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Characterization of Bioactive Actinomycetes Isolated from Kadolkele Mangrove Sediments, Sri Lanka

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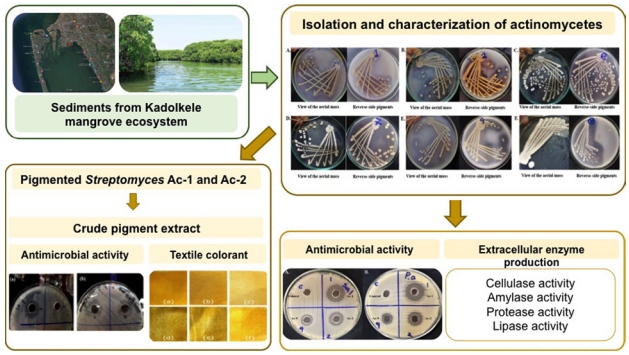
Abstract

Exploring untapped microbial potentials in previously uncharted environments has become crucial in discovering novel secondary metabolites and enzymes for biotechnological applications. Among prokaryotes, actinomycetes are well recognized for producing a vast range of secondary metabolites and extracellular enzymes. In the present study, we have used surface sediments from 'Kadolkele' mangrove ecosystem located in the Negombo lagoon area, Sri Lanka, to isolate actinomycetes with bioactive potentials. A total of six actinomycetes were isolated on modified-starch casein agar and characterized. The isolates were evaluated for their antibacterial activity against four selected bacterial strains and to produce extracellular enzymes: cellulase, amylase, protease, and lipase. Three out of the six isolates exhibited antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus cereus*, but not against *Listeria monocytogenes*. Five strains could produce extracellular cellulase, while all six isolates exhibited amylase activity. Only three of the six isolates were positive for protease and lipase assays separately. Ac-1, Ac-2, and Ac-9, identified as *Streptomyces* spp. with the 16S rRNA gene sequencing, were used for pigment extraction using four different solvents. Acetone-extracted crude pigments of Ac-1 and Ac-2 were further used in well-diffusion assays, and growth inhibition of test bacteria was observed only with the crude pigment extract of Ac-2. Further, six different commercially available fabrics were dyed with crude pigments of Ac-1. The dyed fabrics retained the yellow color after acid, alkaline, and cold-water treatments suggesting the potential of the Ac-1 pigment to be used in biotechnological applications.



K e y w o r d s: mangrove sediment bacteria, bioactive actinomycetes, extracellular enzymes, bacterial pigments

Introduction

The order Actinomycetales of the phylum of Actinobacteria, commonly known as actinomycetes, comprises of filamentous Gram-positive bacteria with high G+C (> 55%) content, which are found ubiquitously in both aquatic and terrestrial habitats. Actinomycetes exhibit considerable diversity and are often placed as an intermediate group between bacteria and fungi (Subathra Devi et al. 2022). Many actinomycetes are saprophytic, aerobic, or anaerobic bacteria and act as an inexhaustible environmental reservoir of secondary metabolites and industrially important enzymes. It makes them one of the most economically, medically, and agriculturally important groups of prokaryotes (Subramani and Aalbersberg 2012; Nawani et al. 2013; Mukhtar et al. 2017; Subathra Devi et al. 2022). Bioactive natural products derived from actinomycetes are extensively used as medically important antibiotics, anti-tumor agents, immune suppressors and vitamins; antibacterial/antifungal agents in agriculture; and for various other biotechnological applications as hydrolytic enzymes, enzyme-inhibitors, etc. (Gomes et al. 2000; Kumar et al. 2019; Pagmadulam et al. 2020; Song et al. 2020). Their ability to decompose plant biomass by producing various lignocellulolytic enzymes and chelate

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soil minerals makes their contribution to improving agricultural productivity and mineral recycling in soil compelling (Saini et al. 2015; Javed et al. 2021; Shanthi 2021). Further, actinobacterial endophytic associations are found to be beneficial to their plant counterparts, as actinobacteria can produce plant growth regulators, solubilize minerals, and fix nitrogen (Franco-Correa and Chavarro-Anzola 2016; Bhatti et al. 2017; Yadav et al. 2018; AbdElgawad et al. 2020).

Among the actinomycetes found in different environments, actinomycetes derived from marine and marine-associated habitats are widely acknowledged for their importance in medical, agricultural, and other industrial applications (Sebak et al. 2019; Hernández-Bolaños et al. 2020; Swarna and Gnanadoss 2020; De Simeis and Serra 2021; Jagannathan et al. 2021). Although most bioactive metabolites currently being used are isolated from different actinomycetes, the discovery of novel compounds from this bacterial group has encountered a decline in the last few decades, compelling the scientific community to explore more habitats for new groups of actinomycetes. Hence, different marine-associated territories have garnered scientific interest. Therefore, with their unique physical and chemical properties, mangrove sediments are often studied as a rich source for novel actinomycetes with the potential to yield new useful products (Law et al. 2020).

Mangrove forests are highly productive, dynamic, and relatively rare ecosystems, mainly confined to coastal areas of the world's tropical and sub-tropical regions (Spalding et al. 2010; Liu et al. 2019). The varying physicochemical parameters of mangrove forests influenced by the tidal actions of the sea throughout the day determine their characteristic biological diversity (Chen et al. 2016; Wang et al. 2019). Marine-derived sediment actinomycetes are well known for their ability to synthesize novel antibiotics with unusual structures and properties than other ordinary soil actinomycetes (Sangkanu et al. 2017; Gong et al. 2018; Hu et al. 2018; Muhammad et al. 2022). This property has been associated with the harsh living environment with high salts concentrations they inhabit producing these secondary metabolites. These compounds have the potential to exhibit their bioactive properties under extreme conditions, making them beneficial in various medical and biotechnological applications (Jensen et al. 2005). Actinomycetes, producing many different antimicrobial compounds, have been isolated from different mangrove sediments in India (Baskaran et al. 2011; Sengupta et al. 2015). Other than antimicrobial compounds, various bioactive compounds like anti-tumor and antiviral agents, antioxidants, enzymes, and anti-fibrotic agents have been isolated from mangrove actinomycetes (Xu et al. 2014; Azman et al. 2015; Indupalli et al. 2018).

Sri Lankan mangrove systems are interspersed along the coastline of the country and are estimated to cover an area of more than 15,000 ha (Edirisinghe et al. 2012; Arulnayagam et al. 2021). Although Sri Lankan mangroves have been extensively studied for their faunal and floral diversity (IUCN 2010; Amarasinghe and Perera 2017; Arulnayagam et al. 2021; Fernando et al. 2022), mangrove sediment microbiology is a relatively new field in Sri Lanka and a largely untapped area for the isolation of new microorganisms that can produce new active secondary metabolites. In the present study, six cultivable actinomycetes isolated from Kadolkele mangrove sediments associated with the Negombo lagoon, Sri Lanka, have been characterized using their phenotypic and genotypic characteristics. Further, these isolates' extracellular enzyme production and antibacterial activities have been explored, along with the potential use of the pigments produced by selected isolates in biotechnological applications.

Experimental

Materials and Methods

Sampling sites and collection of mangrove sediments. Mangrove sediments were collected from 'Kadolkele' mangrove forest associated with the Negombo lagoon area in May 2018. The sampling area is maintained by the NARA (National Aquatic Resources Research and Development Agency) – Regional Research Center, Sri Lanka. Two mangrove sediment samples were collected from two sites (7°11′44.6″N 79°50′38.0″E and 7°11′46.3″N 79°50′37.3″E), and at each site five surface sediment samples were collected at 0–15 cm depth within an area of 1 m^2 and homogenized to obtain a composite sample. Sediment samples were separately collected into sterile polythene bags using sterile scrapers. Collected samples were transported to the laboratory on ice and stored at 4°C until further processing.

Isolation of actinomycetes from mangrove sediments. A suspension of mangrove sediment was prepared by using a sediment soil sample (10 g) and sterile distilled water: sterilized mangrove water (1:1 v/v) mixture (100 ml). The mangrove water obtained from the respective sampling site was sterilized and used to prepare the suspension of each sediment sample. The resulting sediment suspension was shaken well and was serially diluted up to 10^{-7} using sterile distilled water: mangrove water (1:1 v/v) mixture as the diluent. Seawater (50% v/v) – Starch Casein Agar (SCA) plates supplemented with 80 μg/ml cycloheximide and 75 μg/ml nalidixic acid were inoculated with 0.5 ml of each serially diluted sediment sample, separately. Plates

were incubated at 28°C for 1–2 weeks, and typical actinomycete colonies were isolated with three consecutive single colony isolations.

Morphological identification of actinomycete isolates. All isolated actinomycetes colonies were tested for Gram's reaction and catalase test. Their aerial mass color and reverse-side pigments were recorded. The starch casein agar was poured on a sterile slide to obtain a thin agar layer and allowed to solidify. Then, the actinomycete cultures were streaked separately and incubated at 30°C for 2–3 days. After the appearance of growth on the slide-agar layer, several drops of methylene blue dye were added to the growth and observed under the microscope.

Antibacterial activity of actinomycete isolates. The actinobacterial isolates were screened for their antibacterial activity using the previously described 'cross streak method' (Lemos et al. 1985). A single streak of the isolate was made on Mueller-Hinton agar plates and incubated at 30°C for 2–4 days. After observing a plentiful ribbon-like growth of actinomycetes on the plate, four selected bacterial pathogens, namely *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 10876), and *Listeria monocytogenes* (NCTC 11994) were cross-streaked near the actinobacterial growth. After incubation at 30°C for 24–48 h, the presence of inhibition zones near the growth of actinomycete was recorded.

The actinobacterial isolates that showed any antibacterial activity in the cross-streak assay were selected and grown in starch-casein broths at 30°C at 100–150 rpm for 5–7 days. After the incubation, 2 ml aliquots of each actinobacterial culture ($OD_{405} \approx 0.2$) were centrifuged at 10,000 rpm for 10 minutes, and the resulting supernatant was filtered. The cell-free supernatant of each selected actinobacterial culture was tested against five different test pathogens (*Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *B. cereus* ATCC 10876, and *P. aeruginosa* ATCC 27853) using standard well-diffusion assay (Baskaran et al. 2011). Briefly, separate bacterial lawns were prepared on Mueller-Hinton agar plates with overnight grown bacterial pathogens ($OD_{500} \approx 0.6$), and wells were made in agar medium using a sterile cork-borer. The cell-free supernatant of each actinobacterial isolate (100 µl) was added to a separate well against each test pathogen. After the incubation at 30°C for 24–48 h, the diameter of the inhibition zone around each well was measured. The diameter of the zone was measured, across the center of the well. The average diameters of the inhibition zones were calculated using three independent replicates for each cell-free supernatant.

Extracellular enzyme production by actinomycetes isolates. ***Cellulase activity.*** The isolated actinomycete strains were screened for the production of cellulase by inoculating them. The organisms were spot inoculated on Carboxymethyl Cellulose agar medium (CMC) and incubated at 28°C for seven days. After the incubation, the presence of clear zones was observed by flooding the growth with 1% Congo red dye (w/v) followed by 1N HCl for 15–20 min.

Amylase activity. Starch casein agar plates were prepared, and the isolated actinomycetes were spot inoculated onto the medium. The cultures were incubated at 28°C for 7 days. The plates were flooded with Gram's iodine stain after the incubation and left for 5 minutes to observe the presence/absence of clearance against the dark blue color background.

Protease activity. Casein agar plates were spot inoculated with isolated actinomycete strains, and the plates were incubated at 28°C for four days. The presence/absence of clear zones was observed by flooding the growth with 0.0015% (w/v) bromocresol green.

Lipase activity. Tween 20 agar plates were spot inoculated with the actinobacterial isolates, and the plates were incubated at 28°C for seven days. After incubation, the bacterial growth was flooded with $CaCl_2$ solution (0.01% w/v), and the presence of a white precipitate surrounding the microbial growth was recorded as a positive result.

Statistical analysis of data. The results of enzyme assays were statistically analyzed using one-way ANOVA and Tukey's pairwise comparison using Minitab 17.1.0.

Molecular characterization of selected actinomycete isolates. For the molecular characterization of the selected actinobacterial isolates, their genomic DNA was extracted using the CTAB method (Magarvey et al. 2004). The 16S rDNA sequence analysis was carried out using fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGT GATCCAGCC-3') universal primer pair. The PCR reaction mixture contained the DNA template (5 µl), Master mix (Promega, USA) (12.5 µl), forward and reverse primer (5 µM each), and nuclease-free water. Amplifications were performed in an Eppendorf Mastercycler Nexus. The thermal cycle consisted of an initial denaturation at 94°C for 10 minutes, followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 90 s, followed by a final extension of 72°C for 10 minutes. The presence of the correct PCR products was confirmed using 1% agarose gel electrophoresis, and the amplified product from each isolate were subjected to bidirectional sequencing using 27F and 1100R universal primers at Macrogen (South Korea). The partial 16S rRNA gene sequences of the selected isolates were compared against the available sequences in the GenBank through BLAST searches. The partial 16S rRNA gene sequences of the actinomycetes isolates Ac-1, Ac-2, and Ac-9 can be accessed under the GenBank accession numbers OL638154,

OL638155, and OL638156, respectively. BioEdit v 7.2.5 (http://bioedit.software.informer.com) was used for the sequence alignment. The neighbor-joining phylogenetic method with default parameters was used to construct the bootstrap – NJ tree. The phylogenetic relationships were inferred with MEGA 7 (Kumar et al. 2016).

Optimization of the growth of pigment-producing actinomycete isolates. Starch casein broth (100 ml) in a 250 ml Erlenmeyer flask was inoculated with the actinomycetes culture and incubated for seven days at 100 rpm on a rotary shaker. A 100 µl-fraction from the overnight culture was transferred to the following sets of culture broths to estimate the growth of pigmented bacteria under different growth conditions.

For the determination of the aeration effect, two sets of starch casein broths (25 ml) in 100 ml Erlenmeyer flasks were inoculated as per the above with the actinomycetes culture. One set was incubated on a rotary shaker at 100 rpm, and the other set was incubated under stationary conditions at room temperature. The significant difference between the dry weights of the biomass resulting from the growth with and without shaking was evaluated separately using a two-tailed, unpaired *t*-test, and *p*-values were calculated.

To determine the effect of different salinity levels on actinomycetes growth, five sets of starch casein broths (25 ml), additionally supplemented with the different concentrations of sodium chloride (0%, 2%, 4%, 6%, and 8% w/v) were used. Similarly, the initial pH of four sets of starch casein broths (25 ml) in 100 ml Erlenmeyer flasks was separately adjusted at 3, 5, 7, and 9. Inoculated flasks were incubated at room temperature on a rotary shaker at 100 rpm. The growth was estimated after seven days of incubation by measuring the dry weight of microbial biomass.

Extraction of crude pigments from actinomycete isolates. Pigmented actinomycete isolates were grown in a 250 ml Erlenmeyer flask containing 100 ml of starch-casein broth for 5–7 days at room temperature on a rotary shaker at 100 rpm. The cells were harvested by centrifuging the cultures at 10,000 rpm for 20 min. The supernatant was discarded, and pellets were resuspended in 95% alcohol, ethyl acetate, acetone, and chloroform separately to determine the suitable solvent for the solvent extraction of the intracellular pigments. Based on the colorimetric analysis of the extracted pigments, acetone was selected as the suitable solvent for the extraction of pigments (data not given). Pellets were re-suspended in acetone and vortex-mixed to obtain cell suspensions. The suspended cell pellet was centrifuged at 10,000 rpm for 10 minutes, and the resulting debris was discarded. The supernatant was collected and filtered through a membrane filter (0.45 µm) to remove any remaining debris. The crude pigment extract was used for the preliminary characterization.

Antibacterial activity of the crude pigment extracts. To determine any possible antimicrobial activity of crude pigment extracts of actinomycetes, the standard well-diffusion method described above was performed against selected test bacterial strains (*E. coli*, *S. aureus* and *P. aeruginosa* – growth standardized using OD_{600}) on Mueller-Hinton agar. The wells were filled with 100 µl of crude pigment extracts. The solvent used for the pigment extraction was used as a control of the tests. The antibacterial activity of each crude pigment extract was determined by measuring the diameter of the zone of inhibition after 24–48 hours of incubation at 30°C.

Screening for the potential industrial applications of actinomycetes pigments. ***As a fabric colorant.*** Six different types of fabrics (satin, polyester, terin, poplin, silk-cotton blended fabric, and Khushubu) were used to determine the potential effect of microbial pigments on dyeing fabrics. Before dyeing, the scouring process was carried out to remove impurities in the fabric and increase the pigment's absorbance. Fabrics were treated with soap water (18 ml/l) and 25% w/v sodium carbonate in hot water, and then the mixture was heated to 60°C. In a conical flask, the fabrics were washed with water at ambient temperature and then dyed with pigment extracts (40 ml of pigment extract : 1 g of fabrics). The flask containing pigment extract and fabrics was heated at 80°C for 30 mins to better adsorb the pigment (Morales-Oyervides et al. 2017).

Results

Isolation and preliminary characterization of actinomycetes. Dry, powdery colonies with filamentous appearance grown on the modified-SCA plates were selected according to their colony morphologies and purified through a series of single colony isolations. A total of six actinomycetes of different colony morphologies were isolated from the two composite samples obtained from the two selected sampling sites of Kadolkele mangrove sediment, Sri Lanka (Table I). All the isolates were Gram-positive with filamentous cellular morphology and gave positive results for the catalase test. The aerial mass color and the reverse side pigment color of isolates were recorded (Table I and Fig. 1). Isolates Ac-1, Ac-2 and Ac-9 had whitish-grey aerial mass color and produced different shades of yellow-colored reverse side pigments. The isolates with white color aerial mass did not show any pigment production. Ac-1 and Ac-9 produced open, spiral-type spore chains, while Ac-2 spore chains were biverticillate with spirals. Flexuous spore-bearing structures were observed for Ac-6 and Ac-7 isolates (Table I and Fig. 2).

Extracellular enzyme production. The ability of the actinomycete isolates to produce four extracel-

Table I
Characteristics of actinomycete isolates.

Isolate name	Colony morphology		Gram's reaction	Spore chain morphology	Catalase production
	Aerial mass color	Reverse side pigment color			
Ac-1	greyish white	yellowish green	+	open spirals	+
Ac-2	greyish white	yellowish orange	+	biverticillate with spirals	+
Ac-6	white	–	+	flexuous	+
Ac-7	white	–	+	flexuous	+
Ac-9	greyish white	yellowish grey	+	open spirals	+
Ac-10	white	–	+	pleomorphic bacilli	+

lular enzymes: cellulase, amylase, protease, and lipase, was evaluated separately. The 'Enzymatic Index' of the strains for the production of each enzyme was expressed as a ratio between the average diameter of the enzymatic activity zone and the average diameter of the colony (Fig. 3). Five out of the six actinomycete strains were able to produce extracellular cellulase, while strain Ac-10 produced no clear zone on CMC medium when flooded with Congo Red and HCl after the incubation. All seven isolates exhibited amylase activity, whereas the highest amylase activity was observed with Ac-6.

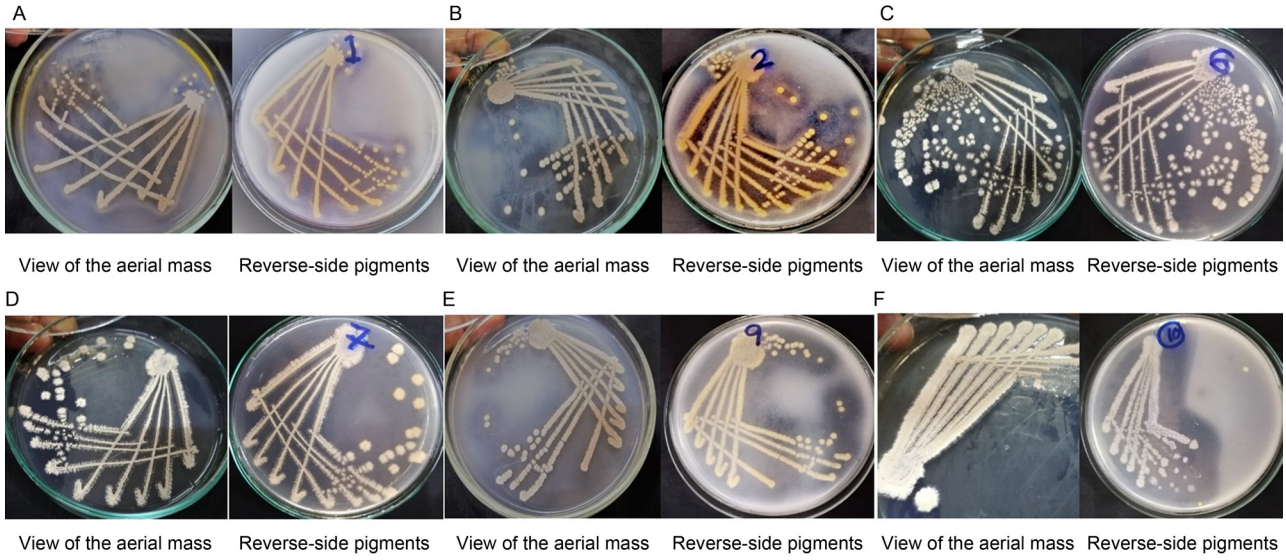

Fig. 1. Colony morphologies of actinomycete isolates. The view of the aerial mass and reverse-side pigment colors of isolates Ac-1 (A), Ac-2 (B), Ac-6 (C), Ac-7 (D), Ac-9 (E), and Ac-10 (F) on modified-starch casein agar after 1–2 weeks of incubation at 28°C.

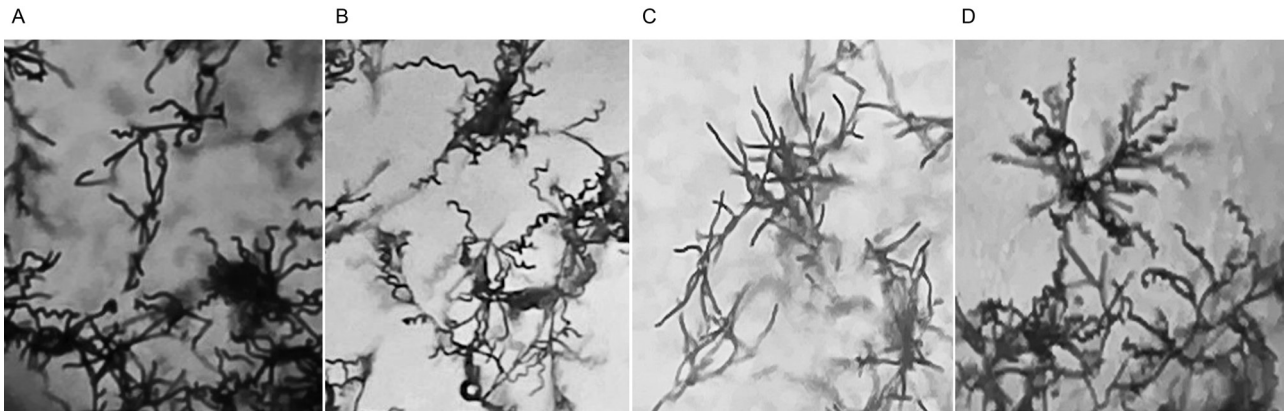

Fig. 2. Spore chain morphologies of actinomycete isolates. Spore chain morphologies of actinomycetes Ac-1 (A), Ac-2 (B), Ac-6 (C), and Ac-9 (D) as observed under a light microscope (magnification 100×).

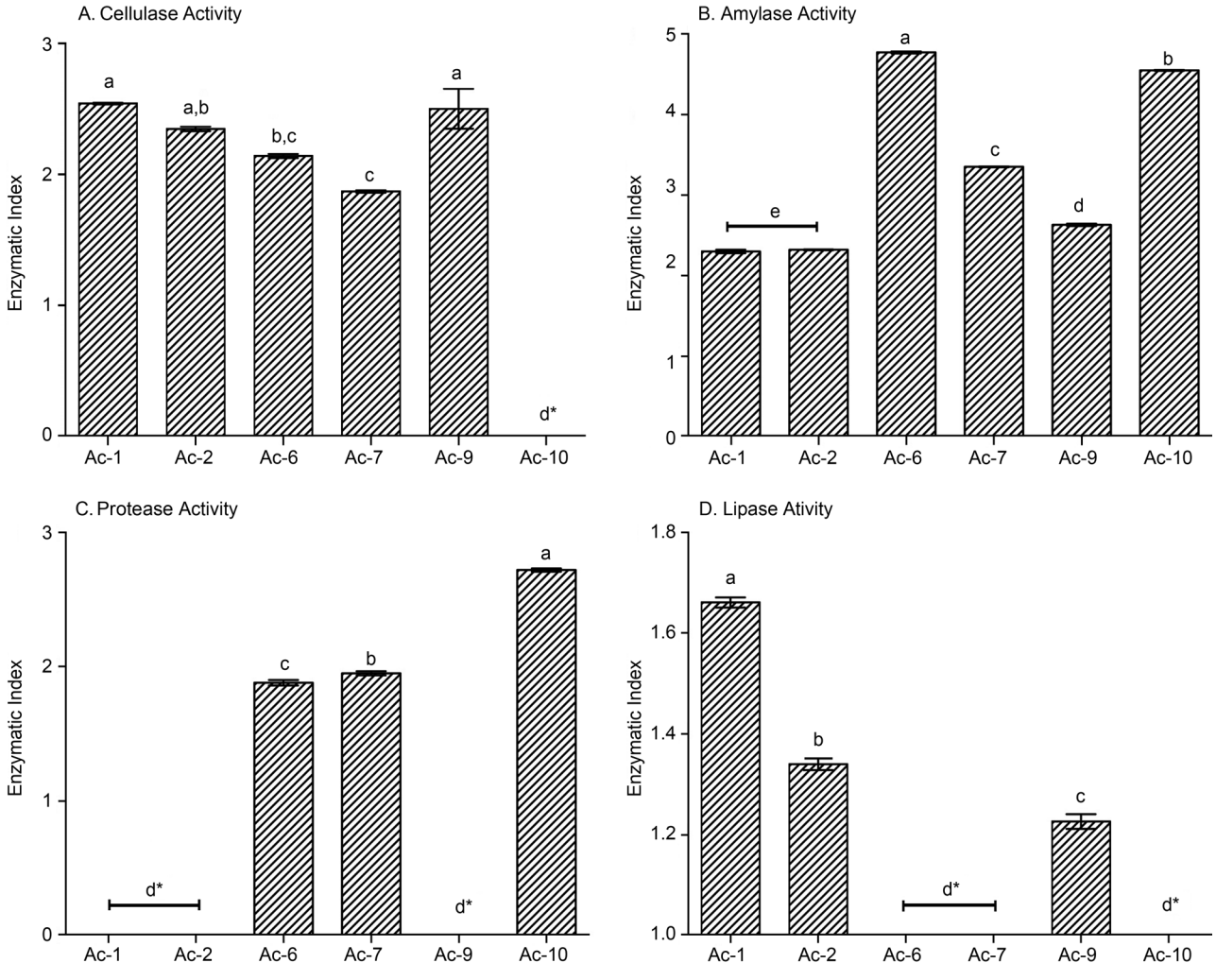

Fig. 3. Extracellular enzyme production of actinomycete isolates. Actinomycete isolates were tested for the production of cellulase (A), amylase (B), protease (C), and lipase (D)separately on solid agar media. The 'Enzymatic Index' for each enzyme assay was expressed using the ratio between the average diameter of the halo/clear zone around the colony and the average diameter of the colony. Error bars represent the standard error of the mean (n = 3). The bars indicated with the same lower case letter are not significantly different from those in the same graph (Tukey's test, $p < 0.05$).

* – no visible enzymatic activity

Only three of the six isolates gave positive protease and lipase assays results. Interestingly, strains Ac-1, Ac-2, and Ac-9, which were negative for protease production, exhibited lipase production. Similarly, the three strains that produced protease (Ac-6, Ac-7, and Ac-10) showed no lipase activity. Among the isolates, Ac-10 showed the highest enzymatic index for protease, while Ac-1 had the highest enzymatic index for lipase assay (Tukey's test, $p < 0.05$).

Antibacterial activity of actinomycete isolates. The primary screening of antibacterial activity of all isolated actinomycete strains was done using a cross-streak assay (Table II). Isolates Ac-1, Ac-2, and Ac-9 exhibited antibacterial activity against *S. aureus*, *E. coli*, and *B. cereus*, but not against *L. monocytogenes*. Strains Ac-6, Ac-7, and Ac-10 did not show any inhibitory effect against any tested pathogens during the cross-streak assay. Strains that exhibited positive results in the cross-streak assay were further tested in a well-diffusion assay. The cell-free supernatants of strains Ac-1, Ac-2, and Ac-9 were used against test bacterial pathogens in a standard well-diffusion assay (Fig. 4). Cell-free supernatants of all three actinomycete isolates exhibited inhibitory activities against the five tested bacterial pathogens, while Ac-1 showed the highest antibacterial activity.

Molecular characterization of the strains. Three isolates (Ac-1, Ac-2, and Ac-9) were selected for further characterization based on the antibacterial activities and other characteristics. The PCR amplification of the16S rRNA gene using fD1 and rD1 primer pair resulted in ~ 1,500 bp products for all the three isolates, and the PCR products were sequenced using 27F and 1100R universal primers at Macrogen (South Korea). BLAST searches of the partial 16S rRNA gene sequences

Table II
Antibacterial activity of actinomycete isolates against the test strains in the cross-streak assay.

Strain ID	Test strain			
	Staphylococcus aureus	*Escherichia coli*	*Bacillus cereus*	*Listeria monocytogenes*
Ac-1	++	++	+	–
Ac-2	++	+	+	–
Ac-6	–	–	–	–
Ac-7	–	–	–	–
Ac-9	+	+	+	–
Ac-10	–	–	–	–

(+)/(++) – the presence of a zone of inhibition near the growth of actinomycete strain, (++) indicates that the inhibition zone was higher compared to (+)
(–) – the absence of a zone of inhibition near the growth of actinomycete strain

of Ac-1, Ac-2, and Ac-9 showed similarities towards several *Streptomyces* spp. in the database, confirming their identity as belonging to the genus *Streptomyces*. Ac-9 as a query had 99.9% similarity to *Streptomyces vinaceusdrappus* NRRL 2363 (query coverage – 99%, E-value – 0), while the sequence of Ac-1 showed the highest similarity to *Streptomyces enissocaesilis* strain NRRL B-16365 (identity% – 97.48%, query coverage – 94%, E-value – 0). Ac-2 showed 91.51% percent identity to *Streptomyces avidinii* KSR1 (query coverage – 97%, E-value – 0). Further, the partial 16S rRNA gene sequences of Ac-1, Ac-2, and Ac-9 were compared with several *Streptomyces* strains, and the relationship with their closest phylogenetic neighbors were inferred using a neighbor-joining phylogenetic tree (Fig. 5).

Growth optimization and crude pigment extraction. The optimum conditions required for the growth of pigment-producing actinomycete strains Ac-1 and Ac-2 were analyzed by varying growth parameters and measuring the dry weight of their biomass. According

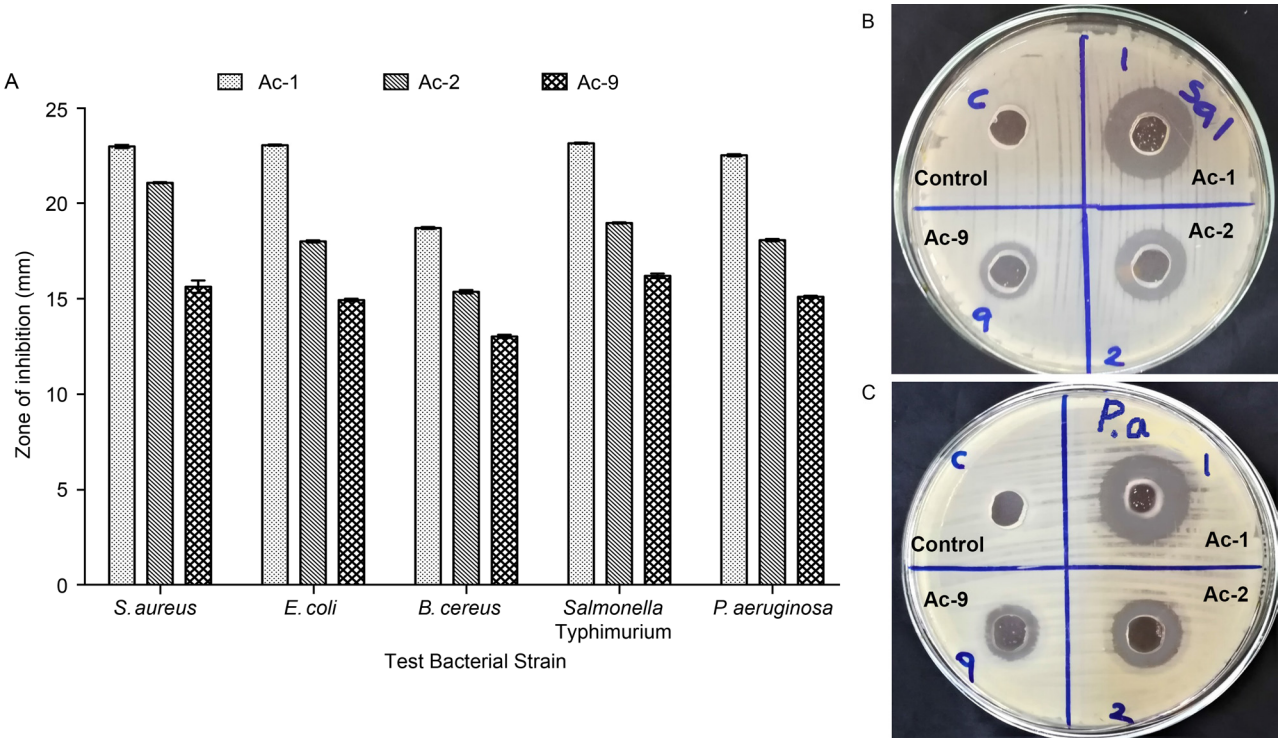

Fig. 4. Antibacterial activity of selected actinomycete isolates against test strains. The cell-free supernatants of selected actinomycete isolates were tested against selected test bacterial pathogens using a standard well-diffusion assay. The average diameter of the zones of inhibition for each cell-free supernatant was calculated using three independent replicates. Error bars indicate the standard error of the mean (A). Plates show the inhibition zones given by cell-free supernatants of Ac-1, Ac-2, and Ac-9 against *Salmonella* Typhimurium (B) and *Pseudomonas aeruginosa* (C) on Mueller-Hinton agar after the incubation.

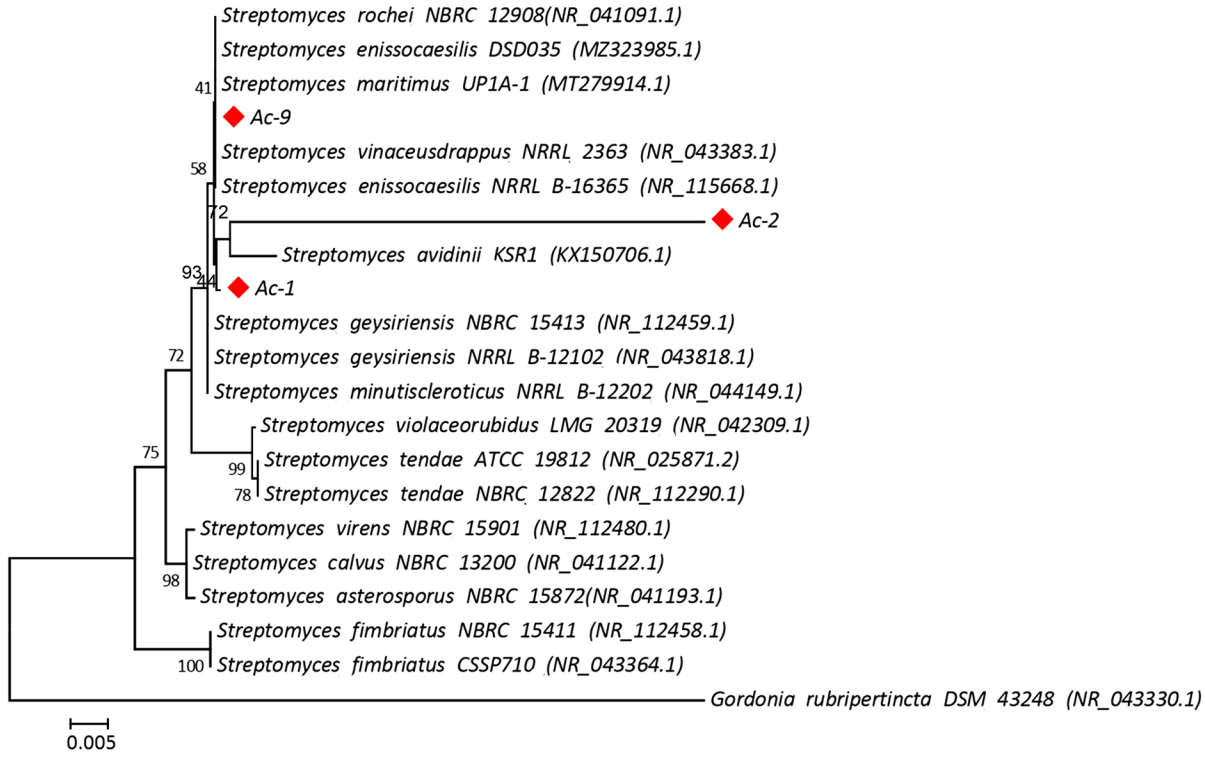

Fig. 5. Phylogenetic relationship based on the partial 16s rRNA gene sequences. The evolutionary relationships of *Streptomyces* isolates (Ac-1, Ac-2, and Ac-9) were inferred via a neighbor-joining method. Strains isolated in this study are indicated with a red diamond (◆). Bootstrap support values from 1,000 replicates are shown next to the branches. *Gordonia rubripertincta* was used as the out-group of the analysis. The scale bar represents 0.005 nucleotide substitutions per character.

to the experimental results, the maximum dry weight of the Ac-2 isolate's biomass was observed in the starch casein broth containing 4% sodium chloride (Fig. 6) and at pH = 9 (Fig. 7), separately. Ac-1 had the highest biomass dry weight when grown in the starch casein broth supplemented with 2% NaCl. Similarly, Ac-1 produced the highest biomass when the pH of the medium was slightly acidic (pH = 5). Color of the pigment produced by each strain varied with different pH values. A significant difference between the dry weights of the bacteria grown with and without shaking was observed (Table III). Ac-1 showed no or very slight growth when incubated under a steady-state, while the growth was higher in aeration. However, the Ac-2 strain's most significant biomass weight was observed when it was cultured at a steady state, whereas the larger proportion of the biomass was due to the production of black-colored spores on the liquid-air interface of the broth medium.

Four different solvents: 95% alcohol, ethyl acetate, acetone, and chloroform, were separately used to determine the suitable solvent for extracting pigments from isolates Ac-1 and Ac-2. Extraction of pigments with acetone resulted in the intense yellow colored pigment solution compared to pigments extracted with other solvents. Therefore, acetone-extracted pigments of Ac-1 and Ac-2 were used for further studies. Although the

Table III
Comparison of growth of actinomycete isolates Ac-1 and Ac-2 under shaking and non-shaking conditions.

Growth condition	Dry weight of the biomass (g)[#]	
	Ac-1	Ac-2
With shaking (at 100 rpm)	0.023 ± 0.003**	0.043 ± 0.006*
Without shaking	0.000**	0.057 ± 0.006*

[#] – The biomass was obtained by drying the culture resulting from inoculation 25 ml of SCB and incubation for seven days at room temperature under shaking and non-shaking conditions. The values given are the means of three individual replicates (n = 3) with a standard error of mean (± SEM). Means followed by an asterisk/asterisks within a column, are significantly different (unpaired *t*-test, ** $p < 0.005$, * $p < 0.05$).

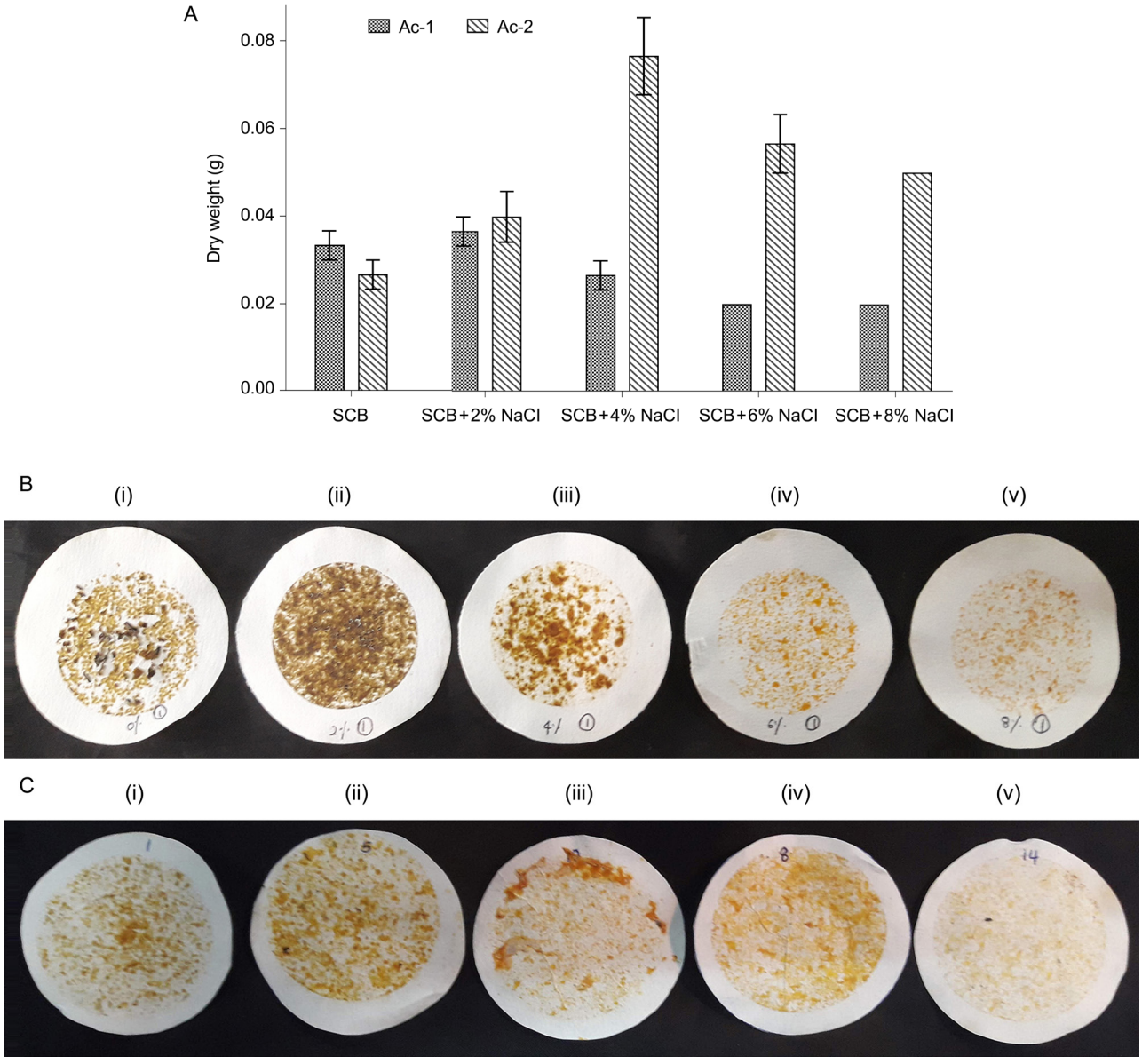

Fig. 6. Effect of salt concentration of the medium for the growth of actinomycete isolates. Isolates Ac-1 and Ac-2 were grown in modified-starch casein broth (SCB) supplemented with different NaCl concentrations, and the dry weight of the resulting biomass was measured after the incubation. Values represent the mean of three independent replicates (n = 3) , and error bars represent the standard error of the mean (A). The biomass of Ac-1 (B) and Ac-2 (C) grown in modified-SCB supplemented with (i) 0%, (ii) 2%, (iii) 4%, (iv) 6%, and (v) 8% NaCl were filtered and dried on a sterile filter paper.

strain Ac-9 produced yellow-colored pigments, the yield and the color intensity of the Ac-9 crude pigment extract were not satisfactory for further evaluations. Primarily, as the resulting pigment extract of Ac-9 had a very faint yellow color that could not be improved through the initial experimental conditions, the crude pigment extract of Ac-9 was not evaluated further.

The antibacterial activity of crude microbial pigment extracts was assessed using a standard agar well diffusion assay against *E. coli*, *P. aeruginosa*, and *S. aureus* on Mueller-Hinton agar. No zones of inhibition were observed with crude pigment extracts of Ac-1, whereas pigment extracts of Ac-2 showed an inhibitory effect against all the organisms tested (Table IV).

Table IV
Antibacterial activity of actinomycete crude pigment extracts against test strains in well-diffusion assay.

Test organism	Average diameter of the zone of inhibition (mm) ± SEM*	
	Ac-1	Ac-2
Escherichia coli	–	21.0 ± 0.5774
Pseudomonas aeruginosa	–	18.0 ± 0.0
Staphylococcus aureus	–	22.5 ± 0.2881

* – Values given are the means of three individual replicates (n = 3) with a standard error of mean (± SEM). Acetone was used as the negative control for the assays.

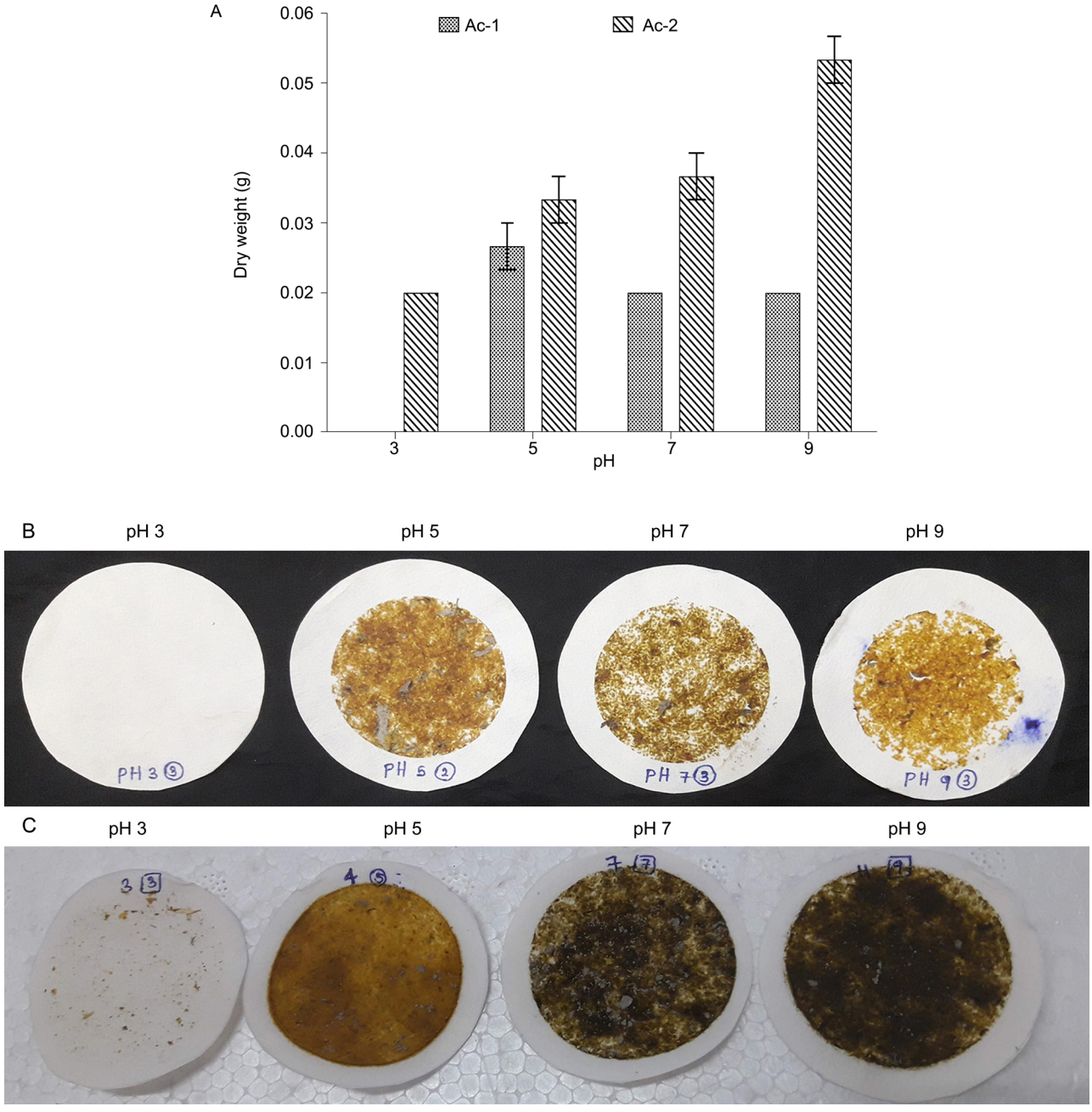

Fig. 7. Effect of initial pH of the medium for the growth of actinomycete isolates. Isolates Ac-1 and Ac-2 were grown in modified-starch casein broth (SCB) adjusted to different initial pH, and the dry weight of the resulting biomass was measured after the incubation. Values represent the mean of three independent replicates (n = 3) and error bars represent the standard error of the mean (A). The biomass of Ac-1 (B) and Ac-2 (C) grown in modified-SCB with different initial pH values (3, 5, 7, and 9) were filtered and dried on sterile filter paper.

The potential of yellow-colored pigment of Ac-1 as a fabric colorant was evaluated using six different commercially available fibers: satin, polyester, terin, poplin, silk-cotton-blended fabric, and Khushbu. Depending on the fiber type, different dyeing performances were observed (Fig. 8). However, all the resulting color tones had a good visual quality for all types of fibers, and subsequent cold-water, acid, and alkaline treatments of the dyed fabric resulted in no visible color loss indicating its potential suitability in future biotechnological applications.

Discussion

Tropical mangrove forests are not only acknowledged as one of the most productive global ecosystems, they also serve as a significant pool of untapped microbial populations with the ability to produce novel secondary metabolites to be used in biotechnological applications (Baskaran et al. 2011; Law et al. 2020). Sri Lankan mangrove systems are interspersed along the coastline of the country. Although the richness of Sri Lankan mangrove forests concerning the floral and

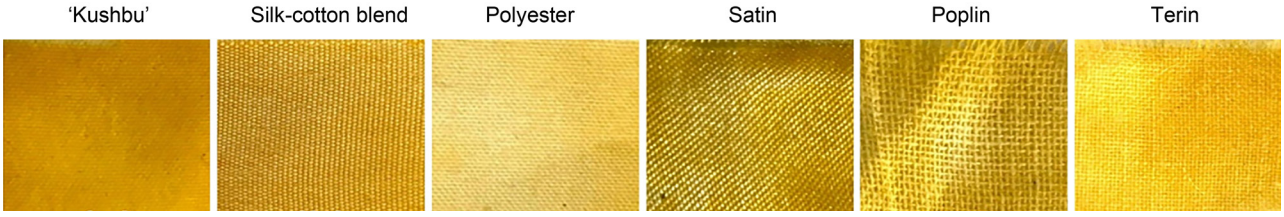

Fig. 8. Application of yellow-colored pigment of Ac-1 as a fabric colorant. Six different commercially available fabric types, 'Kushbu', silk-cotton blend fabric, polyester, satin, poplin, and terrine, were dyed using acetone-extracted crude pigments of *Streptomyces* spp. Ac-1.

faunal diversity has been acknowledged widely (Pinto and Punchihewa 1996; Amarasinghe and Perera 2017; Fernando et al. 2022), the microbial potentials of these unique ecosystems remain elusive. Moreover, mangrove forests are known to harbor diversified actinomycete populations (Baskaran et al. 2011; Zainal Abidin et al. 2016). Actinomycetes are a group of Gram-positive bacteria with greater economic importance due to their potential ability to produce various bioactive secondary metabolites (Hong et al. 2009; Janardhan et al. 2014; Gong et al. 2018). In the current study, we have used surface sediment samples from two sites within the Kadolkele mangrove ecosystem to isolate six actinomycete strains. The sampling sites are located within the area maintained by the Regional Research Centre of the National Aquatic Resources Research and Development Agency, Sri Lanka. 'Kadolkele' mangrove forest is associated with Negombo lagoon in the west coast of Sri Lanka and dispersed over an area of 14 ha. Actinomycetes were isolated using modified-SCA. Filter sterilized-sea/mangrove water (50% v/v) obtained from the sampling site or the closer by area of the sampling site was used to prepare diluent and the isolation medium. Further, the isolation medium was supplemented with cycloheximide and nalidixic acid to inhibit the growth of fungi and Gram-negative bacteria from the sediment samples, respectively. SCA is commonly used for the isolation of saccharolytic bacteria, especially actinomycetes, from marine and other environmental samples (Mackay 1977; Baskaran et al. 2011). SCA is considered as one of the most suitable media for the isolation of actinomycetes (Lee et al. 2014; Waheeda and Shyam 2017).

Many studies have shown that actinomycetes isolated from various terrestrial and aquatic ecosystems can produce economically significant components (Subramani and Aalbersberg 2012, 2013; Singh and Dubey 2015). Different *Streptomyces* spp. can produce industrially important extracellular enzymes such as lipase, cellulase, keratinase, and amylase (Mukhtar et al. 2017; Swarna and Gnanadoss 2020). Moreover, new and under-explored habitats such as deserts, biomes, and marine ecosystems are known to be very rich sources of novel actinomycetes, which can produce new bioactive compounds, including antibiotics (Hong et al. 2009; Aftab et al. 2015; Davies-Bolorunduro et al. 2019; Subramani and Sipkema 2019; Zamora-Quintero et al. 2022). These enzymes and bioactive compounds are of great value in industrial applications, as many of them possess important properties such as heat tolerance and stability under alkaline conditions (Grover et al. 2016; Nandimath et al. 2017). In the present study, actinomycete isolates were explored for cellulase, lipase, protease, and amylase production. All isolates produced amylase with varying enzymatic indices, while only five strains out of six showed positive results in cellulase assay. Three isolates exhibited the ability to produce lipase and protease separately.

Preliminary screening for the antibacterial activities of the isolates using a cross-streak assay showed that the three pigment-producing isolates, Ac-1, Ac-2, and Ac-9 could inhibit three of the four test organisms. In contrast, no growth inhibition was observed for *L. monocytogenes*. These three isolates were further tested using their cell-free supernatants in well-diffusion assays to determine their antibacterial activities. Based on the average diameters of the resulting inhibitory zones, the inhibitory effects of cell-free supernatant of Ac-1 against *Salmonella* Typhimurium, *E. coli*, and *S. aureus* were determined as significantly higher than against the other test organisms (Tukey's test, $p < 0.05$). Moreover, the highest inhibitions by cell-free supernatant of Ac-2 and Ac-9 were observed against *S. aureus* and *Salmonella* Typhimurium, respectively. All three tested strains showed the lowest inhibitory effect against *B. cereus*. Many actinomycetes isolated from marine-derived sediments produce novel antibiotics with unusual structures and properties different from those of ordinary soil actinomycetes due to the habitat stress unique to mangrove ecosystems. It makes them excellent candidates for biotechnological applications (Lam 2006; Subramani and Aalbersberg 2012).

Isolates Ac-1, Ac-2, and Ac-9 were identified as *Streptomyces* spp. based on the partial 16S rRNA gene sequencing. The morphological and biochemical characteristics of these isolates were consistent with the genus. Although the BLAST searches and subsequent phylogenetic analysis of these isolates supported identifying their closest neighboring species with the highest percent identities, the results were inconclusive in accurately placing them in an existing species of the

genus. It is worth noting that the genus *Streptomyces* harbors a diverse range of species. Therefore, the 16S rRNA gene sequence analysis alone is insufficient for the accurate species delineation of an organism, and these data should be supported by multi-locus sequence analysis (MLSA) to establish the species identity of a strain. MLSA of several house-keeping genes such as *atp*D, *rec*A, and *rpo*B is needed for their classification in a well-delineated clade or for describing them as a novel species of the genus.

Many genera belonging to the actinomycete group are known to produce pigments of different colors (Parmar and Singh 2018; Vasanthabharathi and Jayalakshmi 2020; Gupta et al. 2022). The majority of the 41 actinomycete strains isolated from soil samples collected from different locations in Nepal were capable of producing pigments of different colors, such as blue, green, yellow, and violet on SCA (Sapkota et al. 2020). Further, many studies have shown that these pigments may harbor bioactive properties such as antibacterial, antifungal, antioxidant, and anticancer properties (Prashanthi et al. 2015; Azman et al. 2018; Mesrian et al. 2021).

The pigments of microbial origins are preferred in industrial applications for various reasons. A yellow-colored pigment, extracted from *Streptomyces hygroscopicus* subsp. *ossamyceticus* stain D10 using ethyl acetate, showed antibacterial activity (Selvameenal et al. 2009). The intracellular pigment of *Streptomyces bellus* strain was extracted using ethyl acetate and characterized for its bioactive properties (Srinivasan et al. 2017). The crude pigment extracts of strains Ac-1 and Ac-2 were prepared with acetone and tested for their antibacterial effect. Crude extracts of yellow-colored pigment of Ac-2 showed an inhibitory effect against bacterial test strains. Although the cell-free supernatant of Ac-1 exhibited an antibacterial effect against tested pathogens, its crude pigment extract lacked any inhibitory effect. It could be suggested that the bioactive compound may be secreted and present in the cell-free supernatant; hence, when the supernatant is discarded to extract the intracellular pigment, no inhibitory effect was observed with the resulting crude extract. Also, the Ac-1 crude pigment was successfully used in dyeing different fabric types, and the visible color retention after water, acid, and alkaline washes of the dyed fabric suggests that the pigment may have the potential to be developed into an eco-friendly textile colorant.

Conclusion

Actinomycete strains isolated from 'Kadolkele' mangrove sediments in Sri Lanka, exhibited the potential of producing several extracellular enzymes and the ability to inhibit the growth of selected Gram-negative and Gram-positive bacteria. Three isolates were identified as belonging to the genus *Streptomyces* using the 16S rRNA gene sequencing; however, more studies are needed to classify them further. Also, the range of enzymes and other bioactive secondary metabolites produced by these isolates and the stability and efficiency of these compounds should be further evaluated to determine their potential in industrial applications.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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