.001
Production	E x I	0.034	9	0.004	$5.889 \times 10^{+30}$	<0.001

Note: F=Fisher's test, p=Significance level, df= Degrees of freedom, E= Enzyme effect (Different in enzyme activity), I= Isolates (enzyme activity across all the bacterial isolates), E x I= Interaction between isolates and enzymes

each respective media were: cutinase (OD 3.72)>lipase (OD 2.68)> PEG dehydrogenase (OD 0.29)>esterase (OD 0.2) (Fig. 4). Statistical analysis using One-way ANOVA showed significant variation in enzyme activity between the bacterial consortium and each of the individual isolates (*Bacillus cereus* 1MM04, *Priestia megaterium* 1MB40 and *Priestia megaterium* SXC01) indicated very low *p*-value (< 0.001) for all the analyses conducted in the present study (Table 3). The post-hoc Bonferroni test confirmed higher enzymatic activity by bacterial consortium than each of the individual isolates ($p < 0.001$).

FTIR spectral analysis revealed that treated plastic films began to degrade by exhibiting shift on the peaks (Fig. 5), which was used to compare the degradation patterns in PVC treated

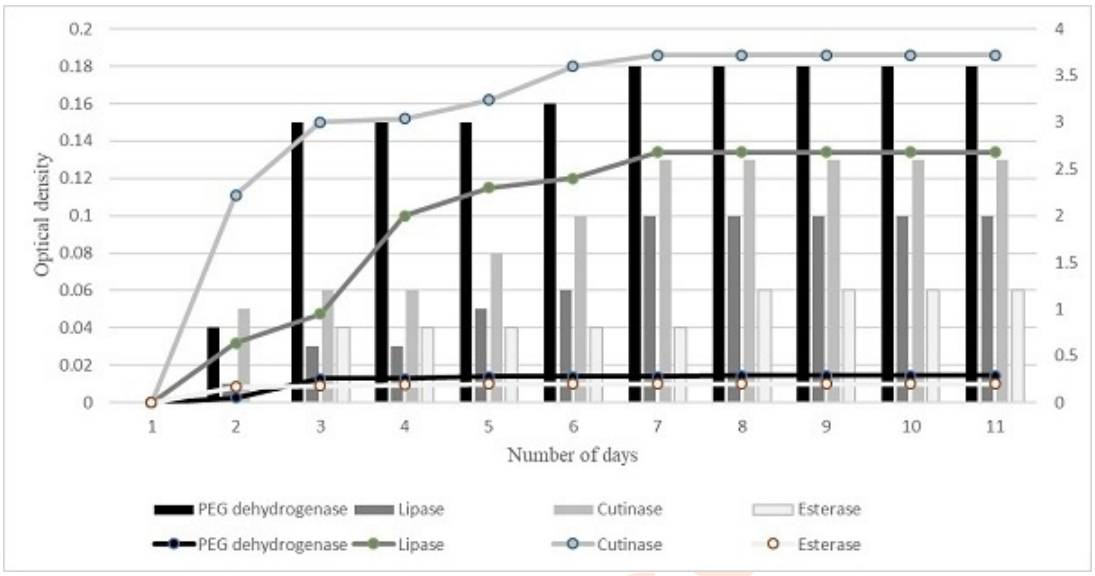

Fig. 4: Enzyme production (bars) and growth curve (lines) analysis by bacterial consortium for PEG dehydrogenase, esterase, lipase and cutinase.

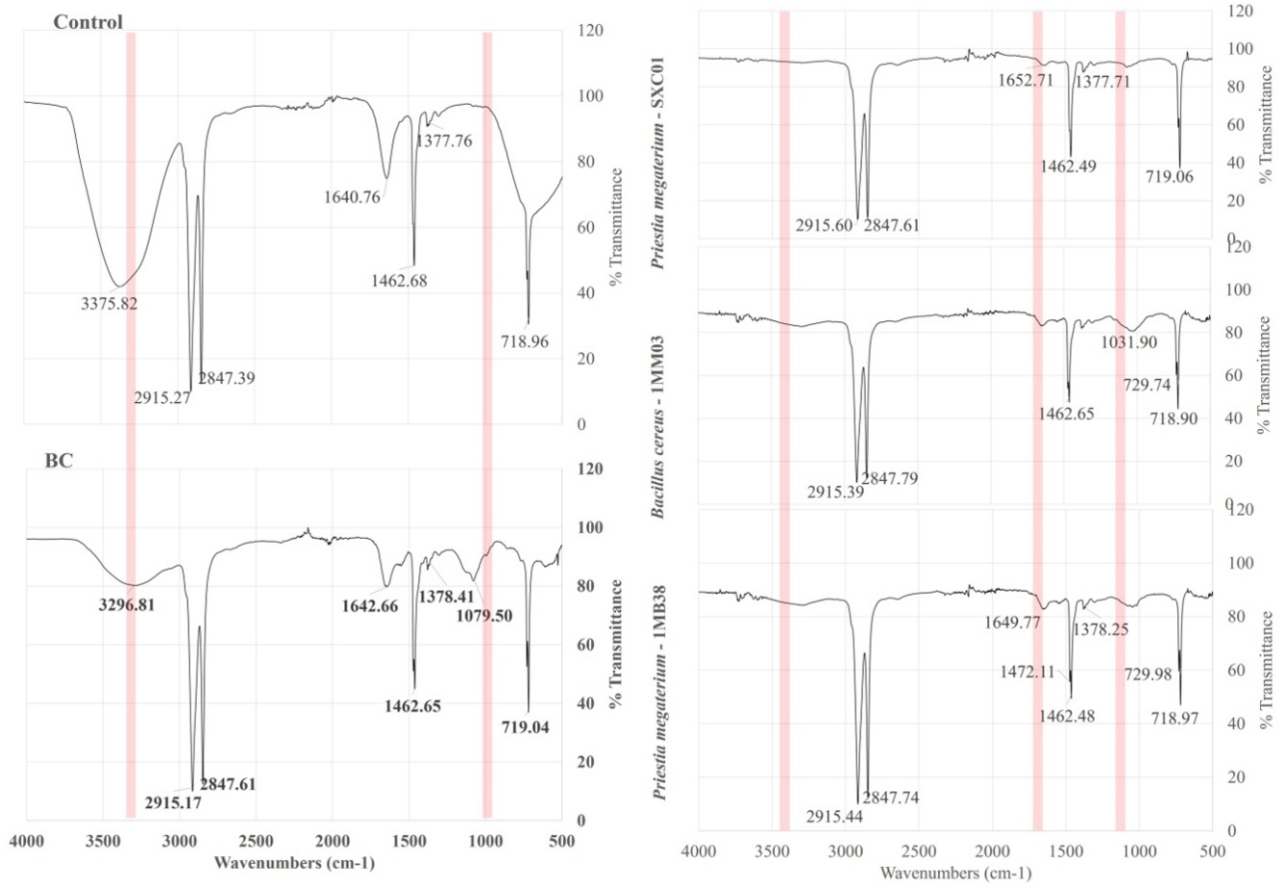

Fig. 5: FTIR data of control and biodegraded PVC plastic films.

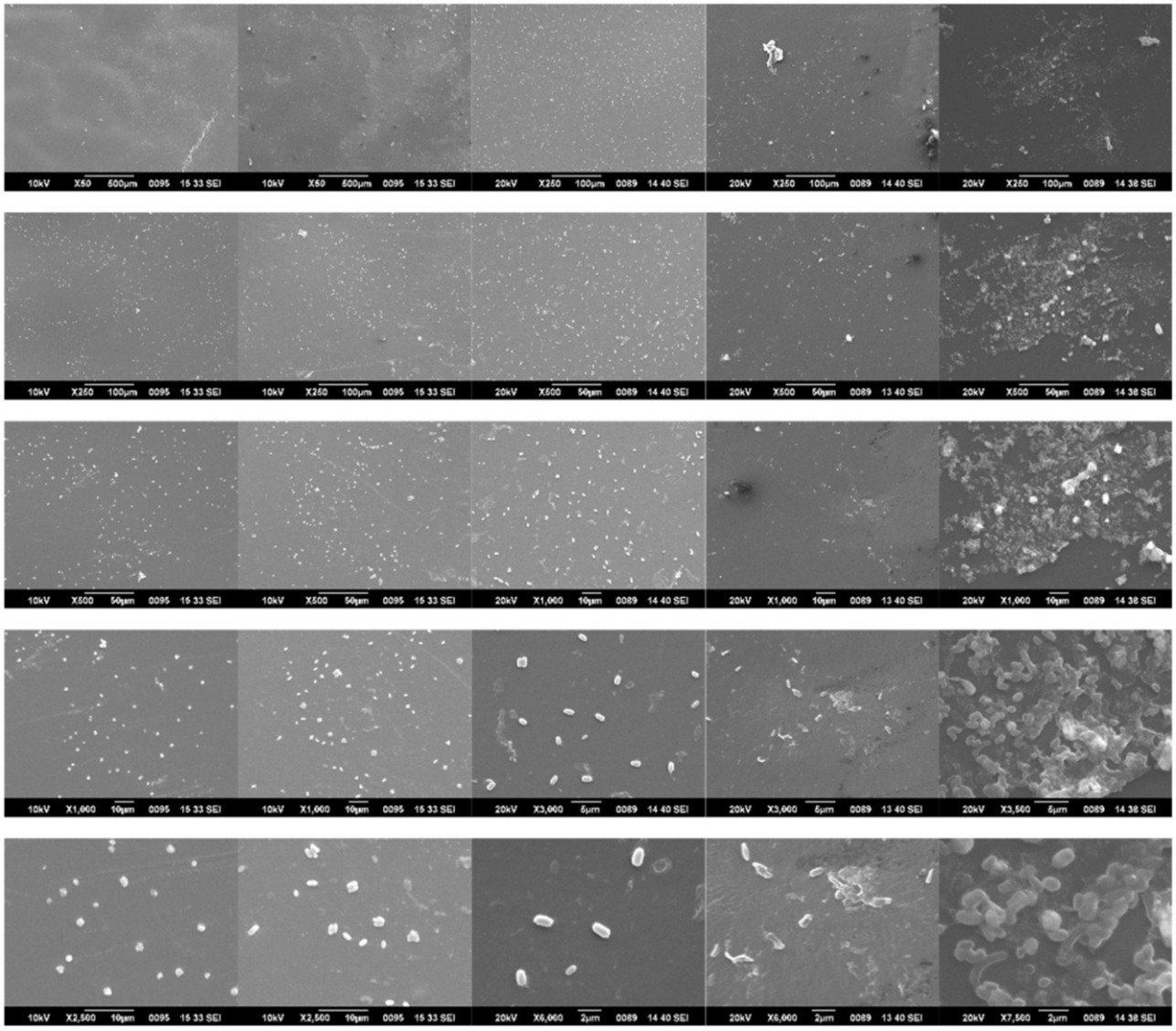

Fig. 6: SEM images of biodegraded PVC by the rhizobacterial strains 1MM03, 1MB38, SXC01, Bacterial consortium (BC), and untreated PVC (Control) after 30 days of incubation.

with individual strains (*Bacillus cereus* 1MM03, *Priestia megaterium* 1MB38 and *Priestia megaterium* SXC01) and bacterial consortium. The degradation of PVC films has been reflected by lowering the wave numbers (cm^{-1}) denoted on each shift when compared with the control sample (Anwar *et al.,* 2016). In the present investigation, a degradation study was conducted after incubation period of 30 days for each sample. Based on the results, FTIR showed different peaks at fingerprint regions and functional groups. Generally, for PVC, these peaks comprised three regions namely, C-Cl stretching region (600-700 cm^{-1}), C-C stretching (900-1200 cm^{-1}), and numerous CH modes (1250-2970 cm^{-1}) (Rajendran *et al.,* 2008). Function-wise for PVC, the first region of spectra 4000 to 1000 cm^{-1} deals with the plasticizer content, and the second region of spectra beyond 1000 cm^{-1} denotes degradation of C-Cl bonds. Furthermore, additional wave numbers on spectra 4000 to 1000 cm^{-1} correspond to OH functional groups and a reduction in the transmittance of C=O (Anwar *et al.,* 2016). Wave numbers (cm^{-1}) of untreated PVC was at 3375.82 (O-H stretching- vOH), 2915.27 (C-H asymmetric stretch-$v_{as}CH_2$), 2847.39 (C-H symmetric stretch- vsCH2), 1640.76 (C=C bending - vC=C), 1462.66 (C-H bending - $\delta_{as}CH_3$), 1377.76 (C-H bending - δs CH3) and 718.96 (vC-Cl) (Fig. 5).

Similarly, for each test sample, the wave number is shown in Table 2 with the functional groups. On comparative analysis, the vibrational peaks of plastic film (PVC) for control sample (3375.82, 2915.27, 2847.39, 1640.76, 1462.66, 1377.76, 718.96) were shifted to (2915.6, 2847.61, 1652.71, 1462.49, 1377.71, 719.06) for *Priestia megaterium* SXC01, (2195.39, 2847.79, 1462.65, 1031.9, 729.74, 718.9) for *Priestia megaterium* 1MM03, (2915.44, 2847.74, 1649.77, 1472.11, 1462.48, 1378.25, 729.98, 718.97) for *Priestia megaterium* 1MB38, and (3296.81, 2915.17, 2847.61, 1642.66, 1462.65, 1378.41, 1079.5, 719.04) for bacterial consortium, respectively. In test samples, the formation of new peaks and shifting indicate structural abruptions due to microbial degradation (Anwar *et al.,* 2013). Each isolate has its signatures in the degradation pattern. Most of the shift caused by bacterial consortium had the lowest wave numbers and two extra peaks in the region of 3296.81 and 1079.5 when compared with the degradation patterns of different bacteria. Hence, the relative potential peaks on FTIR data reflected that the spectral range of PVC biodegradation is in the range of 1700 to 500 cm^{-1} and 3400 to 3000 cm^{-1}. Hence, Fourier transform infrared spectral studies evidenced larger vibration at hydroxyl (–OH) regions and C-Cl stretching regions (Table 2).

Similarly, degradation analysis of PVC degrading bacteria from the insect's gut after 90 days of incubation was documented with large vibration at -OH and -C=C- functional groups (Zhang *et al.,* 2022). Further, slight stretching at hydroxyl (–OH) regions of carboxylic acid in the range of 3300-2500 cm^{-1} was due to the oxidative activities of bacteria. This was reported for *Bacillus* sp., after 90 days of incubation (Kumari *et al.,* 2019). In addition to the above vibrations, decreased chlorine contents were also analyzed in the bacterial consortium test after 30 days of incubation (Xu *et al.,* 2023). The decreased peak intensity at the terminal chlorine group located at 1000–1300 cm^{-1} resulted in dechlorination. This was also reported in marine bacteria *Vibrio* sp., *Alteromona*s sp., and *Cobetia* sp., respectively after 60 days of incubation (Khandare *et al.,* 2021). Further, in a bacterial consortium analysis, the bacterium *Acanthopleurobacter* sp., and *Bacillus* sp., resulted in the reduction of wave number of vC-C-C groups, and another bacterium *Pseudomonas* sp., *Bacillus* sp., *Acanthopleuribacter* sp., and *Bacillus* sp., strains increased shift at v C-Cl, v C-C-Cl and δ C-C-C (Anwar *et al.,* 2016). In contrast, changes were also reported in the range of 1450-1050 by *Pseudomonas* sp., and *Bacillus* sp., after 70 days of incubation (Giacomucci *et al.,* 2019).

Scanning electron microscopy was used to examine the biodegradation of polyvinyl chloride plastic film by the bacterial isolates under study (Fig. 6). After 30 days of incubation, the SEM images revealed the interaction between bacteria and PVC film. *B. megaterium* has previously demonstrated efficiency in degrading polystyrene, exhibiting surface cracks and voids in the PVC film after 30 days of incubation (Meng *et al.,* 2021). In comparison to individual isolates, the consortium exhibited remarkable alterations in the formation of a biofilm and development of fissures on thin PVC sheet. Bacteria isolated from diverse sources which included *Bacillus* sp. *Vibrio* sp., *Alteromonas* sp., *Cobetia* sp., *Enterococcus* sp., *Klebsiella* sp., and *Achromobacter* sp., have been reported to exhibit increased roughness, cracks and deterioration in PVC film surfaces upon SEM analysis (Kumari *et al.,* 2019; Khandare *et al.,* 2021; Zhang *et al.,* 2022; Saeed *et al.,* 2022). Additionally, a consortium comprising *Pseudomonas otitidis*, *Bacillus aerius,* and *Bacillus cereus* isolated from plastic waste displayed cavities with wrinkles on PVC after 70 days of incubation (Anwar *et al.,* 2013). Similarly, another consortium consisting of *Microbacterium* sp., *Pseudomonas* sp., Bacterium Te68R, *Bacillus* sp., and *Acanthopleurobacter* sp., demonstrated significant disruption in the texture of PVC polymer (Sah *et al.,* 2011). Thus, in the present study SEM analysis, revealed bacterial growth on the plastic film surfaces, providing evidence of initiation of polymer degradation.

Microplastics accumulated in marine ecosystems remain an emerging concern in the current era. Mangroves, which form the interphase between land and sea, also face adverse consequences of microplastic pollution. However, bacterial communities thriving in the rhizosphere sediments produce a range of enzymes to facilitate the degradation of microplastics, thus supporting the mangroves. Hence, it is imperative to recognize and harness the potential of these rhizobacteria to address the challenge posed by microplastics worldwide.

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