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Biodegradation of low density polyethylene (LDPE) by a new biosurfactant-producing thermophilic *Streptomyces coelicoflavus* NBRC 15399^T

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In the present study, we tested 83 actinomycete isolates for low density polyethylene (LDPE) degradation in the laboratory, and these isolates were previously isolated for biosurfactant production from the oil contaminated soil sample near Naval Dockyard in Visakhapatnam. Among them, 20 were positive using 2,3,5-triphenyl tetrazolium chloride (TTC) as a viability indicator and able to grow in a mineral salts medium containing LDPE as a sole source of carbon. Of these, NDYS-4 isolate showed prominent result with redox probe 2,3,5-triphenyltetrazolium chloride (TTC) as a viability indicator, which forms pink colour insoluble triphenylformazan (TPF) on mineral salts media containing emulsified polyethylene as a carbon source within five to seven days, and it was also grown prominently on mineral salt agar plates containing LDPE in comparison to other isolates, and it was selected for detailed analysis. The weight loss of polyethylene films was approximately 30% after four weeks of incubation with selected isolate. The viability and metabolic activity of this isolate growing on the polyethylene surface was confirmed using a TTC reduction test. The metabolic activity was also correlated with a concomitant increase in the protein density of the biomass on the surface of the polyethylene. The microbial degradation of LDPE was also analyzed by the change in pH of the culture media and microscopic analysis. Based on the results, this degradation may be mediated by enzymatic activity and may also involve cell-surface hydrophobicity. It was also able to grow on other polymers such as polyvinyl acetate (PVA), polycaprolactone (PCL), polyethylene oxide (PEO) and polyethylene glycol (PEG) using TTC as a viability indicator and also showed prominent growth on mineral salts agar plates containing polyethylene as a source of carbon. Based on the results of phenotypic characteristics, phylogenetic analysis, biochemical characteristics and 16S rRNA gene sequence homology, the isolate NDYS-4 was identified as *Streptomyces coelicoflavus* NBRC 15399^T. It could be concluded that the PE degrading actinomycete selected in this study showed diverse and varying capacities to degrade polyethylene and other polymers and can be exploited for cleaning up polyethylene contaminated sites.

Key words: Biosurfactant, low density polyethylene, 2,3,5-triphenyl tetrazolium chloride, mineral salts media, hydrophobicity, sequence homology, phylogenetic analysis.

INTRODUCTION

Biosurfactants are heterogeneous group of secondary metabolites with surface active properties, and are synthesized by a variety of microorganisms (Chandrasekaran et al., 1978). Biological surfactants possess a number of potential advantages over their chemically manufactured counterparts, including lower toxicity, biodegradability (Zajic et al., 1983) and effectiveness at extreme temperatures, pH and salinity (Kretschmer et al., 1982).

The use of synthetic plastic by any means has changed the nature of waste in last three to four decades (Sheavly, 2005). Over this period, it has replaced natural material in various aspects of human life and is non-degradable thereby causing serious environmental and human health problems. These plastics are used extensively because of their availability, durability and light weight and low cost. According to the Central Pollution Control Board (CPCB), New Delhi, India, 8 million tons of plastic products are consumed every year in India. A study on plastic waste generation in 60 major cities in India revealed that approximately 15,340 tons per day of plastic waste is generated in the country (CPCB, New Delhi, India, 2013). The UV irradiation (photo-oxidation) (Esmaeili et al., 2013), thermal and chemical oxidation of PE prior to its exposure to a biotic environment enhances biodegradation (Volke-Sepuleveda et al., 2002). These pre-treatments increase surface hydrophilicity of the polymer by forming polarisable groups such as carbonyl groups that can be utilized by microorganisms (Albertsson, 1978; Albertsson, 1980; Cornell et al., 1984). Among these plastics, low density polyethylene (LDPE) is used mainly as carrier bags, in agricultural and other industrial applications constitutes the major portion of waste problem. LDPE is chemically inert and microbial attack resistance as it possess hydrophobic nature and absence of carbonyl and hydroxyl groups.

The generation of biodegradable polyethylene requires modifying the properties that are responsible for the PE resistance to degradation. A standard test to determine the biodegradation of plastic materials when exposed to soil was developed by the ASTM D 5988 (2003). Moreover, plastics pollute beaches and oceans, and kill marine fauna. These environmental issues raise concerns regarding the use of plastics. Since, the plastics have become an integral part of modern life; it is not possible to discontinue the use of these waste materials. Therefore, there is an emergency requirement of biotechnological approaches to solve these environmental challenges related to plastics. Disintegration of LDPE and subsequent degradation by microorganisms using only polymers as sole source of carbon is the main strategy (Roy et al., 2008).

Furthermore, actinomycetes also play an important role in polyester degradation (Tokiwa and Pranamuda, 2001). Most of the studies about high temperature of PES-degradation were focused on bacteria. A thermophilic bacterium, for example, is able to degrade PES at 50°C (Tansengco and Tokiwa, 1998). Pranamuda et al. (1997) had isolated a PLA-degradable actinomycete, *Amycolatopsis* strain HT-32, and Jarerat and Tokiwa (2003) had isolated the *Saccharothrix wayanrdensis* and *Kibdelosporangium aridum*. Sanchez et al. (2000) had found the polycaprolactone (PCL)-degradable fungi, *Aspergillus* sp. However, the studies about the PES degradation by actinomycetes on high temperature are still rare in the literature. The use of thermophilic actinomycetes for biodegradation of polymers such as polyethylene succinate, polyhydroxybutyrate and polycaprolactone at different temperatures, have gained notable importance because the inefficiency of the physical and chemical methods for disposal of these materials in the environment (Kim-Chi et al., 2007). Biodegradability of plastic wastes under natural conditions is required in the management of these solid wastes (Orhan and Buyukgungor, 2000). Therefore, the present study focused mainly on the biodegradation of LDPE by actinomycetes and its cultural and morpho-logical characteristics.

MATERIALS AND METHODS

Low density polyethylene

The low density polyethylene granules (size range between 35-65 mm) were provided by Sai Polymers, Auto nagar, Visakhapatnam (India). The granules were dissolved in xylene (Fisher Scientific, 97% pure) by heating for 15 min; then, the residue was crushed while it was warm followed by filtration. The powder so obtained was washed with ethanol to remove residual xylene and allowed to evaporate to remove ethanol. The powder was dried in hot air oven at 60°C overnight to remove residual solvent and obtain dry fine powder.

Collection of soil

According to Kalyani et al. (2014), the mixture of soil with petrochemicals produces actinomycetes, with biosurfactant production, which requires further screening of actinomycetes for degradation of polyethylene and other polymers.

Screening of actinomycetes for polyethylene degradation

The biosurfactant producing actinomycetes from oil contaminated

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soil were isolated on humic acid vitamin agar (Hayakawa and Nonomura, 1987) (1.0 g humic acid, 1.0 g yeast extract, 1.7 g KCl, 0.5 g Na_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.02 g $CaCO_3$, 50 mg nalidixic acid, 50 mg cyclohexamide, 0.25 mg biotin, 0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxin-HCl, inositol, Ca-pantothenate, p-aminobenzoic acid and agar 18.0 g, distilled water 1.0 L, pH 7.2) plates using dilution technique and incubated at room temperature for one week, then observed in long working distance objective lens. The powdery colonies withbranched hyphae were picked and streaked on Bennett's agar [1.0 g beef extract, 1.0 g yeast extract, 2.0 g N-Z amine A (enzymatic digest of casein), 10 g glucose, 15.0 g agar, distilled water 1.0 L, pH 7.3] plates, then maintained as glycerol suspensions (20% v/v) at -80°C.

These isolates were then assayed for their ability to utilize polyethylene as the sole source of carbon and energy. They were grown in basal medium containing (per liter): yeast extract, 0.1 g; $FeSO_4.7H_2O$, 10 mg; $MgSO_4.7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1 g; $CaCl_2.2H_2O$, 20 mg; NaCl, 0.1 g; $Na_2MoO_4.2H_2O$, 0.5 mg; $NaWO_4.2H_2O$, 0.5 mg; $MnSO_4.H_2O$, 0.6 mg with a pH 7.2. Then, 20 µl of 1% TTC solution was added to 10 mL of medium as an indicator of viability (Alef and Nannipieri, 1999).

Furthermore, the screening actinomycetes were performed by comparing their growth ability in solid media containing 2% liquid oligomer as the sole source of carbon. The actinomycetes with the ability to grow in the presence of 5% ethylene oligomer were transferred to synthetic mineral medium containing 0.1% LDPE powder as the sole source of carbon for the final screening step (Atefeh et al., 2013).

Morphological and cultural studies

The colour of aerial mycelium, substrate mycelium and soluble pigment when grown on different media were observed. The macro and micro morphological features of the colonies and the colour determination of the aerial mycelium, substrate and soluble pigment were examined every 24 h for 7 to 14 days of incubation. Macro morphology was observed by the naked eye and also by using 10x, 40x magnifying lens.

Micro morphology (spore morphology)

Study of the aerial mycelium and its sporulation characteristics was carried out by inclined cover slip method (Williams and Davies, 1967). Sterile cover slips were placed at an angel 45° into solidifying agar medium in Petri plate such that a part of the cover slip was in the medium. Inoculum was spread along the line where the upper surface of the cover slip meets the medium. After full sporulation, the cover slips were removed and examined directly under the microscope (Labo America Inc., LABOMED USA CXR3 9122100).

Identification of actinomycetes

The molecular identification and characterization of the actinomycete was carried out by 16S rRNA gene sequencing which was performed at Institute of Microbial Technology (IMTECH), Chandigarh (India). The similarity search was conducted *in-silico* using the basic local alignment search tool (BLAST) database of NCBI. The scanning electron microscope of the actinomycete was done by RUSKA Laboratory's College of Veterinary Sciences, SVVU, Rajendra nagar, Hyderabad (India).

Physiological and biochemical characterization

The ability to grow at various temperatures (12-42°C), pH (5.0-10.0) and different concentrations of NaCl (2-10% w/v) on medium was also tested. The organism was also tested for its ability to utilize carbon sources such as D-glucose, galactose, L-arabinose, D-fructose, raffinose, Meso-inositol, D-mannitol, sucrose, salicin and rhamnose in modified Bennett's broth. Cultural characteristics of this strain were determined following incubation for 10-15 days at 28-42°C on various media. After incubation, the growth and colour of spore mass and diffusible pigment production were observed.

Chemotaxonomy

Isomers of diaminopimelic acid (DAP) and sugars present in hydrolysates of whole cell actinomycete were determined by thin-layer chromatography following the standard methods of Waiksman and Henrici (1943) and Boone and Pine (1968).

Polyethylene film biodegradation assay

The biodegradation tests were performed on samples of low-density polyethylene film (that is, 2×1 cm pieces of polyethylene bags) that had been dried overnight at 60°C, weighed, disinfected (autoclaved at 105°C for 1 h) and added to each flask (approximately 50.0 mg of polyethylene film per flask) containing 50 mL of basal medium. The flasks were inoculated with 3 mL of a mid-exponential phase culture that were maintained in Bennett's agar medium. Before inoculation, the culture was washed with basal medium to remove medium and cellular soluble debris. The cell densities of the inoculums were adjusted to 1.5×10^6 colony forming units (CFU) per mL. The flasks containing non-inoculated basal medium supplemented with polyethylene film served as the control.

Weight loss measurements

To facilitate accurate measurement of the residual polyethylene weight, the bacterial film colonizing the polyethylene surface was removed by supplementing the cultures with a 2% (v/v) aqueous sodium dodecyl sulfate (SDS) solution. The flasks were then incubated for 4 h at 50°C and further washed with warm distilled water (Sivan et al., 2006). The polyethylene samples were collected on filter paper, rinsed with distilled water and then dried overnight at 60°C before they were finally weighed. The initial weights of the pre-incubated polyethylene samples were measured following the same procedure mentioned above.

Viability and metabolic activity of the isolates

The viability and metabolic activity of the surface-attached actinomycete was measured with the redox probe 2,3,5-triphenyltetrazolium chloride (TTC), which facilitates direct monitoring of actively respiring actinomycete. The colorless TTC is readily reduced by the microbial electron transport system (ETS) to pink-colored insoluble triphenylformazan (TPF). The respiration was monitored by measuring the level of TPF (Alef and Nannipieri, 1999). Surface adhered cells were monitored for their viability. Cell pellets were washed twice with 50 mM phosphate buffer at a pH of 7.6 and centrifuged at 5000 rpm for 10 min. Then, the pellets were re-suspended in 4.5 mL of buffer solution, and 0.5 mL of TTC (0.1 g/L) was added. The mixture was incubated at 37°C for 15 min in a water bath. Then, 5 mL of 96% cold methanol was added to stop the reaction and to begin the extraction of TPF. The enzyme activity was quantified at the absorption spectra of 480 nm in the spectro-

photometer. Ice-cold methanol and a phosphate buffer solution at a 1:1 ratio served as a blank.

Estimation of protein content of the surface attached actinomycetes

The total protein content of the surface-attached biomass was determined after alkaline hydrolysis, as follows. The polyethylene pieces were sampled from flasks containing mineral salt cultures of actinomycete isolates, washed gently with water to remove medium debris and boiled for 30 min in 4.0 mL of 0.5 M NaOH. The extracts were centrifuged to precipitate cell-debris fragments. The supernatants were collected, and the pellets were subjected to the same procedure to minimize the experimental error. The collected supernatants were combined, and the protein content in the extract was determined spectrophotometrically at 280 nm.

Determination of pH change

Study of pH change was adopted to make sure any metabolic activity of the NDYS-4 in supplemented medium, as metabolism shown by microbial cells may greatly support the evidence of degradation. The pH of the medium inoculated with actinomycete was measured daily during the study. The pH probe was inserted in the medium to measure the pH. Initial pH of the medium was ensured to be 7.2 ± 0.3. The medium uninoculated with microbe served as negative control.

Evaluation of cell surface hydrophobicity

The method used to determine bacterial cell-surface hydrophobicity: the bacterial adhesion to hydrocarbon (BATH) test (Rosenberg et al., 1980) was carried out. The BATH assay for bacterial hydrophobicity is based on the affinity of bacterial cells for an organic hydrocarbon such as hexadecane. The more hydrophobic the bacterial cells, the greater their affinity for the hydrocarbon, resulting in a transfer of cells from the aqueous suspension to the organic phase and a consequent reduction in the turbidity of the culture. For this assay, bacteria were cultured in starch casein medium until the mid-logarithmic phase, centrifuged and washed (twice) with PUM buffer containing (per liter): 17 g K_2HPO_4, 7.26 g KH_2PO_4, 1.8 g urea and 0.2 g $MgSO_4 \cdot 7H_2O$. The washed cells were resuspended in PUM buffer to an optical density at 400 nm (OD_{400}) value of 1.0 to 1.2. Aliquots (1.2 mL each) of this suspension were transferred to a set of test tubes, to which were added increasing volumes (range 0 to 0.2 ml) of hexadecane. The test tubes were shaken for 10 min and allowed to stand for 2 min to facilitate phase separation. The OD_{400} of the aqueous suspension was measured. Cell-free buffer served as the blank.

Microscopic observation of the polyethylene

The untreated and treated samples after two weeks days of duration were subjected to microscopic analysis (after washing thrice with 2% (w/v) aqueous sodium dodecyl sulphate and warm distilled water repeatedly through mild shaking for few minutes and additionally flushed with 70% ethanol with the objective of removal of cells so as to get maximum surface to be exposed for visualization, air dried overnight and then, the degradation was observed with a trinocular microscope (America Inc., LABOMED USA CXR3 9122100).

Table 1. Screening of polyethylene degrading actinomycetes using TTC as a viability indicator and their growth on mineral salts agar plates with polyethylene as a carbon source.

Strain	Viability and growth
NDYS-4	+++
NDYS-6	++
NDYS-8	+
NDYS-17	+

+ Indicates intensity of pink colour and growth.

RESULTS

Isolation and screening of PE degrading actinomycetes

A total of 83 strains of biosurfactant producing actinomycetes were tested for biodegradation; only 20 isolates shown positive result with TTC as a viability indicator. Of these, four samples displayed intense pink colour (Supplementary Figure 1), which indicates that the occurrence of degradation microorganisms in the polyethylene. It is the simplest method for investigating microbial degradation of aliphatic polymer since the formation of pink colour in the mineral salts medium indicates solubilisation of the polymer by the enzyme secreted from the microbes (Nishida and Tokiwa, 1993; Pranamuda et al., 1995). The pink colour was formed when strains secrete extracellular enzymes in order to breakdown compounds into soluble materials (Fields et al., 1974). Out of 20 strains, only four isolates of NDYS had shown intense pink colour (Table 1 and Supplementary Figure 1) and of these, NDYS-4 had shown PE degradation efficiently by forming intense pink colour.

In addition, the screening of these 20 isolates was performed by comparing their growth ability in solid media containing synthetic mineral salts medium supplemented with 2% ethylene oligomer, resulting in the selection of four isolates. Of these isolates, one actinomycete isolate was selected as the final strain by comparing their growth ability in mineral salts agar medium containing LDPE powder as the sole source of carbon (Table 1). This result agrees with the TTC reduction test, which was used to test the viable actinomycetes on mineral salts media.

Identification and characterization of the isolate NDYS-4

Morphology

Outer surface of colonies were perfectly round initially, but later developed thin wavy mycelium. The colour of the

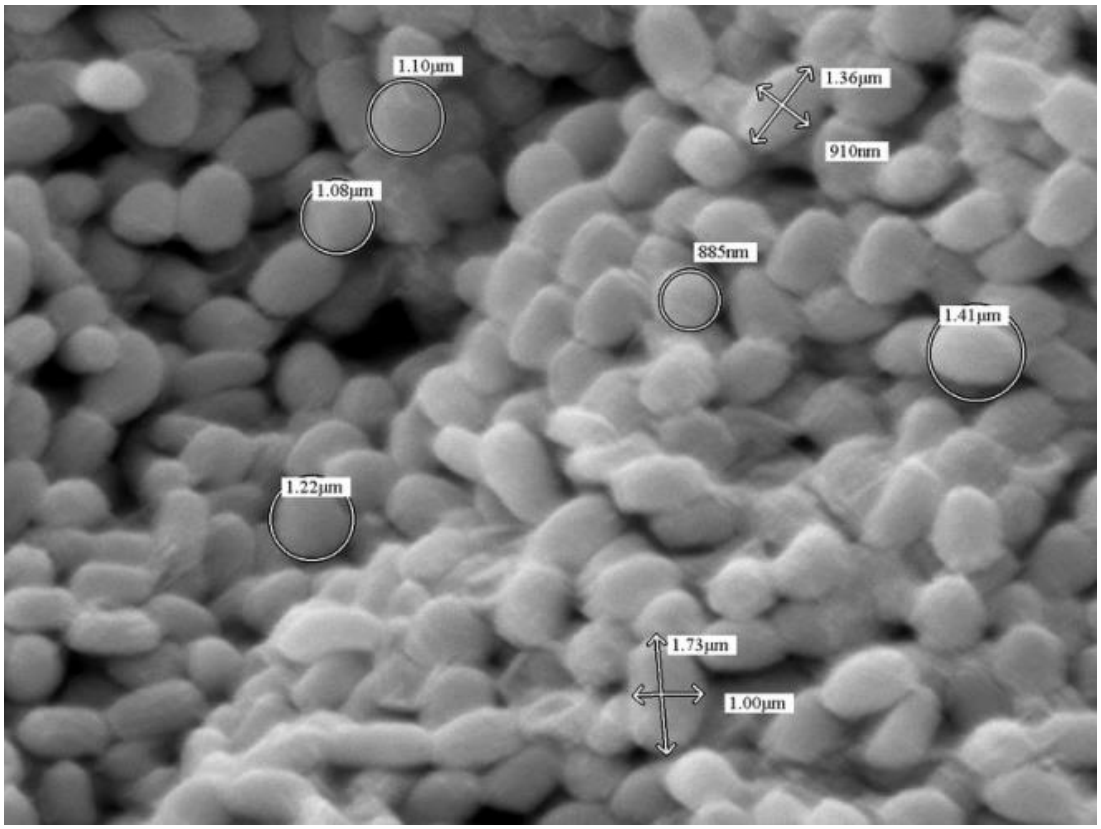

Figure 1. Scanning electron microscopy image of strain NDYS-4.

aerial mycelium observed was white and colour of the substrate mycelium was pink by studying the morphology (Supplementary Figure 2), SEM (Figure 1), 16s rRNA gene sequencing (Supplementary Figure 3), homology (Supplementary Table 1) and phylogenetic tree (Figure 2); the isolated strain was found to be *Streptomyces coelicoflavus.*

Growth on different media

The cultural characteristics of strain NDYS-4 are shown in Supplementary Table 2. The growth was prominent on most of the media and the substrate mycelia on most media tested were light pink. Aerial mycelia were white powdery in color. Diffusable pigments were adsent in all of the media.

Physiological and biochemical characteristics

Citrate utilization, methyl red test, catalase reduction and urea reaction were positive. Starch hydrolysis, casein hydrolysis, gelatin liquefaction, voges-prauskouer test, nitrate reduction, indole production, hydrogen sulphide production, tyrosine reaction and oxidase utilization were not observed. Only LL-diaminopimelic acid and glycine were detected in whole cell hydrolysates, no diagnostic sugars were present, indicating NDYS-4 has a chemo type I cell wall and can be grouped under the genus *Streptomyces* (Supplementary Figure 3 and Table 3).

The chemical and physiological characteristics of strain NDYS-4

Utilization of carbon source was observed in mannitol, rhamnose and fructose. However, arabinose, glucose, galactose, raffinose, salicin, sucrose and meso-inositol were not utilized. Moderate to good growth was observed with L-histidine, sodium nitrate, yeast extract and L-arginine but no growth was observed with valine. Sodium chloride tolerance range was between 0-10% and optimum concentration was between 2 and 7% (Supplementary Table 4a, b). The temperature and pH tolerance range were 12-42°C and 5.0 to 10.0, respectively. The optimum temperature was 28 ± 2°C.

Determination of weight loss

The selected actinomycete was allowed to degrade the polyethylene under shaking condition for four weeks to observe the percent weight loss of polyethylene films

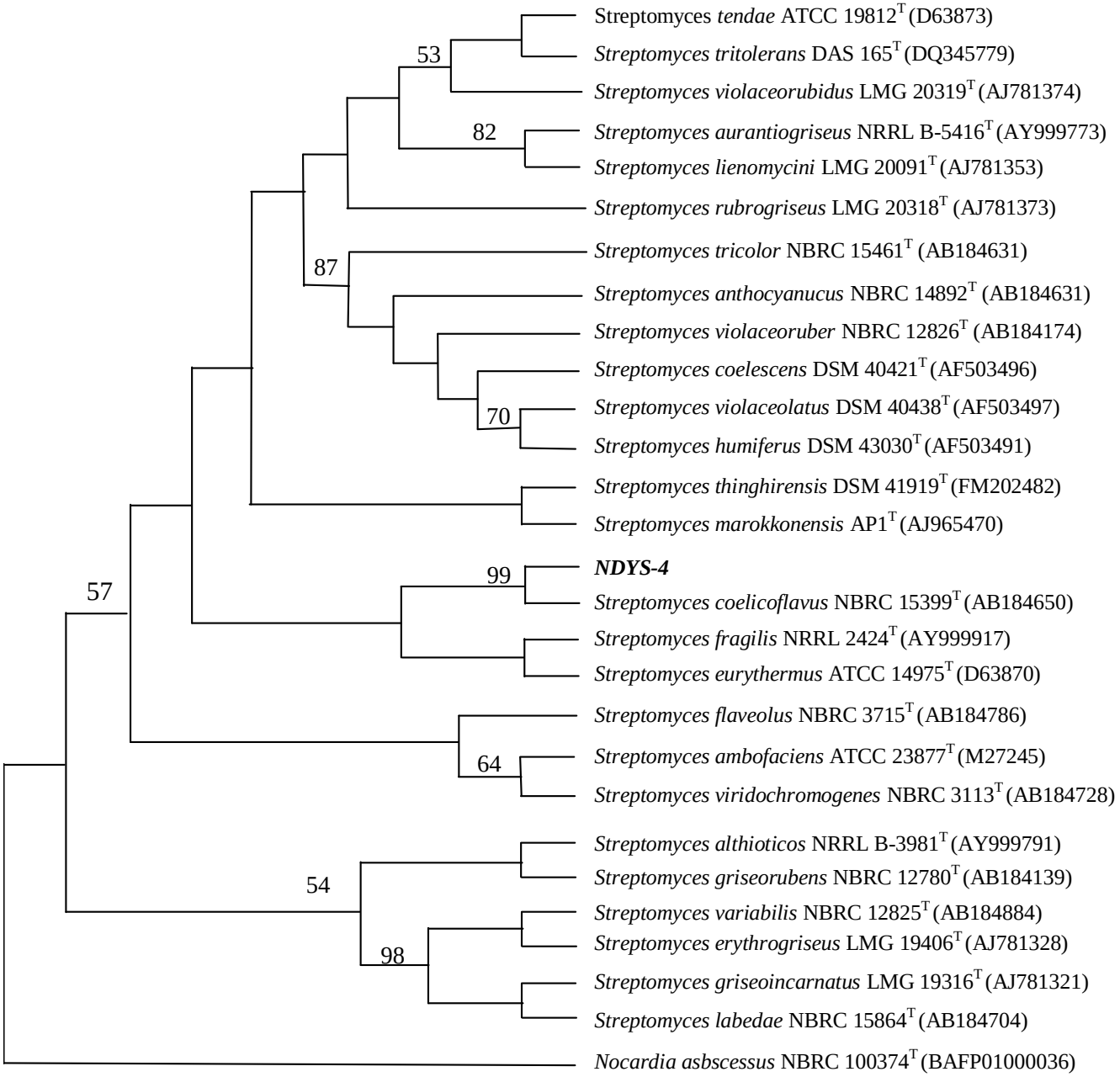

Figure 2. A phylogenetic dendrogram showing the relationship between the 16S rRNA gene sequences. The evolutionary relationship was inferred using the Neighbor-Joining method.

weekly because the complete sporulation of actinomycetes usually takes one week. The percentage of weight reduction was 3, 10, 19 and 30% after incubation for 1, 2, 3 and 4 weeks, respectively with S. coelicoflavus. The low percent weight loss after one week may be due to the complete maturation of actinomycetes which takes six to seven days. The weight loss of the polythene films can be attributed to the breakdown of carbon backbone due to enzymatic degradation by this actinomycete.

Viability and metabolic activity of the actinomycete

The respiration of the surface-attached actinomycete on the polyethylene films was monitored regularly for 30 days. The reduction of TTC to TPF, driven by the ETS, was used to determine the respiration level of the surface-attached bacteria. The formation of TPF increased sharply on the seventh day of incubation, followed by a constant increase until the 6th week (Figure 3). This observation indicates a regular increase in the

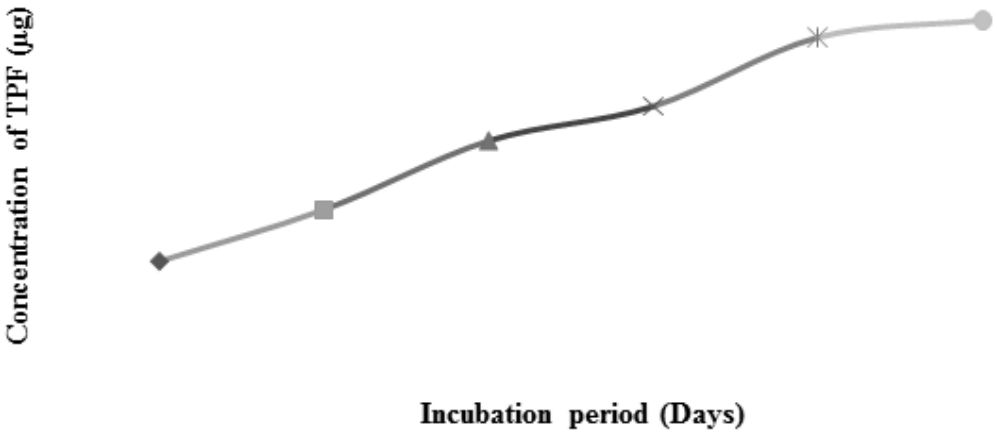

Figure 3. The respiration of surface attached actinomycete culture *Streptomyces coelicoflavus* NBRC 15399^T in basal medium supplemented with polyethylene as a sole source of carbon. The respiration was measured by the intracellular reduction of TTC by cytochrome of the electron transport system to TPF.

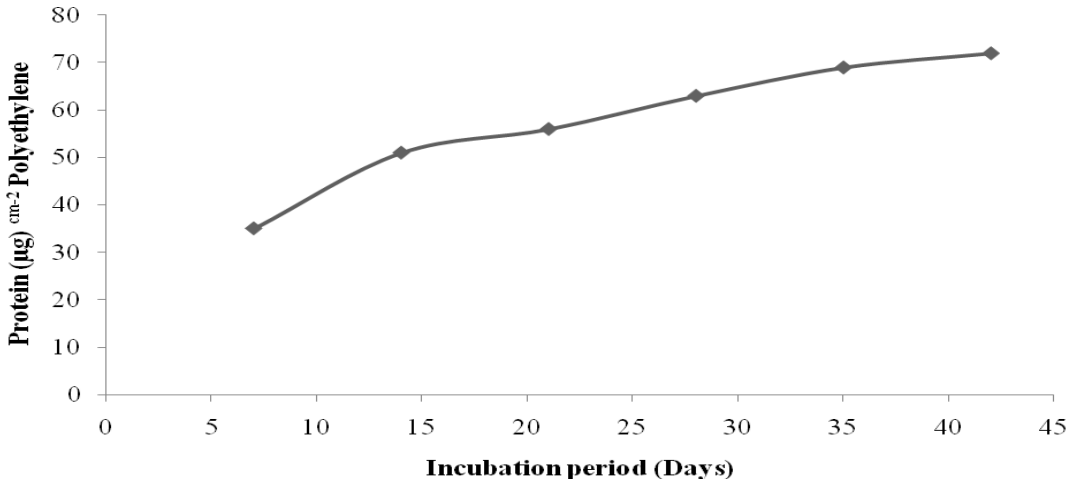

Figure 4. The protein content of the surface attached actinomycete culture *Streptomyces coelicoflavus* in mineral salts medium supplemented with polyethylene as a sole source of carbon.

respiration rate and suggests isolate viability and growth indicating it utilizes the polyethylene as a carbon source.

Growth kinetics of surface attached actinomycete

The growth kinetics of actinomycete on the polyethylene film surface was monitored by quantifying the total protein extracted from the surface of the film. The data depicted in Figure 4 shows increase in protein content over six weeks of incubation and is reflected by an increase in the surface-attached biomass. The continuous increase in extractable protein suggests a growth of isolates on the polyethylene. This data may also suggest that the biomass on the polyethylene is proliferating. This result agrees with the TTC reduction analysis, which was used to measure the metabolic activity of the actinomycete.

pH change

The pH value is a key factor for the survival and activity of microorganisms. Generally, the pH should be between 6 and 8 (ASTM D 5988, 2003). The daily measurements of the medium pH are presented in Figure 5. The change in pH of the medium indicates the metabolic activity of the actinomycete in the mineral salts medium containing polyethylene films as a sole source of carbon. The pH of the medium starts decreasing on 3rd day of incubation (Figure 5).

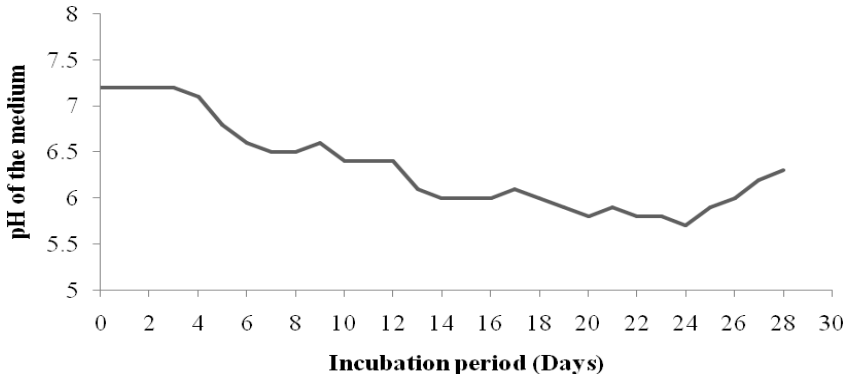

Figure 5. Variation in pH level during biodegradation due to microbial activity.

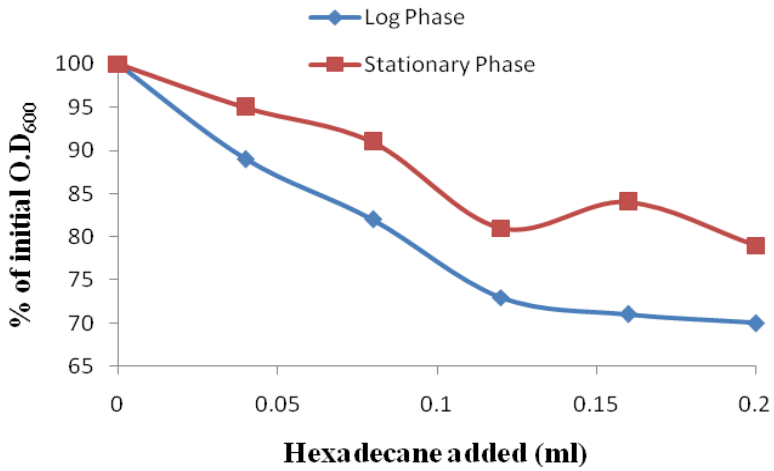

Figure 6. Hydrophobicity of NDYS-4 was determined by the bacterial adhesion to hydrocarbon test. Aliquots of logarithmic and stationary cell suspensions were supplemented with increasing concentrations of hexadecane. The transfer of hydrophobic cells from the aqueous phase to the hexadecane is reflected as a decrease in the turbidity (optical density at 600 nm, $O.D._{600}$) of the bacterial suspension.

Bacterial hydrophobicity

To determine the bacterial interaction with polyethylene, the bacterial cell surface hydrophobicities of the positive isolates, in both log and stationary phases, were assayed using a BATH test and the results are shown in Figure 6. The isolate showed only an 11% reduction in turbidity at the lowest concentration and an approximately 30% reduction at the maximum concentration. The logarithmic cells were more hydrophobic than those of the stationary phase. The greater affinity to hydrocarbon (hydrophobicity) suggests a higher colonization interaction of the isolate with the polyethylene surface.

Microscopic analysis of polyethylene films

Polyethylene films were incubated in liquid cultures as a substrate to examine their degradability. The films would conform to degrade at day five and the quick degradation was observed after eight days. The surfaces of PE films became rough and small cracks were formed in the inoculated culture after 15 days (Figure 7b). On the contrary, the film surfaces were smooth in the system without cell inoculation cultures (Figure 7a).

Degradation of other polymers

The appearance of pink colour in basal medium indicated that the polymer could be hydrolyzed by the enzyme into water soluble products (Nishida and Tokiwa, 1993). The pink colour formed not only on PE-emulsified broth but also on PCL, PVA, PEG and PEO indicating that the actinomycete *Streptomyces coelicoflavus* utilize these polymers for its growth. The results are shown in Table 2.

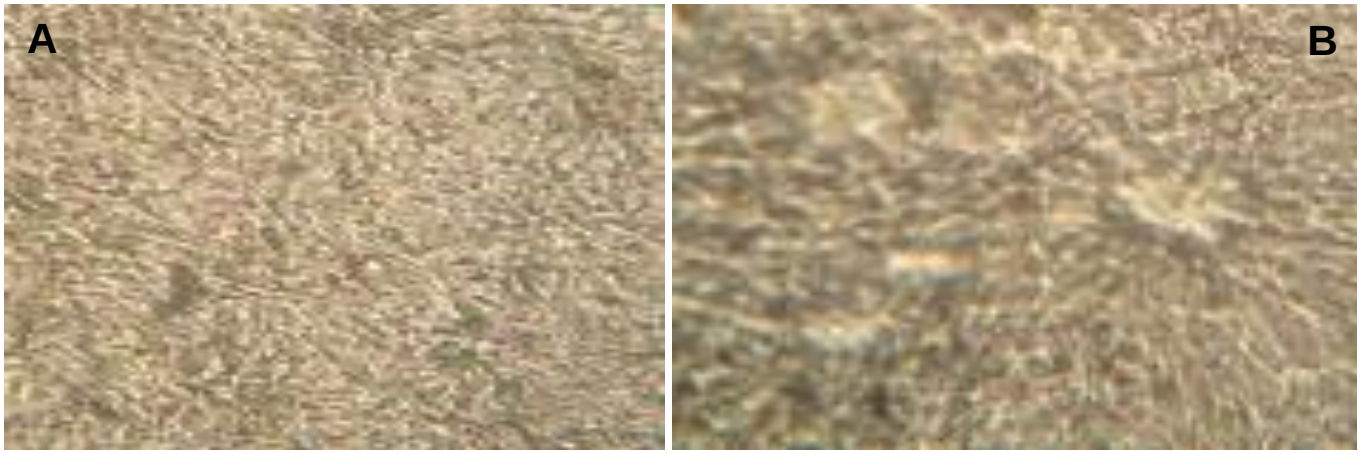

Figure 7. Surface structures of polyethylene films on microscope. (A) Without cell inoculation; (B) With cell inoculation NDYS-4.

Table 2. Viability of the isolate NDYS-4 on other polymers using TTC as a viability indicator.

Polymer	Viability (intensity of pink colour)
Polyethylene	+++
Polycaprolactone	+
Polyvinyl acetate	+
Polyethylene glycol	++
Polyethylene oxide	++

+ Indicates the intensity of pink colour

DISCUSSION

Degradation of LDPE by microorganism had been known for several years and there is no report on biodegradation of LDPE by *S. coelicoflavus* so far. This is the first experimental report on low density polythene (LDPE) utilization as a carbon source under laboratory conditions by showing effective ability of *S. coelicoflavus*.

The selected isolate NDYS-4 showed 100% homology with *Streptomyces graminearus*, 99% homology with *Streptomyces fradiae* and 99% homology with *Streptomyces violaceoruber.* The basic local alignment search tool (BLAST) result and phylogenetic analysis confirmed that their similarity to the respective species. The viability was found on basal medium containing LDPE powder as a sole source of carbon within five to seven days of incubation in all the four strains of NDYS; however the growth development was rapid in the case of NDYS-4. In the present study, the colour of the viable cells increased steadily during the first 7 days to 42 days of incubation in basal media. The formation of pink colored insoluble TPF increased sharply on the seventh day of incubation, followed by a constant increase until the 6^{th} week. This indicates a regular increase in the respiration rate and suggests viability and growth of actinomycete but in case of bacteria the formation of TPF increased sharply on the first day of incubation, followed by a constant increase until the fourteenth day (Kumar and Bhavanth, 2013). Due to their actively increasing metabolism after 42 days of incubation supporting the additional use of this actinomycete for biodegradation of low density polyethylene; dehydrogenases, a group of intracellular enzymes, are involved in microbial oxidoreductases metabolism. These enzymes have been frequently used as an index of microbial activity in soil (Alef and Nannipieri, 1999). The activities of these enzymes are linked to respiratory and energy processes in the cell, which depend on the metabolic state of microorganisms. To determine dehydrogenase activity, tetrazolium salts such as TTC were used as artificial electron acceptors. TTC replaces oxygen as the final H^+/e acceptor and is reduced by the aerobic cytochrome system to water-insoluble, red-colored formazans by microbial activities (Alef and Nannipieri, 1999; Schimmel and Morrison, 1989). From this, it is clearly understood that the viability and growth are occurring conventionally in the presence of LDPE as a carbon source in this study. These results are also supported by the growth kinetics of actinomycete on mineral salts medium containing polyethylene.

The application of biosurfactants in bioremediation process is currently an ambiguous topic. The ability of biosurfactant producing actinomycete for degradation of low density polyethylene in the present study indicates that these two processes are closely related. Several studies confirm that biosurfactants exhibit higher biodegradability compared to surfactants of synthetic origin (Lima et al., 2011a, b). Albertsson et al. (1993) showed that Tween 80 increased the adhesion and biodegradation of polyethylene by *Pseudomonas aeruginosa*, and Yamada-Onodera et al. (2001) reported that the nonionic surfactant Triton X-100 improved the growth of *Penicllium simplicissimum* in a medium contain-

ing polyethylene without being utilized by the fungus.

A simple and quick way to measure the biodegradation of polymers is by determining the weight loss. Microorganisms that grow within the polymer lead to an increase in weight due to accumulation, whereas a loss of polymer integrity leads to weight loss. Weight loss is proportional to the surface area since biodegradation usually is initiated at the surface of the polymer. The isolate NDYS-4 shows approximately 30% reduction in weight loss of polymer after 4^{th} week of incubation. Kumar and Bhavanth (2013) had also reported the loss was 1 ± 0.033%, 1.5 ± 0.038% and 1.75 ± .06% for *K. palustris* M16, *B. pumilus* M27 and *B. subtilis* H1584, respectively after 30 days of incubation.

Figure 5 shows the variation in pH of the medium during this biodegradation study. Microorganisms secrete a variety of enzymes into the soil water, which begin the breakdown of the polymers. Two types of enzymes are involved in the process, namely intracellular and extracellular depolymerases.

Exoenzymes from the microorganisms first breakdown the complex polymers giving short chains or monomers that are small enough to permeate through the cell walls to be utilized as carbon and energy sources by a process of depolymerization (Dey et al., 2012). The similar results were also reported on *Bacillus amyloliquefaciens* (Merina and Santosh, 2014). The isolate NDYS-4 showed the production of some enzymes and metabolites with the indication of pH change supporting the metabolic activity of this isolate on the LDPE substrate and also its degradation.

The ability of a microorganism to utilize any substrate depends on its growth and adherence to that substrate. Bacterial adhesion to either a hydrophilic or hydrophobic substrate is governed by many factors, including the forces by which the bacterium attaches to the surface and the properties of the substrate and microorganism. Generally, a hydrophobic bacterium prefers a hydrophobic surface for attachment, whereas the opposite is true for bacteria with hydrophilic properties.

As the polyethylene surface is hydrophobic in nature, it has been suggested that the more hydrophobic the bacterial cell surface, the higher the interaction with polyethylene. A 20% maximum reduction in turbidity has been reported for *Rhodococcus ruber* (Gilan et al., 2004), and increase (32%) in the hydrophobicity of two marine bacteria, *K. palustris* and *B. subtilis* (Kumar and Bhavanth, 2013). In the present study, we report a significant increase (30%) in the hydrophobicity of *S. coelicoflavus*.

While the growth, viability, weight reduction and pH change and cell hydrophobicity provide solid evidence of polymer biodegradation, whereas the changes of surface of LDPE films were elucidated by microscope. However, our study supports the occurrence of enzymatic activity on polyethylene as microscope photographs of the polyethylene film showed some localized degradation (Figure 7a, b). There are examples in the literature confirming the ability of the genus *Streptomyces* to degrade PE. The *Streptomyces* shows the degradation of disposable polyethylene containing 6% starch (Hanaa et al., 1998) and *Microbispora* shows the disappearance of PES film within six days in liquid cultures at 50°C (Kim-Chi et al., 2007).

Polyethylene, which has a wide range of applications, is accumulating in the environment. Its inert properties that resist deterioration and degradation are creating a serious environmental concern. This *in vitro* biodegradation study suggests the suitability of *S. coelicoflavus* for the degradation of environmental hazardous materials such as polyethylene. Based on the viability, growth results on the polyethylene surface, hydrophobicity, metabolic activity and we were able to determine that *S. coelicoflavus* is more efficient than the other actinomycetes. Hence, further study on microbial enzymes from actinomycetes in degradation of the LDPE and plastic will pave way for finding technology for degrading these environmentally hazardous plastic materials.

Furthermore, there is a need for the relationship between the biosurfactant production and biodegradation, and investigate the mechanism of polymer degradation and screen for other reported genes to establish the mechanism of biodegradation. There is also need to determine the optimum growth requirements of the isolate, bioaugmentation studies in the field and compare the *S. coelicoflavus* with well characterized actinomycetes and other microbes.

Conflict of interests

The author(s) have not declared any conflict of interests.

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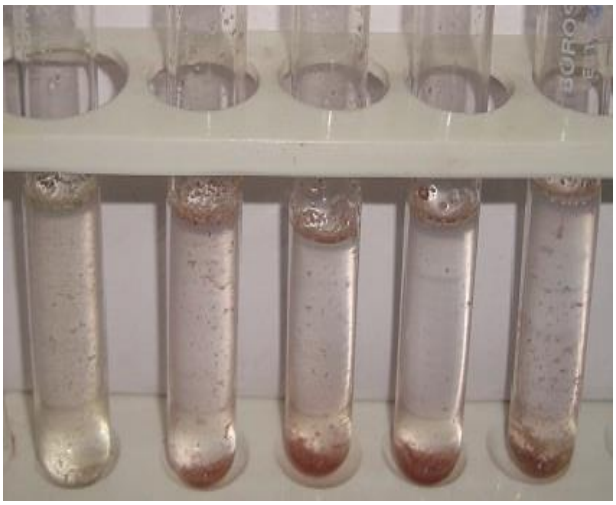
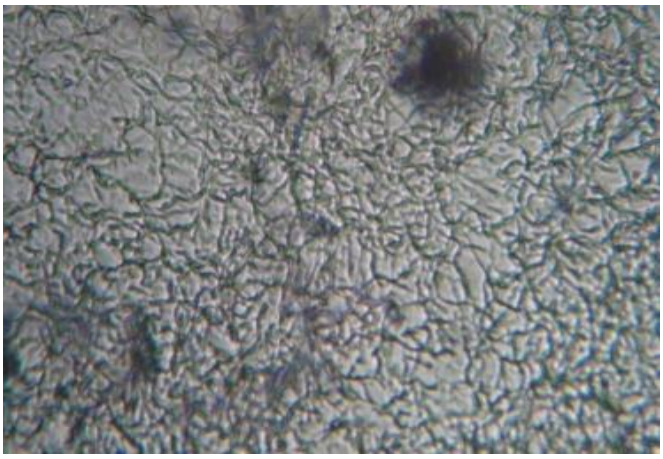


Negative control NDYS-6 NDYS-4 NDYS-8 NDYS-17

Supplementary Figure 1. Growth of actinomycetes in basal media containing TTC as redox indicator and polyethylene as a sole source of carbon.



Supplementary Figure 2. Microscopic morphology of the strain NDYS-4.

CATGCAAGTCGAACGATGAACCACCTTCGGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCT
GGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGACCTGCCAAGGCATCTTGGCGGGTCGAAAGCTCCGGCGGTGAAGG
ATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGC
GACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAA
GCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG
GCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGA
GATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGA
CGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGC
AACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGG
AAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGT
CAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAT
GAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCG
GAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTC
GGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGGGACGAAGTC

Supplementary Figure 3. 16S rRNA gene sequence of strain NDYS-4.

Supplementary Table 1. BLAST result showing significant homology with other sequences.

Accession	Description	Max score	Total score	Query coverage (100) (%)	E value	Max identy (%)
AB548687.1	*Streptomyces coelicoflavus* gene for 16S rRNA, partial sequence, strain: USF-6280	2641	2641	100	0.0	100
GQ395243.1	*Streptomyces* sp. XAS589 16S ribosomal RNA gene, partial sequence	2641	2641	100	0.0	100
EF371437.1	*Streptomyces graminearus* strain 14318 16S ribosomal RNA gene, partial sequence	2641	2641	100	0.0	100
NR_041175.1	*Streptomyces coelicoflavus* strain NBRC 15399 16S ribosomal RNA gene, partial sequence	2641	2641	100	0.0	100
JN969016.1	*Streptomyces* sp. CPE291 16S ribosomal RNA gene, partial sequence	2639	2639	99	0.0	100
FJ261963.1	*Streptomyces* sp. 210408 16S ribosomal RNA gene, partial sequence	2636	2636	100	0.0	99
EU201137.1	*Streptomyces* sp. ZG0656 16S ribosomal RNA gene, partial sequence	2595	2595	100	0.0	99
EU257234.1	*Streptomyces* sp. A309Ydz-QZ 16S ribosomal RNA gene, partial sequence	2595	2595	100	0.0	99
FJ267616.1	*Streptomyces* sp. 216701 16S ribosomal RNA gene, partial sequence	2590	2590	100	0.0	99
JF682780.1	*Streptomyces fradiae* strain GOS1 16S ribosomal RNA gene, partial sequence	2586	2586	99	0.0	99
AB184369.1	*Streptomyces violaceoruber* gene for 16S rRNA, partial sequence, strain: NBRC 13385	2586	2586	100	0.0	99
AJ781365.1	*Streptomyces violaceolatus* 16S rRNA gene, type strain LMG 20293	2584	2584	100	0.0	99
AF503495.1	*Streptomyces caesius* 16S ribosomal RNA, partial sequence	2584	2584	100	0.0	99
NR_027222.1	*Streptomyces coelescens* strain AS 4.1594 16S ribosomal RNA gene, partial sequence	2584	2584	100	0.0	99

Supplementary Table 2. Cultural characteristics of the isolate NDYS-4 on various media.

Medium	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigment
Yeast extract malt extract (ISP-2)	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Oat meal agar (ISP-3)	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Inorganic salt starch agar (ISP-4)	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Glycerol asparagine agar (ISP-5	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Nutrient agar	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Starch casein agar	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Bennett's agar	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None

Supplementary Table 3. Physiological and biochemical characteristics of the isolate NDYS-4.

Character	Result
Melanin production:	
ISP-1	-
ISP-6	-
ISP-7	-
Nitrate reduction	-
Starch hydrolysis	-
Casein hydrolysis	-
Gelatin liquefaction	-
Hydrogen sulphide (H_2S) production	-
Tyrosine production	-
Catalase reduction	+
Citrate utilization	+
MR	+
VP	-
Indole production	-
Oxidase utilization	-
Urea	+
Cell wall composition	+
Gram staining	G+
Spore staining	-

+, Yes; -, No

Supplementary Table 4a. Carbon source utilization of the isolate NDYS-4.

Carbon source utilization (1% w/v)	Result
D-Glucose	-
Galactose	-
L-Arabinose	-
D-Fructose	+
Raffinose	-
Meso-Inositol	-
D-Mannitol	+
Sucrose	-
Salicin	-
Rhamnose	+

Supplementary Table 4b. Nitrogen utilization, NaCl, temperature and pH tolerance of the isolate NDYS-4.

Nitrogen Source (0.1% w/v)	Result	NaCl tolerance (w/v) (%)	Result	Temperature tolerance (°C)	Result	pH tolerance
L-Histidine	+	2	+	12	+	5.0
Sodium nitrate	+	5	+	25	+	8.0
Yeast extract	+	7	+	37	+	9.0
L-Valine	-	10	+	42	+	10.0
L-Arginine	+					

+, Present; -, absent.