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Hydrocarbon and Dye Degradation by Actinomycetes from Flooded Soils

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Hydrocarbon and Dye Degradation by Actinomycetes from Flooded Soils

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Abstract. Hydrocarbon and dye pollutants could exert adverse effects to the polluted surroundings and their occurrences are often anthropogenic. The presence of both pollutants could endanger the environment if there are no preventive and corrective measures taken. Fifteen actinomycete strains were used for potential application on hydrocarbon degradation and 10 strains used for dye degradation study. Kerosene, petrol and diesel were introduced to the actinomycetes in separate media as source of carbon and energy. Seven actinomycete strains were able to grow well on hydrocarbon growth medium and they showed notable changes in terms of number and size of colonies formed, formation of mycelia and the colour of colonies formed. Four actinomycetes that grow well on hydrocarbon medium were further cultivated in MSM liquid media supplemented with hydrocarbon. Optical density (OD) of culture media was measured as an indicator of actinomycete growth. Strain D053 and D25.2 showed higher trend of overall OD measurement. Although OD measurement is lower, the growth of Strain KSJ12.7 and D13.5 were observed where tiny cell cluster or cellular flocs were formed. Kerosene and diesel were found to be good growth substrate for the actinomycetes. For dye degradation, the decrease in absorbance will determine the percentage of decolorization of Congo Red achieved by actinomycete strains. Highest decolorization of Congo Red recorded by Strain D4 in ISP-1 at 79.22%, whereas Strain D008 in MSM at 8.88%. These two different media indicated that actinomycete strains need additional sources of carbon and nitrogen and did not only depend on the dye as their sole carbon and nitrogen sources. This study showed that actinomycetes could potentially be used in hydrocarbon and dye degradation given their ability to grow in both.

1. Introduction

Petroleum hydrocarbons are naturally hydrophobic and are considered as recalcitrant pollutants that pose great danger and harm to the environment. Some petroleum hydrocarbons are proved to exert possible mutagenic and carcinogenic properties to many living organisms. Whereas, textile industry utilizes a lot of water and recently produce high discharge rate of wastewater contains many loads of contaminants. The dyes are discharged into the environment during the fibre dyeing and finished dyeing processes causing the main source of water pollution. Such pollutants, which contaminate the environment, require immediate remedial action in order to avoid further complications to the surroundings. Rendering them technically difficult to be removed from the environment and it requires vast amount of capital to do so. Physical and chemical methods such as coagulation-flocculation, activated carbon, oxidation and electrochemical methods can be used in



removing the dye colour. However, these conventional methods are more expensive, have operational problems and generate huge amounts of sludge that may cause secondary pollution [1]. These conventional modes of treatment can lead to the formation of some adverse effect of the products. The impacts of hydrocarbon and dye pollutants are immeasurable and would cause harm to the ecosystem once it is being polluted. Thus, affordable and effective technology is a necessity in remediating the contamination caused. Bioremediation using hydrocarbon and dye degrading bacteria is an alternative solution, which is non-invasive, and it requires lower cost, thus reducing its harm to the environment. Microorganisms that are able to degrade hydrocarbons and dyes naturally are ubiquitous, however, not all these microorganisms have been studied and their efficiency in hydrocarbon degradation has yet to be fully discovered. Actinomycetes are ubiquitous and can be found in both marine and terrestrial environments. Organic compounds can be degraded by actinomycetes through their extracellular enzyme produced. It also capable to degrade polymers with complex structures. Therefore, a total of 25 selected actinomycete strains isolated from previous study were tested and investigated in this study. For instance, the data and findings collected in this study could helped to identify potential of the actinomycetes in hydrocarbon and dye degradation.

2. Experimental

2.1. *Actinomycetes strain*

Fifteen actinomycete strains isolated from flooded soils of Kelantan, Malaysia [2] were randomly selected for hydrocarbon degradation study, and sub-cultured on Nutrient Agar (NA). The plates were incubated at 30 °C for one week and maintained for further use. Whereas, for dye degradation study, only 10 actinomycetes strain were used.

2.2. *Determination of actinomycetes growth on hydrocarbon*

Mineral salt medium (MSM) (0.18% dipotassium hydrogen phosphate, 0.001% iron (II) sulfate heptahydrate, 0.02% magnesium sulphate heptahydrate, 0.4% ammonium chloride, 0.01% sodium chloride) agar plates added with different hydrocarbon were used as the medium in determining the presence of actinomycetes growth. Three main hydrocarbons used in this study were kerosene, petrol and diesel. Actinomycete strains were inoculated onto MSM agar plates supplemented with 2% of each hydrocarbons; kerosene, petrol and diesel, and incubated at 30 °C for 2 weeks. Growth of actinomycetes was assessed based on the formation and sizes of colonies formed, formation of mycelia and the colours of colonies formed. The growth of each actinomycetes on different hydrocarbon-added medium were observed and recorded daily.

2.3. *Determination of actinomycetes growth in hydrocarbon broth*

The actinomycete strains were first inoculated in 15 mL MSM using 50 mL falcon tube and incubated at 30 °C, 120 r.p.m for 1 week. The inoculum were added into 100 mL MSM broth (in 250 mL conical flask) supplemented with 2% of each hydrocarbons; kerosene, petrol and diesel. Inoculum without hydrocarbon served as control. Cultures were incubated at 30 °C with agitation at 120 r.p.m for 3 weeks. Optical density (OD) of the culture media was measured at 600 nm. OD measurement of culture media was obtained on the first day of incubation, while subsequent OD measurements were collected after each week of incubation.

2.4. *Dye degradation test on actinomycetes*

Dye degradation test was carried out using Congo Red dye in 2 liquid mediums; ISP-1 and MSM. Dye stock solution was prepared by dissolving 1 g of Congo Red in 1 L of distilled water. The freshly grown actinomycetes lawn were used to prepare the inoculum in 10 mL of ISP-1 broth (5 g/L tryptone, 3 g/L yeast extract) and incubated overnight at 30 °C. 2 mL of inoculum suspension was transferred into 2 different 250 mL conical flask containing 100 mL of ISP-1 and MSM. 100

mg/L concentration of dye was added into each flask and incubated at 30 °C for 48 hours. A control flask containing only dye was used as control for abiotic decolourization.

2.5. Decolorization assay

Dye Decolourization assay was expressed in terms of percentage. 6 mL of decolourize samples withdrawn periodically (every 12 hours) were centrifuged at 10,000 rpm for 10 minutes to obtain the culture supernatant. The degradation activity was determined by measuring the absorbance of the culture supernatant (2 mL) at 502 nm using UV spectrophotometer. The uninoculated dye free medium was used as blank and the samples were analysed in triplicates. ISP-1 or MSM medium containing dye without inoculum was used as control, respectively. The percentage of decolourization by actinomycete strains was calculated using the following equation (1):

$$\text{Percentage of decolourization (\%)} = (A_0 - A_t) / A_0 \times 100 \quad (1)$$

Where A_0 is the initial absorbance; A_t is observed absorbance.

3. Results and Discussions

Severe floods hit state of Kelantan, Malaysia during December 2014 and January 2015. The disaster however has given the opportunity for researchers to study potential of isolating novel actinomycete from soil of flooded area. Soil saturation occurred during and after flood, resulting in depletion of oxygen in soil. This has created the anaerobic conditions in soil, normally microorganism that uses the available oxygen in soil to survive. However, shift of microbial community structure in flooded soil is expected when saturated anaerobic conditions happened.

Actinomycetes decompose a wide array of substrates, but are especially important in degrading recalcitrant (hard-to-decompose) compounds, such as chitin and cellulose, and the microorganism is active at high pH levels. In this study, actinomycetes isolated from flooded soils of Kelantan, Malaysia were used for hydrocarbon and dye degradation. Under specific condition and through the optimization of limiting conditions, bioremediation is a non-destructive, economical cost and effective treatment to clean up the wastewater. This will accelerate the naturally occurring biodegradation of contaminants with the use of naturally occurring bacteria and fungi in degrading or detoxify substances that are dangerous to human health or environment [3]. The microorganisms may be isolated from somewhere else and brought to the contaminated site or may be indigenous to a certain contaminated area which can secrete useful enzymes for helping the hydrocarbon and dye degradation process. The catabolic diversity of microorganisms to transform contaminants into less harmful compounds can be achieved through bioremediation. This includes the isolation of microorganisms that exhibit the required biodegradable activities towards the organic compound [4].

3.1. Actinomycetes growth on hydrocarbon agar

Several growth patterns and characteristics were observed on hydrocarbon MSM agar as the indicator of growth. The growth patterns and characteristics of colonies formed varied among different actinomycete strains. Significant aspects that were used to distinguish actinomycetes growth on hydrocarbon MSM agar were the presence and size of colonies formed, formation of mycelia as well as colour changed of the colonies formed. Based on observation, the growth of certain actinomycetes started to be observed on the fifth day of incubation, while some actinomycete strains started to form visible growth after 1 week of incubation. Few actinomycetes were observed with significant colour changed of the colonies formed. Differences of growth pattern or characteristics of colonies formed among the actinomycete strains were notable. The results of actinomycetes growth recorded in table 1 were obtained after 2 weeks of incubation.

Table 1. Growth of actinomycete on hydrocarbon msm agar.

Actinomycete strains	Growth on hydrocarbon MSM agar		
	Kerosene	Petrol	Diesel
KSJ12.4	Absent	Absent	Absent
D056	Very poor	Absent	Very poor
D034	Very poor	Very poor	Poor
D087	Poor	Very poor	Poor
D044	Poor	Poor	Poor
D8.1.2	Poor	Poor	Poor
D009	Poor	Very poor	Good
KSJ3.6	Poor	Poor	Good
D13.4	Good	Good	Good
D1.1	Good	Good	Good
D25.2	Good	Good	Good
D094	Good	Good	Good
KSJ12.7	Good	Good	Good
D053	Good	Good	Good
D13.5	Good	Good	Good

'Absent' indicates no growth, 'Very poor' indicates little or few microcolonies, 'Poor' indicates some colonies formed and 'Good' indicates numerous colonies formed on the agar surface.

Strain D009 and KSJ 3.6 had poorly grown colonies on kerosene and petrol but both were shown to grow well on diesel MSM agar. The other 7 actinomycete strains showed better growth in terms of large colonies on all 3 hydrocarbon MSM agar plates. Among these 7 strains, Strain D1.1, D25.2, KSJ12.7, D053 and D13.5 (figure 1) showed greater growth on hydrocarbon plates.

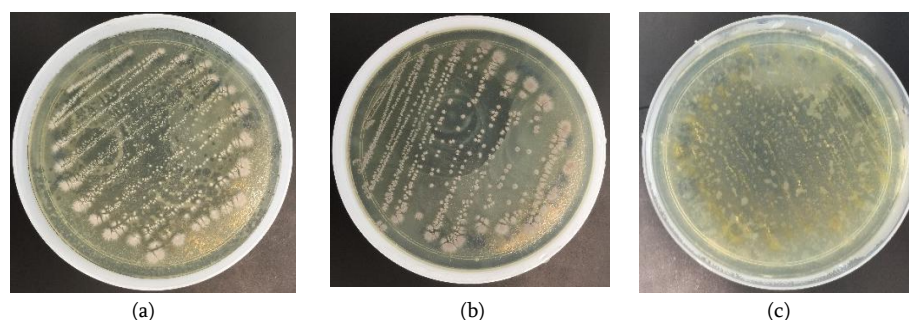


Figure 1. Growth of Strain D13.5 observed on MSM agar supplemented with kerosene (a), petrol (b) and diesel (c) respectively.

Out of 15 actinomycete strains tested, only Strain KSJ12.4 showed no sign of growth on all 3 hydrocarbon MSM agar plates. Strain D056 was observed with very poor growth on MSM agar plates added with kerosene and diesel but it was not able to grow on MSM agar added with petrol. Microorganisms are only able to degrade and utilize certain hydrocarbons due to their metabolic affinity towards certain chemical structures [5]. Metabolic capabilities of microorganisms are one of the major requirements in hydrocarbon degradation. Rate of hydrocarbon degradation can be optimized when microorganisms with desired metabolic capabilities are ensured with sufficient

nutrients and suitable growth or surrounding conditions [6]. The absence of growth of actinomycete in this study was due to the incompatibility between the actinomycete and the hydrocarbons used. Little or poorly formed microcolonies were spotted on all 3 hydrocarbon MSM agar plates for Strain D087, D044 and D8.1.2.

Strain D009 and KSJ 3.6 had poorly grown colonies on kerosene and petrol but both were shown to grow well on diesel MSM agar. The other 7 actinomycete strains showed better growth in terms of large colonies on all 3 hydrocarbon MSM agar plates. Among these 7 strains, Strain D1.1, D25.2, KSJ12.7, D053 and D13.5 showed greater growth on hydrocarbon plates. The presence of colonies and mycelia formed in some actinomycete strains was an indicator of growth on hydrocarbon MSM. Microorganisms like bacteria and fungi are able to hydroxylate aromatic rings in hydrocarbons to form diols. Subsequent degradation of diols will take place and be taken up by microorganisms as the source of carbon and energy. Even though the volatile hydrocarbons will be vaporized as time passed by, the hydrocarbon vapors were remained within the atmosphere of petri dish. The actinomycetes able to grow in hydrocarbon and possibly utilise the hydrocarbons when it was added as source of carbon and energy, suggesting that the bacteria to be a potent candidate in hydrocarbon degradation [7].

3.2. Actinomycetes growth on hydrocarbon broth

Significant differences were observed among flasks with different actinomycete strains. Culture media for Strain D053 and D25.2 slowly turned turbid on the first week of incubation. The degree of turbidity increased slowly, and it persisted after 3 weeks of incubation. Culture media for all sample flasks of Strain KSJ12.7 and D13.5 had lower turbidity in comparison. It can be deduced that the growth of actinomycetes Strain D053 and D25.2 in culture media were dispersed throughout the liquid media. In contrast, Strain KSJ12.7 and D13.5 had cellular growth that is attached or clumped into tiny clusters suspended at the bottom of flasks. The degree of turbidity for all sample flasks of Strain D053 and D25.2 is notably higher than all samples flasks for Strain KSJ12.7 and D13.5. Tiny cell growth in the form of clusters can be seen suspended at the bottom of flasks for Strain KSJ12.7 (figure 2 (a)) and Strain D13.5 (figure 2 (b)). Unlike the other 2 strains, the culture media of Strain D053 (figure 3 (a)) and Strain D25.2 (figure 3 (b)) were turbid with few cellular flocs formed after 3 weeks of incubation.

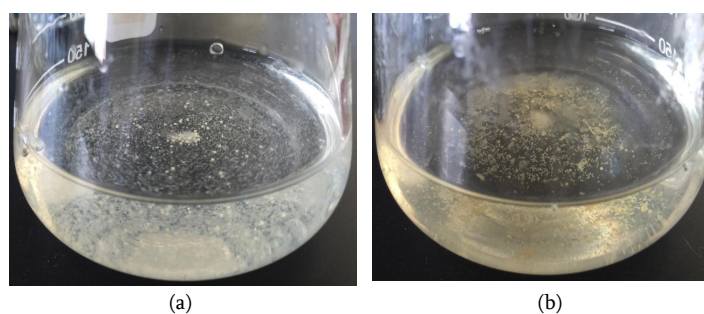


Figure 2. Diesel flask for Strain KSJ12.7 (a) and D13.5 (b) observed on the 3rd week of incubation.

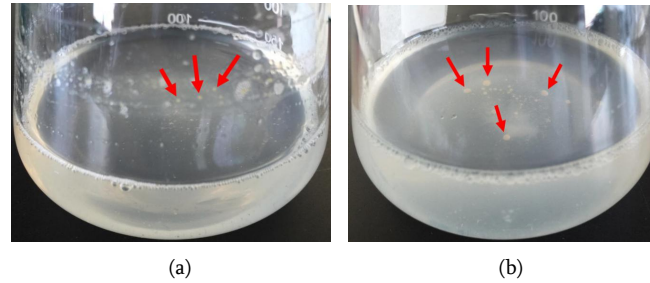


Figure 3. Diesel flask for for Strain D053 (a) and kerosene flask for Strain D25.2 (b) observed on the 3rd week of incubation. Few tiny cell clumps formed in hydrocarbon media were indicated by red arrows.

An overall trend for OD600 measurement of all sample and reference flasks were tabulated as in figure 4. A comparison using kerosene samples for all 4 strains, Strain D25.2 had the highest OD measurement, whereas the OD measurement for Strain KSJ12.7 and D13.5 were similarly low (figure 5).

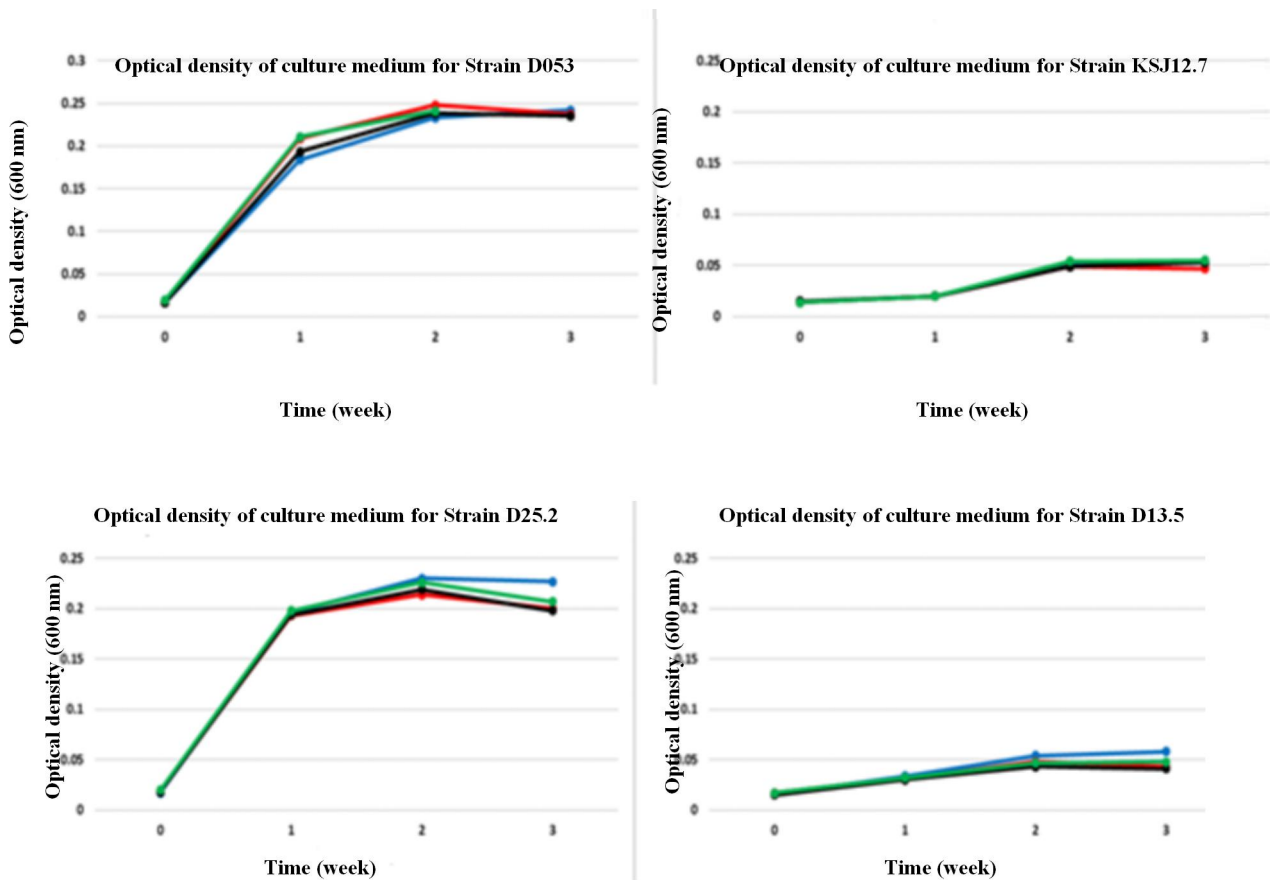


Figure 4. OD measurement for Strain D053, KSJ12.7, D25.2 and D13.5.

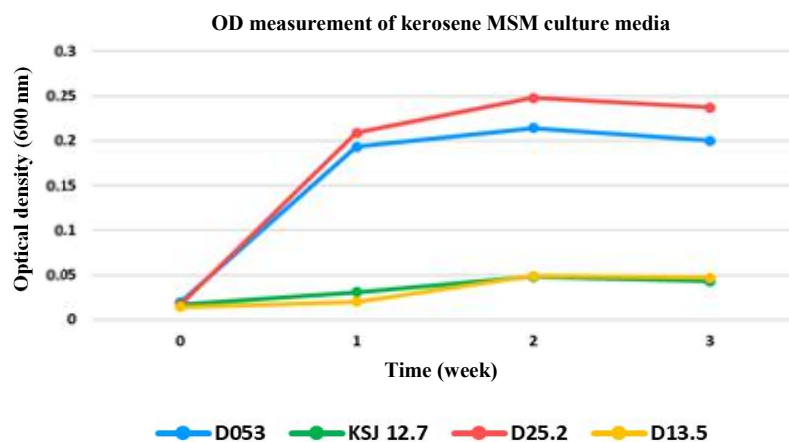


Figure 5. OD measurement of kerosene MSM culture media for Strain D053, KSJ12.7, D25.2 and D13.5.

Formation of cell clusters or cellular flocs were found in all sample flasks for Strain KSJ12.7 and D13.5, while the sizes of cellular flocs slowly increased as the incubation continued. The formation of cellular flocs may be due to the formation of adhesive cell clusters [5]. For instance, the formation of cellular flocs is likely to be the cause of low OD reading for these 2 strains. Cell cluster were heavy and suspended at the bottom of cuvette during OD measurement. Thus, OD measurement is difficult due to the formation and suspension of large cellular flocs [5]. Although Strain KSJ12.7 and D13.5 have lower OD measurement, they were able to grow on hydrocarbon agar. The cellular flocs formed or cells that grew in clumps within hydrocarbon culture media was another indication of grow in hydrocarbon.

3.3. Dye degradation by actinomycetes

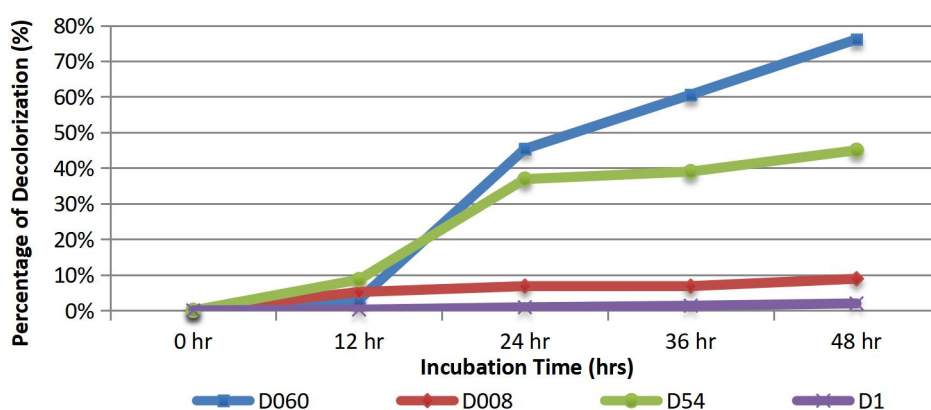
Decolorization of Congo Red by actinomycete strains in 2 different mediums; ISP-1 (table 2) and MSM (table 3) were measured every 12 hrs and recorded as percentage of decolorization. In ISP-1, Strain D4 showed the highest decolorization at 79.22% and lowest by Strain D54 at 44.97%. Whereas, in MSM, the highest decolorization is only at 8.88% by Strain D008, and lowest at 1.96% by Strain D1 (figure 6). At average, all the selected strains have the ability to degrade dye with 4.04% decolourization. The assay was carried out under static condition. The condition of where the test carried out plays an important factor. Under static condition the percentage of decolourization was high compared to agitation condition. With agitation, the presence of oxygen unable the azoreductase enzyme secretes by microorganism from gaining electrons needed for cleavage of azo bond ($-N=N-$) [9]. However, under static conditions, these electrons are available to azoreductase from NADH to decolourize azo dyes.

Table 2. Percentage of Congo Red decolorization by actinomycetes in ISP-1 medium every 12 hours.

Actinomycete strain	Incubation time (hour)				
	0	12	24	36	48
D008	0%	14.04%	35.12%	54.18%	57.08%
D3	0%	5.63%	38.74%	58.06%	59.85%
D5	0%	6.87%	46.24%	59.18%	61.56%
D4	0%	4.31%	32.29%	76.86%	79.22%
D2	0%	28.43%	32.42%	50.96%	53.57%
D060	0%	3.02%	45.34%	60.58%	76.12%
D94	0%	3.18%	34.73%	53.84%	73.81%
D1	0%	7.92%	10.83%	54.79%	55.00%
D54	0%	8.64%	36.86%	38.98%	44.97%
D087	0%	16.64%	30.74%	43.40%	60.76%

Table 3. Percentage of Congo Red decolorization by actinomycetes in MSM medium every 12 hours.

Actinomycete strain	Incubation time (hour)				
	0	12	24	36	48
D008	0%	5.16%	6.81%	6.81%	8.88%
D3	0%	0.42%	2.22%	2.75%	3.17%
D5	0%	1.84%	3.24%	3.35%	4.65%
D4	0%	2.70%	2.70%	3.22%	4.25%
D2	0%	0.43%	1.71%	2.13%	2.67%
D060	0%	0.21%	3.10%	3.63%	3.85%
D94	0%	1.75%	2.57%	2.88%	3.09%
D1	0%	0.22%	0.87%	1.31%	1.96%
D54	0%	0.55%	0.99%	1.32%	4.96%
D087	0%	3.50%	5.09%	5.20%	6.26%

**Figure 6.** Percentage of decolorization of actinomycetes in ISP-1 and MSM.

There are huge differences in the percentage of dye decolourization between the two media. This is because, ISP-1 contained additional sources of carbon and nitrogen such as tryptone and yeast extract while MSM only contained dye as the sole sources of carbon for actinomycete strains.

These differences are due to the fact that bacteria had difficulties to decolourize azo dyes without additional sources of carbon and nitrogen. In some cases, by increasing the concentration of dye, there are some bacteria that can survive only with the availability of dye as their sole carbon sources [9]. Frequently, the process of azo dye biodegradation is relatively slow due to the limited sources of carbon and nitrogen in the complex structure of dyes. Additional nutrients like carbon and nitrogen could improve the efficiency of dye degradation as they provide energy for the growth and survival of the bacteria and also transfer reducing equivalents to the dye for azo bond cleavage [10]. Thus, higher amount of nutrients are needed as the amount of nutrients significantly influences the growth of microorganism. The organic nitrogen sources such as peptone, yeast extract, beef extract and urea seemed to be highly effective in promoting dye decolourization compared to other additional carbon sources because of regeneration of NADH which is the source of electron donor for azo bond reduction [11].

Actinomycetes have good characteristic that make them suitable for bioremediation application of soils contaminated with organic pollutants. They have a significant role in the recycling of organic carbon and are able to decompose complex polymers as they represent dominant group among the degraders in contaminated sites [12]. With the characteristic to perform microbial alterations of organic compounds that are able to decompose a wide variety of hydrocarbons, pesticides, and aliphatic and aromatic compounds, actinomycetes make a great commercial value.

4. Conclusions

Some of the selected actinomycete strains tested were able to grow on hydrocarbon agar with the presence of volatile vapours of hydrocarbon. Growth of actinomycete strains can be observed in terms of visible colonies, mycelia growth and colour changed of colonies formed on the hydrocarbon agar. OD measurement of the hydrocarbon culture media that shown increased turbidity as well as formation of large cellular flocs were the indication for actinomycete growth in culture media. Results obtained suggested that the presence of actinomycetes growth is an indicator to their capabilities or metabolic activities towards utilising hydrocarbon as source or carbon and energy. Although Strain KSJ12.7 and D13.5 had lower OD reading but the formation of cells in clumps or cellular flocs was a different indication of growth. Not all actinomycetes are capable to utilize every hydrocarbon as it is highly dependent on their metabolic activities' affinity towards the chemical structures of certain hydrocarbons. For instance, Strain D053 and D25.2 both exhibit traits as potential bacterial agents for further research in hydrocarbon degradation and bioremediation of hydrocarbon pollutants. Whereas, for dye degradation study, all tested actinomycete strains were able to degrade the Congo Red dye. This indicated that these dye degrading actinomycetes could be used in the microbial degradation of azo dyes. The degradation of industrial dye is complicated as the dye found in wastewater treatment plant is highly resistant to microbial degradation. The used of industrial dye can improve the performance of actinomycetes in dye degradation process. Further research is needed to apply these actinomycetes for potential application in hydrocarbon and dye degradation.

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