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Research Article

Diversity of Actinomycetes Isolated from Peat Soil of Undistrubed Forest and Pineapple Plantation in Sessang, Sarawak

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ABSTRACT
Peatland plays an important role not just as a carbon store but also in facilitating the flux of greenhouse gasses into the atmosphere. Apart from that, peatland is also home to a diverse population of microorganisms such as bacteria, fungi, and actinomycetes. Actinomycetes were known to be one of the most ubiquitous microbes that can be found in most of the soil types including peat soil. In this study, seventy isolates of actinomycetes were isolated from the peat soil using the soil dilution method. The 70 isolates of actinomycetes were later screened for their ability to produce secondary metabolites and antimicrobial activities using the agar diffusion method before the selected potential isolates were identified by targeting their 16S rRNA region. The results obtained showed 34.3% produce cellulase followed by, 12.8, 31.7, 80.0, and 51.4% for mannanase, xylanase, lipase, and protease respectively. The percentage of actinomycetes producing antimicrobial activity was 27.1 and 21.4% for *Ralstonia solanacearum* and *Colletotrichum gleosporioides* respectively. All the selected isolates of actinomycetes were identified as belonging to the genus of Streptomycetes spp. The potential actinomycetes were stored in freeze-dried form for future usage. This study showed that more diverse population of actinomycetes was obtained from the undisturbed forested peat soil area ecosystem compared to the agricultural peat soil area.

INTRODUCTION
Malaysian peatlands were estimated to be about 2.6 Mha, with approximately 70% of these peatlands located in Sabah and Sarawak (Meilling 2016). These peat soils contained microbes that supported the formation of the peat and the carbon and nutrient cycling which is important to the ecosystem (Meilling 2016). One of the most studied peat soil microbes is *Streptomyces* sp which belongs to the family of Actinomycetes.

Streptomyces sp. has been well known to researchers for its ability to produce various beneficial activities such as bioactive compounds producer, decomposer, and plant growth promoter (Abdulla & El-Shatoury 2007; Umi et al. 2019; Sapkota et al. 2020). *Streptomyces* spp. has been known to produce more than 7600 compounds which makes them the largest producers of bioactive compounds for microorganisms (Bérdy 2005). Secondary metabolites obtained from actinomycetes are of special

interest to many researchers due to their diverse biological activities such as antibacterial, antifungal, antioxidant, antitumor, and antiviral (Niyasom et al. 2015). Many studies have been conducted using *Streptomyces* spp. isolated from soil samples for their bioactive compounds. Gopal and Thripathi (2020), noted that *Streptomyces* spp. isolated gave promising bioactive activity towards the plant pathogen *Pseudomonas aeruginosa*. Sapkota et al. (2020), noted that actinomycetes isolated from soil samples from different altitudes in Nepal were found to produce not just enzymatic activities but also antimicrobial activities against *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli*.

Several studies on the isolation and activities of soil actinomycetes isolated from peat soil have been documented (Lestari et al. 2019; Lisa et al. 2022). Peat soil actinomycetes have been known to show good activities in producing bioactive properties. Lestari et al. (2019), observed that actinomycetes isolated from a peat soil sample collected at Tajok Kayong Village, Ketapang Regency a potential antimicrobial producing candidate towards pathogenic microbes such as *Escherichia coli* and *Staphylococcus aureus*. Lisa et al. (2022), also indicated that actinomycetes isolated from soil had the potential to be developed into a biopesticide for *Ralstonia solanacearum*. Apart from that Jeffrey et al. (2011), it was indicated that actinomycetes isolated previously from forested soil at MARDI Peat Land Research Station at Sessang, Sarawak were capable of inhibiting *Ralstonia solanacearum* under *in vitro* screening.

In this study, the authors described the diversity and characteristics of actinomycetes isolated from peat soil obtained from forested and agricultural area at MARDI Peat Land Research Station at Sessang, Sarawak

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected from the MARDI Peat Land Research Station at Sessang, Sarawak. Two types of soil samples that were collected were the undisturbed forest soil (1.91890, 111.23657) where the peat soil has not been used for any agricultural activity, and pineapple-planted soil (1.92500, 111.23762) where it is currently being used for pineapple planting. Soil was collected by digging a hole approximately 10 cm deep using a spade after removing approximately 3 cm of the soil surface. Samples were kept in different sterile zip-lock polyethylene bags during transportation from MARDI Peat Land Research Station at Sessang, Sarawak to MARDI Headquarters at Serdang, Selangor.

Isolation and enumeration of actinomycetes

Soil samples were air-dried for about a week to reduce the growth of gram-negative bacteria. The dried soil samples were then ground to powder form using a pestle and mortar. The ground samples were added to the sterilised distilled water (sH_2O) at the ratio of 10 g of soil in 100 ml of sH_2O. The suspensions were later agitated at 280 rpm for 1 h. Serial dilution of 10^{-4} to 10^{-7} was later prepared for each soil sample collected. Hundred and fifty microliters of each suspension were later pipetted onto the Starch Casein Agar plate (SCA) containing soluble starch, 10.0 g; Casein (vitamin free), 0.3 g, KNO3, 2.0 g, MgSO4.7H2O, 0.05 g, K2HPO4, 2.0 g, NaCl, 2.0 g; CaCO3, 0.02 g; FeSO4.&H2O, 0.01 g and agar, 18.0 g in 1000 ml of distilled water with pH adjusted to 7 and lawned using a spreader stick (Zareenkousar et al. 2022). The agar plates were later incubated at room temperature (28 ± 2°C) for 10-14 days for the actinomycetes to emerge. Total plate counts were calculated from average counts of three replicates and expressed as colony-forming units (c.f.u.) per gram

of dry soil. The emerging colonies of actinomycetes were later transferred onto a fresh SCA plate and used as working cultures (Jeffrey et al. 2011).

Screening of extracellular enzyme activity

Screening for extracellular hydrolysis enzyme activities (mannanase, xylanase and cellulase) were carried using minimal medium agar containing bacteriological peptone, 1.0 g; yeast extract, 1.0 g; KH_2PO_4, 0.5 g; $(NH_4)_2HPO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 15.0 g; and substrate, 1.0 g which contained Megazyme: AZO-CM-Cellulose, AZO-Carob-Galactomannan and AZO-Xylan (oat) in 1000 ml of distilled water with pH adjusted to 7 (Jeffrey et al. 2011). The protease test was conducted using gelatin hydrolysis assay (Jing et al. 2020) while the lipase test was conducted using the method used by Sinha et al. (2014). Plug of pure culture of Actinomycetes isolates were later inoculated onto these substrate mediums. Results were obtained on day 5 after inoculation (Figure 1).

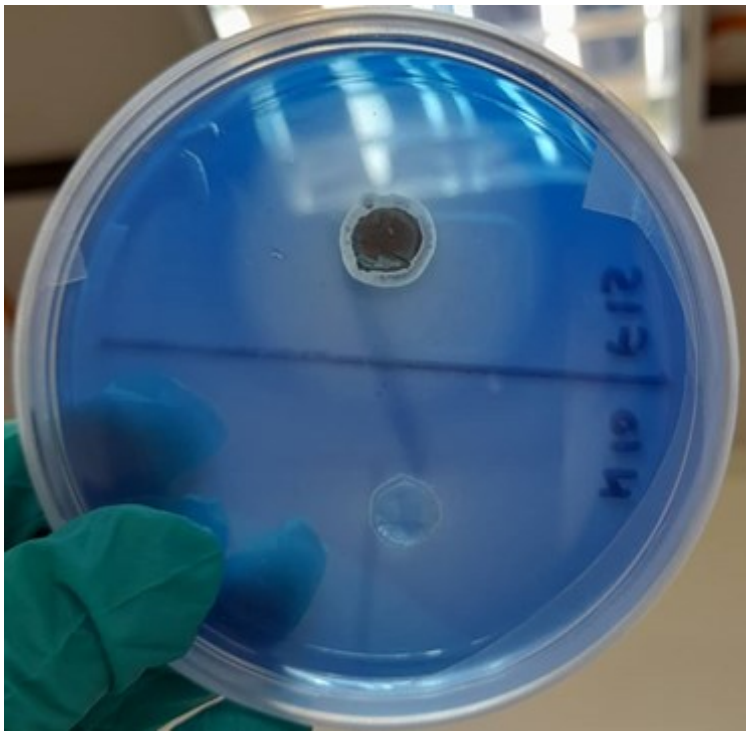

Figure 1. Clear zone forming indicating actinomycetes with the ability to produce enzyme to degrade cellulose.

Screening of antimicrobial activity

Plate diffusion method as propoesed by Bauer et al. (1966) with the sverals modification suggested by Barakate et al. (2000) was used in screening of antimicrobial activities. Under this protocol, Actinomycetes isolates were removed using a sterile cork bore (5 mm in diameter) to make agar stabs and placed onto tested pathogenic bacteria lawn plates. Formation of clearing zone was observed and measured after 5 days of incubation. Inhibition zone was measured from diameter of clear zone as observed from the antagonistic reaction (Figure 2). For anti-fungal activity screening, the fungal plug was placed on the middle of Potato Dextrose Agar plate and the actinomycetes agar plug was placed at two opposite side of the agar plate. Halo zone formed indicates antifungal activity between actinomycetes and the fungal test strain (Figure 3). Pathogenic microbes strains used for in the screening were *Ralstonia solanacearum* and *Colletotrichum gleosporioides*.

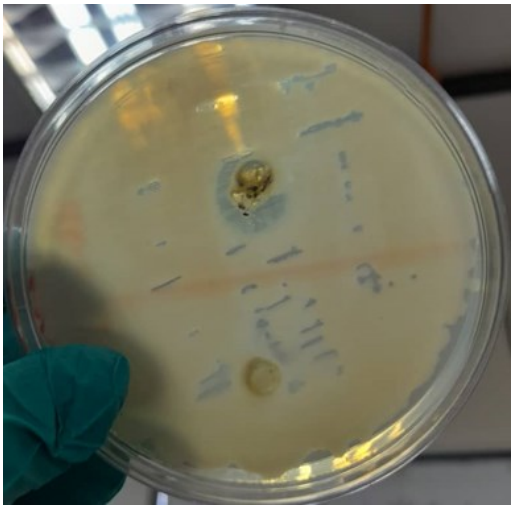

Figure 2. Halo zone indicating antibacterial activity between actinomycetes and *Ralstonia solanacearum*.

Figure 3. Halo zone forming indicating antifungal activity between actinomycetes and *Colletotrichum gleosporioides*.

Isolation of genomic DNA

Genomic DNA (gDNA) was performed for 5 potential isolates of actinomycetes using MagAttract Microbial DNA Kit. Protocol used was as indicated by the manufacturer (Qiagen 2022a).

Polymerase chain reaction (PCR) amplifications

Five of the best producing actinomycetes selected were identified using primers flanking at their 16S rRNA region. Polymerase chain reaction was carried out using 15.5 µl sterile distilled H_2O, 2.5 µl 10X PCR buffer, 2.0 µl 25 Mm MgCl, 0.6 µl dntps, 0.4 µl Taq polymerase, 1.0 µl of 20 pmol of each primers COM1 (5′-CAGCAGCCGCGGTAATAC-3′) and COM2 (5′-CCGTCAATTCCTTTGAGTTT-3) (Congestri et al. 2020) which were used to identify the variable region of V4 and V5 of the ribosomal DNA and 2.0 µl of DNA per single reaction. Protocol for the thermal cycler was as follows; initial denaturing at 94°C for 3 mins, followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 62°C for 45 s, and elongation at 72 °C for 2 mins and a final elongation of 72 °C for 10 mins. PCR products obtained were loaded into the wells of a 1.0 % agarose gel and ran at 80 V for 50 mins. The gel was later viewed using Gel Documentation System from Biorad. PCR products were later purified using QIAquick PCR & Gel Cleanup kit according to the protocol suggested by the manufacturer (Qiagen 2022b).

Sequencing of PCR products

Purified PCR products were later sent for sequencing, at the facilities of Apical Scientific Sdn. Bhd., Selangor using ABI PRISM ® 377 DNA Sequencer (Applied Biosystems). The obtained sequences were then compared to sequences available in the National Centre for Biotechnology Information (NCBI) genebank database using the Basic Alignment Search Tool (BLAST) (Altschul et al. 1990). The phylogenetic tree was then constructed using the sequences obtained using Clustral X software (Thompson et al. 2002). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm was used for the construction of the phylogenetic tree.

Conservation of potential isolates

All the potential microbes were kept in freeze dry form (powder form) for longer term safekeeping at MARDI Microbial Culture Collection (MMCC), MARDI Serdang, Selangor.

RESULTS AND DISCUSSIONS

Isolation and enumeration of actinomycetes

A total of 70 isolates of actinomycetes were isolated from the peat soil. The average colony forming unit of the actinomycetes from undisturbed forest soil was 4.7 x 10^5 however the pineapple planted soil showed only 7.3 x 10^3. In a previous study conducted by Jeffrey et al. (2011), it was reported that cfu/g of actinomycetes obtained from the forest soil was 3.0 x 10^4. There was a slight increase in the cfu of actinomycetes from the same location 10 years ago. This increase of actinomycetes cfu/g of soil indicates that microbial population tends to build up if it is left undisturbed from any development. However, research done on the population of actinomycetes at Sarawak wetland gave a lower cfu count on wetland versus agriculture soil at 6.84 x 10^5 cfu/g and 1.61 x10^6 cfu/g respectively (Ann et al. 2020) which justifies that actinomycetes preferred dry area rather than wet area.

It was observed that approximately 75.7% of the isolated actinomycetes produce grey spores, followed by 20.0% with brown spores and only 4.3% of the actinomycetes produce black spores (Table 1). This finding was almost the same as 10 years ago when Jeffrey et al. (2011) observed that grey spore producer was the highest followed by brown and white spores. However, it was not in line with the observation obtained from Sapkota et al. (2020), where the researchers indicated that 10% of the actinomycetes they isolated produced grey spores and only 3% produced brown spores. Sapkota et al. (2020) noted that 45% of their isolates produce yellow spores, however, there is no yellow spore formation was observed from actinomycetes isolated in this study. It was observed that actinomycetes isolated from the undisturbed forest and pineapple-planted soil gave the highest reading of 37/51 (72.5%) and 16/19 (84.2%) respectively for the grey spore formation (Table 1).

In this study, 65.7% and 28.6% of actinomycetes produce diffusible yellow and brown pigmentation respectively (Table 1). This shows that actinomycetes isolated from Sessang peat soil produce dominantly only yellow and brown diffusible pigment. This is in agreement with Fernandes et al. (2021), where the authors noted that the production of diffusible pigments ia prevalent in soil actinomycetes. According to Gupta et al. (2022), actinomycetes are potent producers of dark-brown coloured melanin pigments however in this study it was observed yellow coloured pigment was the dominant. Both actinomycetes isolated from the undisturbed forest and pineapple planted soil showed that brown pigment pro-

Table 1. Spores colour formation and pigmentation produced by 70 isolates of actinomycetes isolated.

Sampel	Actinomycetes isolates					
	Spore colour			Pigmentation		
	Grey	Brown	Black	Yellow	Brown	No pigment produce
Actinomycetes isolated from undisturbed forest (n=51)	37	11	3	30	18	3
Actinomycetes isolated from pineapple planted soil (n=19)	16	3	-	16	2	1
Total (n=70)	53 (75.7%)	14 (20.0%)	3 (4.3%)	46 (65.7%)	20 (28.6%)	4 (5.7%)

duction was low in comparison to yellow pigment (Table 1). This might be due to the conditions of the environment these actinomycetes grow in (Celedón & Díaz 2021).

Screening of extra-cellular enzyme and anti-microbial activity

Approximately 80.0% of the actinomycetes isolated produce enzyme lipase and 51.4% produce enzyme protease. This is in accordance with the results that were observed 10 years ago at MARDI Sessang. In the previous study, it was observed that lipase (32.5%) was also produced more by actinomycetes compared to protease (12.5%) (Jeffrey et al. 2011). It also observed that the affinity of actinomycetes producing cellulase is higher than xylanase and mannanase (Table 2). This might be due to the reason that peat soil contained generally lignin, cellulose, hemicellulose, and protein thus higher cellulose present required more or better cellulose producer microbes in the surroundings. This is supported by the observation of Veloo et al. (2014), where the authors observed that tropical peat soil contains wood materials in the soil solum which explained why the cellulase producing actinomycetes is more compared to xylanase and mannase producers. It was also noted in this study that *Streptomyces griseus* strain PS42 can be considered the most potent producer of enzymes screened (Table 3). This statement was supported by Talib Saleh et al. (2023), where the researchers observed that *S. griseus* has the ability to produce multiple enzymes such as celllulase, lipase and protease.

Antimicrobial test conducted showed that 27.1% and 21.4% of the actinomycetes has the ability to inhibit the growth of *Ralsonia solanacearum* and *Colletotrichum gleosporioides* respectively (Table 2). *Streptomyces* sp. strain 33 showed good antagonistic activity towards both *Ralsonia solanacearum* and *Colletotrichum gleosporioides* with the average zone size of 1.3 cm and 1.2 cm for both pathogens respectively (Table 3). This showed that actinomycetes isolated from MARDI Sessang, is good at producing antimicrobial activity against *R. solanacearum* because previous study done by Jeffrey et. al. (2011) also indicated that actinomycetes isolated from MARDI Sessang area showed the ability to produce antimicrobial activity against *R. solanacearum* with the average halo zone of 2 cm was produced by *Streptomyces* sp. strain TN06. In a study done by Zhao et al. (2019) *Streptomyces sporangiiformans* was observed to produce antimicrobial activity towards *R. solanacearum*. In a recent study by Lisa et al. (2022), the authors showed that actinomycetes were capable of increasing the fruit yield and decreased *R. solanacearum* the causal agent for bacterial wilt infection on tomato. Bhat et al. (2022) also indicated the potential of actinomycetes as a potential biocontrol agent for *Colletotrichum* spp.

Table 2. Bioactivity produced by actinomycetes isolated.

Samples	Enzyme activity					Antimicrobila activity	
	Cellulase	Mannanases	Xylanase	Lipase	Protease	*Ralstonia solalacearum*	*Colletotrichum gloeosporioides*
Actinomycetes isolated from undisturbed forest	18/70	9/70	17/70	46/70	26/70	16/70	10/70
Actinomycetes isolated from pineapple planted soil	6/70	0/70	9/70	10/70	10/70	3/70	4/70
Total	24/70 (34.3%)	9/70 (12.8%)	26/70 (31.7%)	56/70 (80.0%)	36/70 (51.4%)	19/70 (27.1%)	14/70 (21.4%)

Table 3. Bioactivity of selected 4 actinomycetes isolates.

Isolate no.	Enzyme screening (average ± SD cm)					Antagonistic activity (average ± SD cm)	
	Lipase	Protease	Cellulase	Mannanase	Xylanase	*Ralstonia solanacearum*	*Colletotrichum gloeosporioides*
PS1	1.4± 0.100	1.9± 0.153	1.7± 0.153	1.7± 0.231	4.9± 0.058	1.0± 0.058	1.2± 0.132
PS10	1.9± 0.153	2.3± 0.115	3.1± 0.200	1.1± 0.580	3.9± 0.321	1.2± 0.058	1.0± 0.208
PS42	2.2± 0.200	2.4± 0.200	2.6± 0.153	1.7± 0.200	3.9± 0.173	0	0.5± 0.115
PS46	3.9± 0.300	0	3.0± 0.208	2.3± 0.173	2.8± 0.058	1.0± 0.161	0

It was also observed that actinomycetes collected from forested area showed that undeveloped forest land has higher ability to produce extracellular enzymes and also antimicrobial activities (Table 2). It is believed that microbes in the soil were very sensitive to changes in the soil land use (Romaniuk et al. 2017). Several studies suggested that the function of microbial communities in soil are strongly affected by types of tress and the composition of the soil (Li et al. 2014). This supports well the reason why actinomycetes isolated from forested soil have better bioactivity compared to pineapple plantation soil.

Identification of actinomycetes

Streptomyces spp. had been well known as the dominant actinomycetes in the soil. The morphology of the 4 strains was shown in Figure 4. *Streptomyces griseus* strain PS1 was observed to be having black spore colour while *Streptomyces hyproscopicus* strain PS10 *Streptomyces griseus* strain PS41 and *Streptomyces olivaceus* strain PS46 having grey spore colour. Research done by Ann et al. (2020) indicated that out of 578 strain of actinomycetes isolated from Sarawak wetland, 120 isolates belongs to the *Streptomyces* genus (Ann et al. 2020) this showed that *Streptomyces* spp. were prevalent in soil. In this study, it was obesrved that *Streptomyces griseus* accounted for 2 strains of the potential actinomycetes, followed by 1 strain each for *Streptomyces hyproscopicus* and *Streptomyces olivaceus*. The sequence and relatedness of each strain of *Streptomyces* are shown in Table 4 and Figure 5 respectively.

Table 4: Sequence and identification of 4 selected *Streptomyces* strains.

Isolate No.	Sequence	ID	E value	Percentage of identity
PS1	gcctcccgcg acctgggctt cgactcgctc accgccgtcg acctccgtaa ccgcctcaag gccgccaccg gggagcggct gtccgcgacc gtcgtcttcg accacccgac ccccgccgag ctggccgccc acctccacga ccgttgcttc ccggacgccg acggccggcc gcagcggtcg gtcccggccg tgacggctgt cgccgcgctc cacgacgagc cggtcgcgat cgtcggcatg gcctgccggc tgccgggcgg cgtcaccacc ccggaggagc tgtggcagct cctccgggac ggcggagacg cgatcaccgg cttcccggag aaccgcggct gggacctgga cggtctctac gaccccgatc ccgccacccc cggtaagacc tatgcccgcg acggcggatt cctccacgac gcggcggagt tcgacgcggg gttcttcggg atctcgccgc gtgaggcgct ggcgatggac ccgcagcagc ggctgctgct ggagacgtcc tgggaagcga tcgagcacgc cggcatcgac cccacggtcc tcaagggcac ccgcaccggt accttcatcg gcgccaaccc gtcggactac cgggcggcca tgggacaggc gccggtgggc tacgagggcc acctcgtcac cggaggccac aacagcgtcg tctccggccg gatcgcctac accttcggcc tcgaaggccc ggccgtcacc gtcgacaccg cctgctcctc	*Streptomyces griseus*	0.0	100%
PS10	cctggtagtc cacgccgtaa acggtgggaa ctaggtgttg gcgacattcc acgtcgtcgg tgccgcagct aacgcattaa gttccccgcc tggggagtac ggccgcaagg ctaaaactca aaggaattga cgggggcccg cacaagcagc ggagcatgtg gcttaattcg acgcaacgcg aagaacctta ccaaggcttg acatacaccg gaaaaccctg gagacagggt ccccttgtg gtcggtgtac aggtggtgca tggctgtcgt cagctcgtgt cgtgagatgt tgggttaagt cccgcaacga gcgcaaccct tgtcctgtgt tgccagcatg cccttcgggg tgatggggac tcacaggaga ccgccggggt caactcggag gaaggtgggg acgacgtcaa gtcatcatgc cccttatgtc ttgggctgca cacgtgctac aatggccggt acaaagagct gcgataccgt gaggtggagc gaatctcaaa aagccg	*Streptomyces hygroscopicus*	0.0	100%
PS42	cttaacacat gcaagtcgaa cgatgaagcc tttcggggtg gattagtggc gaacgggtga gtaacacgtg ggcaatctgc ccttcactct gggacaagcc ctggaaacgg ggtctaatac cggataacac tctgtcccgc atgggacggg gttaaaagct ccggcggtga aggatgagcc cgcggcctat cagcttgttg gtggggtgat ggcctaccaa ggcgacgacg ggtagccggc ctgagagggc gaccggccac actgggactg acacacggcc cagactccta cgggaggcag cagtggggaa tattgcacaa tgggcgaaag cctgatgcag cgacgccgcg tgagggatga cggccttcgg gttgtaaacc tctttcagca gggaagaagc gaaagtgacg gtacctgcag aagaa	*Streptomyces griseus*	0.0	100%
PS46	gagcatgtgg cttaattcga cgcaacgcga agaaccttac caaggcttga catacaccgg aaacggccag agatggtcgc ccccttgtgg tcggtgtaca ggtggtgcat ggctgtcgtc agctcgtgtc gtgagatgtt gggttaagtc ccgcaacgag cgcaaccctt gtcccgtgtt gccagcaagc tccttcgggg gtgttgggga ctcacgggag accgccgggg tcaactcgga ggaaggtggg gacgacgtca agtcatcatg ccccttatgt cttgggctgc acacgtgcta caatggccgg tacaatgagc tgcgataccg caaggtggag cgaatctcaa aaagccggtc tcagttcgga ttggggtctg caactcgacc ccatgaagtc ggagtcgcta gtaatcgcag atcagcattg ctgcggtgaa tacgttcccg ggccttgtac acaccgcccg tcacgtcacg aaagtcggta acacccgaag ccggtggccc aacccttgt gggagggagc tgtcgaaggt gggactggcg	*Streptomyces olivaceus*	0.0	100%

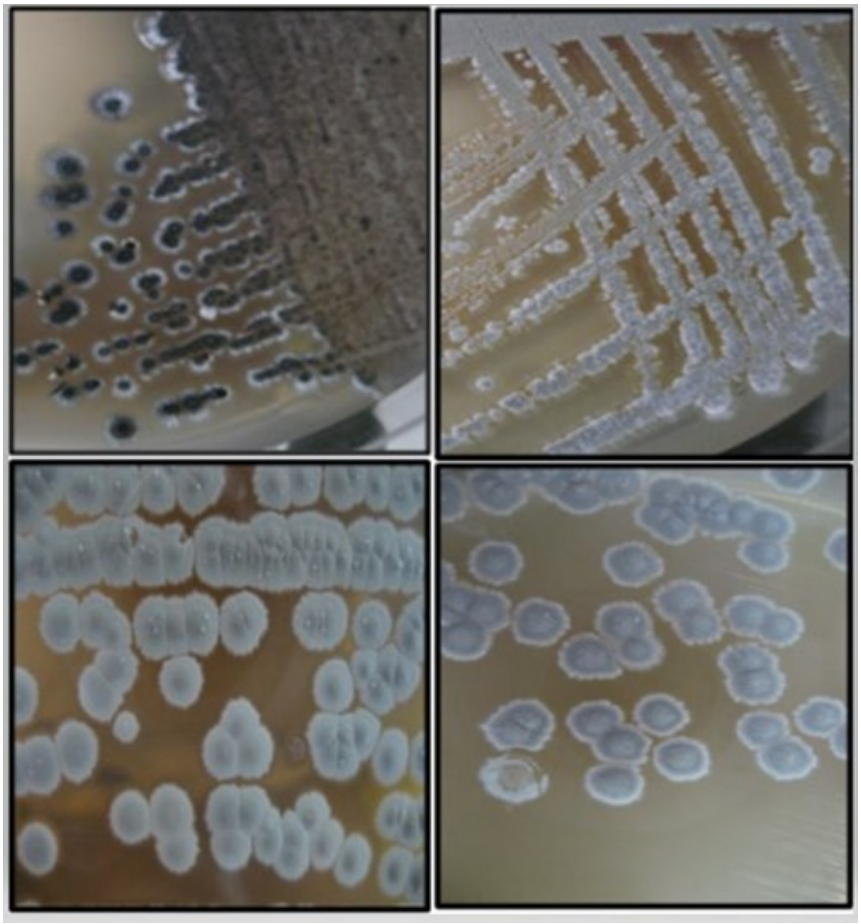

Figure 4: Morphology of 4 selected *Streptomyces* strain, (a) *Streptomyces griseus* strain PS1; (b) *Streptomyces hyproscopicus* strain PS10; (c) *Streptomyces griseus* strain PS42 and (d) *Streptomyces olivaceus* strain PS46.

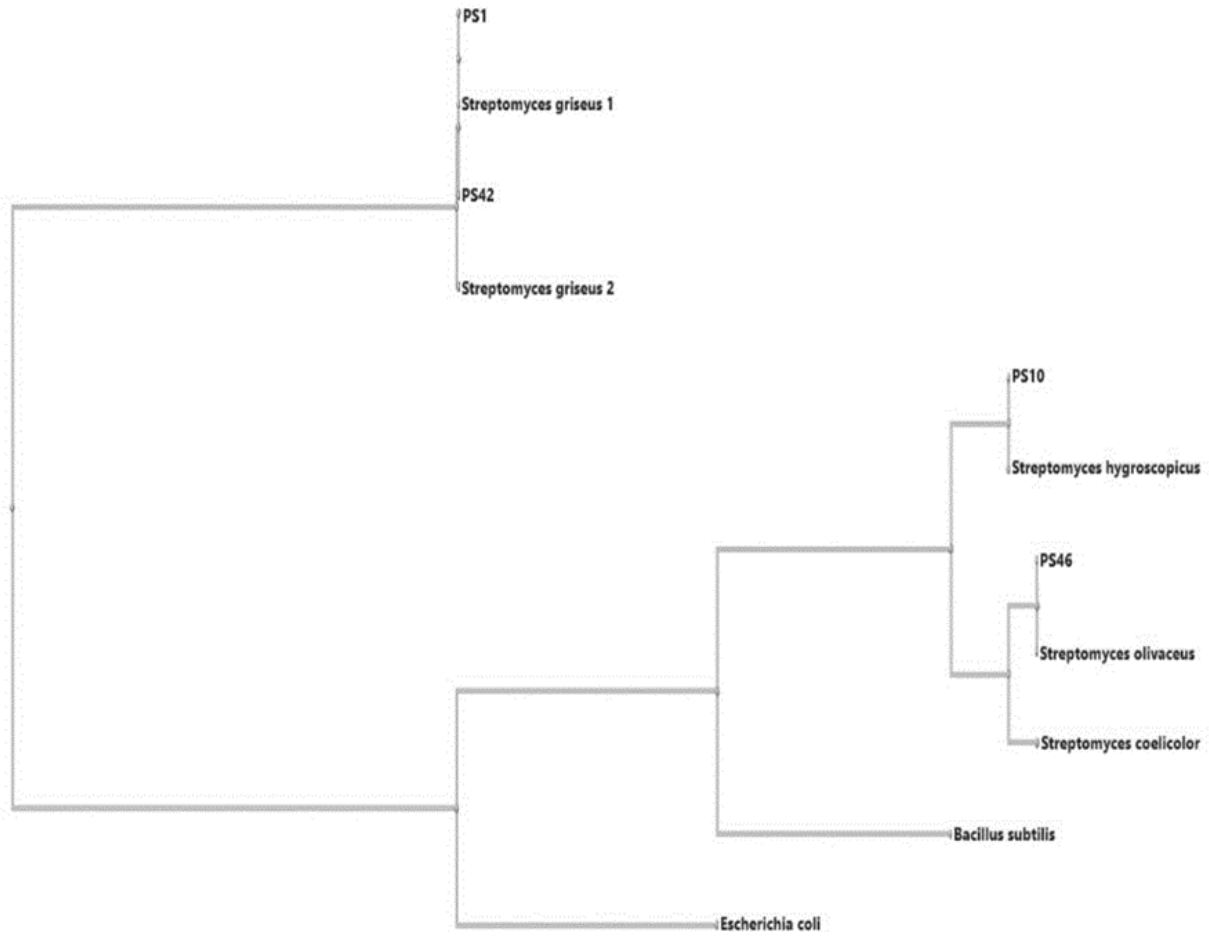

Figure 5: Phylogenetic tree for 4 selected *Streptomyces* strain.

CONCLUSION

Morphological diversity of the actinomycetes was observed from the spore forming colour of grey, brown, and black. The characteristic of the actinomycetes was observed when 34.3%, 12.8%, 31.7%, 80.0%, and 51.4% of the actinomycetes produced cellulase, mannanase, xylanase, lipase, and protease activity respectively. While 27.1% and 21.4% produced antagonistic activity towards *Ralstonia solanacearum* and *Colletotrichum gleosporioides* respectively. All the eight potential actinomycetes isolated were later identified to originate from the genus of *Streptomyces* sp. This study showed the potential usage of peat soil actinomycetes as well as the vast diversity of the actinomycetes in peat soil.

AUTHORS CONTRIBUTION

J.L.S.H. designed and conducted experiment, analysed data and wrote the article. H.H. conducted experiment, collected data and wrote material and methods section. N.A.N. conducted experiment, collected data and wrote introduction section.

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CONFLICT OF INTEREST

There is no conflict of interest regarding to this research.

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