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Hydrocarbon Degradative Potential of *Alcaligenes* sp. strain 3k Under Modified Nitrate Concentrations

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ABSTRACT

Conventional mineral salts medium (MSM) had been used for many decades. A modification of the concentration of nitrate in the MSM was attempted in this work to see its effects or otherwise on microbial cultures. This research investigated the ability of *Alcaligenes* sp. strain 3k to degrade hydrocarbons under modified nitrate concentrations for a period of twenty-seven (27) days. The findings from the growth patterns of *Alcaligenes* sp. strain 3k in the experimental study showed that the organism was capable of utilizing decane as only carbon source as well as the mixture of decane and heptane as its carbon and energy providers. The hydrocarbon utilization test carried out on the mixture of decane and heptane with an increase in nitrate concentration indicated that *Alcaligenes* sp. strain 3k was able to use the hydrocarbon mixture as the major source of carbon and nitrate as the sole source of nitrogen showing a steady increase in growth rate within the period of 27 days. The highest value obtained was 1.84×10^9 cfu/ml on Day 21 and lowest value was 4.80×10^6 cfu/ml on Day 0 thus revealing the efficiency of *Alcaligenes* sp. strain 3k in bioremediation. The findings from the growth patterns of *Alcaligenes* sp. strain 3k in this experimental study revealed *Alcaligenes* sp. strain 3k capability in efficiently utilizing decane solely and the mixture of decane and heptane been modified with nitrate as carbon and energy sources respectively thereby enhancing bioremediation.

Keywords: *Alcaligenes* sp. strain 3k, Biodegradation, Bioremediation, Hydrocarbons, Nitrate

Introduction

Pollution of the ecosystem with petroleum and its derivatives has impacted negatively on sustainable development of the environment globally (Liu *et al.*, 2020). Environmental components, most particularly soil and water are being contaminated with accidental spills of petroleum products containing mainly hydrocarbons. Some of the hydrocarbon compounds have proven to be carcinogenic and neurotoxic to different life forms. Hydrocarbons and heavy metals represent a major portion of pollutants emanating from

anthropogenic activities mostly industrial and domestic activities which when released even in minute quantities into the environment can be lethal (Carla *et al.*, 2020).

Pollution and contamination of components of the ecosystem have led to accumulation of these toxic pollutants in plant and animal tissues thereby resulting in mutation or death. Similarly, toxic constituents of petroleum products reduce the aesthetic quality of the ecosystem as well as lessen

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soil quality on arable lands (Lim *et al.*, 2016). Another disturbing possibility is that some condensed polynuclear components of petroleum that are carcinogenic and relatively resistant to biodegradation may move up marine food chains (Grimaud *et al.*, 2012).

Physical and chemical methods have been used to remediate pollution from hydrocarbons, they were found to be costly, not environmentally friendly and the unpredictable hazards associated with their use further limited their implementation (Johnson and Affam, 2019). Bioremediation which is a biological approach have been found to be a more cost effective and green technique of eliminating pollutants from contaminated sites as it involves the addition of organic or inorganic components to such contaminated sites that will enhance indigenous microbial growth to aid the breaking down process (Agamuthu and Fauziah, 2013).

Agbagwa and Onyemaechi (2014) reported common genera responsible for oil degradation and affiliated with hydrocarbon consumption to comprise mainly: *Nocardia*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Arthrobacter*, *Corynebacterium*, *Achromobacter*, *Rhodococcus*, *Alcaligenes*, *Mycobacterium*, *Bacillus*, *Aspergillus*, *Mucor*, *Fusarium*, *Penicillium*, *Rhodotorula*, *Candida* and *Sporobolomyces* sp. Thus, autochthonous microorganisms have made bioremediation of contaminated sites a generally accepted approach suitable for cleaning up hydrocarbon pollutants (Ali *et al.*, 2020). This research aimed to see the effect of modifying the nitrate concentration in mineral salts medium on the capability of *Alcaligenes* sp, strain 3k in biodegrading heptane and decane.

Materials and Methods

Sterilization and Media Preparation

All microbiological processes were carried out aseptically in accordance with standard procedures described by Fawole and Oso (2007) while the

preparation of Mineral Salts Medium (MSM) was done according to the description of Zampolli *et al.* (2014).

Collection and confirmation of Alcaligenes sp. strain 3k

Pre-isolated and purified bacterial culture of *Alcaligenes* sp. strain 3k was collected in sterile slant bottles from the Environmental Microbiology Laboratory, Department of Microbiology, University of Ilorin, Kwara State, Nigeria. The isolate was preserved at a temperature of 4 °C to arrest its growth. Its identity was confirmed using molecular tools. PCR was carried out with the aid of 16S rRNA primers. The forward reaction was done with Bact 27F (5' AGAGTTTGATCM TGGCTCAG3') while the reverse was with U1492R (5'-TACGGYTACCTTGTTACGACTT-3') as described by Spear *et al.* (2005) for the identification of the isolates. Amplified PCR products were cleaned and sequenced. Amplified 16S rRNA gene sequences were analyzed using the National Center for Biotechnology Information (NCBI) database - BLASTn. The organism was identified based on the percentage similarity of the obtained sequences with those in the NCBI database.

Sub-culturing of organism (Alcaligenes species strain 3k)

One (1) ml from the broth culture containing viable cells of *Alcaligenes* sp. strain 3k was aseptically transferred into McCartney bottles containing 9ml of sterilized nutrient broth and inoculated at 37°C for 24 hours. From the McCartney bottle, 1µl of the inoculum was transferred into eppendorf tubes containing 9µl sterile distilled water. Serial dilution was made up to 10⁻⁶ and the serial dilution of 10⁻⁶ was inoculated into the MSM.

Experimental set-up for studies on the biodegradative potential of *Alcaligenes* sp. strain 3k

These was done according to the method of Adetitun (2017) and Zampolli *et al.* (2014).

Biodegradability examinations of hydrocarbon mixture of decane by *Alcaligenes* sp. strain 3k

In this experimental set-up, the biodegradation of decane was observed at room temperature over a period of 27 days. Twenty-four falcum tubes with 20ml of MSM was prepared. Six (6) tubes were used as control (MSM and organism only), and the remaining eighteen (18) falcum tubes contained 0.1µl MSM, the organism and 27µl of decane.

***Alcaligenes* degradability examinations of hydrocarbon mixture of decane and heptane substrate by *Alcaligenes* sp. strain 3k**

The biodegradation of the mixture of decane and heptane was observed at room temperature over a period of 27 days. Twenty-four falcum tubes with exactly 20ml of MSM was prepared. A total of six (6) tubes was used as control (MSM and organism only), and the remaining 18 falcum tubes contained 0.1µl MSM and the organism as well as 27µl of decane and heptane respectively.

Biodegradability test of decane substrate by *Alcaligenes* sp. strain 3k with 4-fold increase in nitrate concentration

The study of the biodegradation of decane substrate with a modification of a 4-fold increase in nitrate concentration was carried out at room temperature over a period of 27 days. Twenty-four falcum tubes with 20ml of MSM was prepared. Six (6) tubes were used as control (MSM and organism only), and the remaining eighteen (18) falcum tubes contained 0.1µl MSM, the organism and 27µl of decane.

***Alcaligenes* degradability tests of hydrocarbon mixture of decane and heptane substrate by**

***Alcaligenes* sp. strain 3k with 4-fold increase in nitrate concentration**

The degrading ability of *Alcaligenes* sp. strain 3k on the mixture of decane and heptane was studied at room temperature but with a modification of 4-fold increase in nitrate concentration over a period of 27 days. Twenty-four falcum tubes containing 20ml of MSM was prepared. Six (6) tubes were used as control (MSM and organism only), while the remaining 18 falcum tubes contained 0.1µl of MSM and the organism as well as 27µl of decane and heptane respectively.

Enumeration of *Alcaligenes* sp. strain 3k in alkane hydrocarbon substrate for the whole experimental set-up

Routine enumeration of *Alcaligenes* sp. strain 3k in the alkane hydrocarbon substrates as set up in all the experiments was carried out at intervals of 3 days over the period 27 days using the serial dilution and pour plate methods.

Results and Discussion

Confirmation of *Alcaligenes* sp. strain 3k

The sequences are those obtained for *Alcaligenes* sp. strain 3k with 88% Identity

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GGCGAACGACACTTCAGAGTGAGCGCCGTCCTTGGT
AGGCTACCTAGTTCTGGTGAAACCCACTCCCAGGGG
GTGACGGGCGGTGTGTACAGAGCGGGAACGTATTCA
ACGCTACATTCTGATCCGCGATTAACAGCGATTCCC
ACTTCGCGCAGTCGAGTTGCTCACTGGAATCCGGAC
TACGGTCGGGTTTCTGAGATTGGCTCCCCCTCGGGG
GTTGGCGACCCTCTGTCCCGAACATTGTATGACTGGT
GAAGCCCTACCCATAAGGGCCATGAGGACTTGACGT
CATCCCCACCTTCTCCGGTTTGTACCCGGCAATCTC
ATTAGAGTGCTCTTGCGTATCATCTAACGACAAGGG
TTGCGCTCGTTGCGGGACTTAGCCCTACATCTCACGA
CACGAACTGAAGACGGCCATGCAAACTCTGTGTTCC
GGTTCTCTTGCCAGCACGGCCAAATCTTTCAGCTTT
CCGGACATGTCAGGGGTAATAAAGGTTTTTCGCGTT
GCATCCAATTAATCCACATCATCCAGCGCTTGTGCGC
GGTCCCCGTCAGTTCCTTTGAGTTTTAATCTTGCGAC
CGTACTCACTGCGCGGTCAATTTACGCGTTAGCTGC
GCTAATAAGGCCAAAAGTCCCCGAGAAGTACTAGTTGAC
ATCGTTTATGTCGGGAATATCCCGGTGGCTAATCCA
GCTTGCTGGCACGCACTCACGTCTGAACGTCAGTATT
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ATCCGCAGGGAGCTGGCTTCGATATCCGGATTCCCC
 CCCATATATACGAAGTTCCTACTGCCTAGATGTGGCAT
 GTCTAGCCTCCCGTCTGACATAACTCTAGCTCGATAG
 TTAACAATTGCAGGTCGCTAACGCTCGATCCATGAC
 AACTCACATCATATCGTGTACCTGAAGCCAGCCCTA
 CACACGCTGTTTACGTTCTGAGCTATTATTATTGAC
 TGAAGTCTCGCAACGACTACCTTACTTACCTTCTGA
 CTGCTGGCGCGATAGCTTAATAAGATGCGTGACTGC
 TGCTCCGACACCAGATACCGCAATATCCCGTAGTG
 ACTGACTTGCTCTTTCGTTCCGTGCCGATAGAAGTAG
 CTAACAGTACGGACATCCATCATAACCTGCAAGTC
 TGGATCAGCAGTACGCACTGGATCGATATTACCTAC
 CTGACAATGCTTGACGTAAGAAAG

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The variation in growth of *Alcaligenes* sp. strain 3k on decane as the sole source of carbon in the Mineral Salts Medium is depicted in Figure 1. There was a systematic increase in the growth rate within the period of 16 days. The highest value obtained was 5.16×10^8 cfu/ml on Day 12 and lowest value was 1.41×10^6 cfu/ml on Day 0.

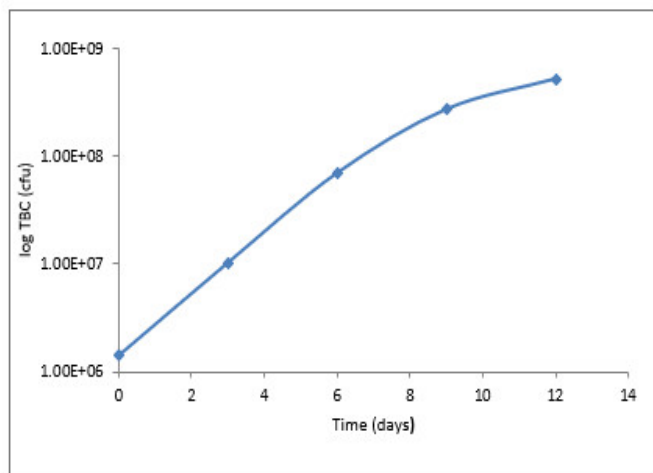


Figure 1: Growth patterns of *Alcaligenes* sp. strain 3k during biodegradability test on decane

Figure 2 shows the growth of *Alcaligenes* sp. strain 3k on a mixture of decane and heptane as the carbon source. It was observed that there was no growth on Day 0, but colonies were observed on Day 3. The growth rate decreased on Day 9 then an immense increase in growth rate was observed from Day 15 – Day 27 with the highest value of 1.12×10^9 cfu/ml recorded on Day 27 and a lowest value of 1.30×10^6 cfu/ml on Day 9.

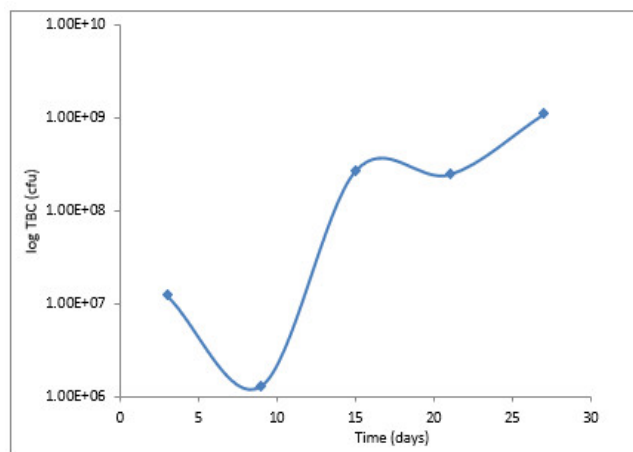


Figure 2: Growth pattern of *Alcaligenes* sp. strain 3k during biodegradability test on hydrocarbon mixture of decane and heptane

Based on the growth pattern observed in Figure 2, there is every possibility that *Alcaligenes* sp. strain 3k is probably devoid of genes responsible for degradation of these types of alkanes [short-chains] or there are factors affecting the rate of biodegradation (Durán *et al.*, 2019). Furthermore, it suggests that the bacteria may be in its lag phase of growth.

Figure 3 explains the growth of *Alcaligenes* sp. strain 3k on decane as the sole source of carbon in MSM but with a 4-fold increase in nitrate concentration (the sole source of nitrogen). A steady increase in growth rate was observed from day 0 to 9. On day 15, there was slight decrease in growth, however from Days 21 – 27, an increase in growth rate was observed. The highest value obtained was 3.24×10^9 cfu/ml on Day 27 and lowest value was 8.00×10^6 cfu/ml on Day 0.

The growth of *Alcaligenes* sp. strain 3k on mixture of decane and heptane but with a 4-fold increase in nitrate concentration is described in Figure 4. There was proportional increase in growth rate of *Alcaligenes* sp. strain 3k within the period of 27 days. The highest value obtained was 1.84×10^9 cfu/ml on Day 21 and lowest value was 4.80×10^6 cfu/ml on Day 0.

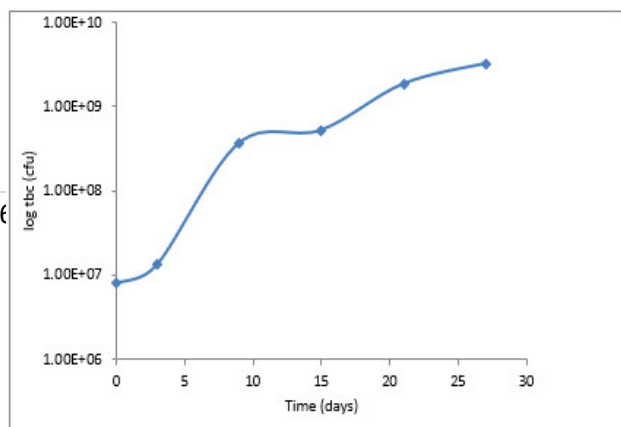


Figure 3: Growth pattern of *Alcaligenes* sp. strain 3k during biodegradability test on decane with 4- fold increase in nitrate concentration

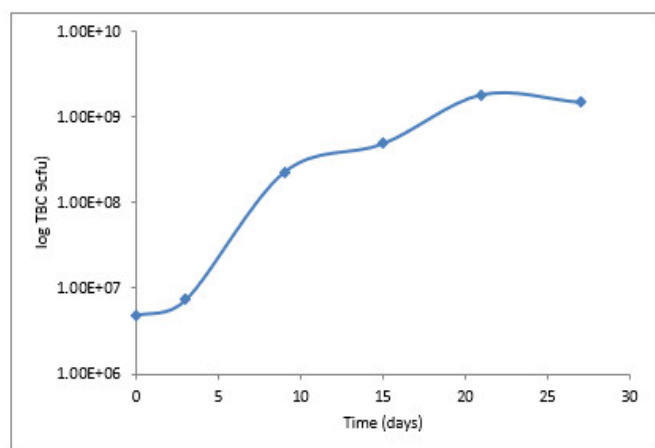


Figure 4: Growth pattern of *Alcaligenes* sp. strain 3k during biodegradability test on hydrocarbon mixture of decane and heptane with 4-fold increase in nitrate concentration

High rates of degradation are largely determined by prevalence of optimal conditions of temperature and pH. Also, the rates by which microorganisms degrade hydrocarbons depend upon their chemical structure and composition. The findings from the growth patterns of *Alcaligenes* sp. strain 3k in this experimental study so far revealed that the *Alcaligenes* sp. strain 3k is capable of utilizing decane and hydrocarbon mixture of decane and heptane as its sole carbon source and energy source as Figures 1, 3 and 4 show the steady increase in the growth pattern of *Alcaligenes* sp. strain 3k in the first nine (9) days for three of the experimental set-ups. This supports the research

findings of Igwo-Ezikpe *et al.* (2009) who studied the ability of *Alcaligenes* sp. specifically *Alcaligenes faecalis* to degrade chrysene and diesel oil with simultaneous production of biosurfactant.

Furthermore, adjustment of carbon/nitrogen ratios stimulates the biodegradation of crude oil and individual hydrocarbons (Abed and Köster, 2005). Various studies have addressed the successful degradation of hydrocarbons by *Alcaligenes* sp. Pathak *et al.* (2008) studied the ability of *Alcaligenes* sp. as a 4T Engine Oil Degradator, and reported that *Alcaligenes* sp. efficiently degraded 4T Engine Oil (mainly composed of medium chain length hydrocarbon). Also, biodegradation of 2-Mercaptobenzothiazole (2-MBT) was investigated by Tebyanian *et al.* (2013) using *Alcaligenes* sp. MH146 strain CSMB1. The results obtained revealed that a pure strain of *Alcaligenes* sp. MH146 strain CSMB1 was able to utilize 2-MBT as sole carbon and nitrogen for its growth under microaerobic conditions. In addition, the study done by Adetitun *et al.* (2019) validated the ability of *Alcaligenes* sp. Strain 3k to make use of kerosene, hexadecane, cyclohexane among others as sole carbon sources and the isolate was able to degrade various lignocelluloses compounds.

Conclusions

The results obtained from this study proves that *Alcaligenes* sp. strain 3k holds good promise in biodegradation and bioremediation of oil.

Conflict of Interest:

The authors declare there are no competing interests associated with this study.

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