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# Assessment of the Plasmid Mediated Biodegradation of Crude Oil under Optimal Growth Conditions

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## Abstract

The present study was undertaken to assess the role of plasmid-mediated biodegradation of crude oil under optimal growth conditions. Enrichment techniques, turbidometric test, antimicrobial susceptibility testing, plasmid curing test and optimal biodegradation conditions were carried out using standard procedures. The results indicated that 22 of the isolates were observed to have high pollutant degrading potentials (A600nm > 0.3) due to their crude oil utilization ability. Isolates designated as $C_1$, $D_1$, $L_2$ and $J_3$ showed various degrees of growth after curing of their plasmids whereas isolates $G_2$, $H_4$, $K_4$ and $I_6$ were unable to grow after plasmid removal. It was observed that 4 plasmid borne cells resisted the antibiotics tested after pre-cured examination but these isolates were 95% sensitive to the same antibiotics after post cured test. The results further indicated that isolates which were able to degrade crude oil belonged to genera *Bacillus*, *Pseudomanas* sp., *Ochrobacterium* sp. and *Enterobacter* sp. Moreso, neutral pH, 35 °C temperature and 3 % crude oil concentration were found to be optimal conditions for crude oil bioaugmentation study. Thus, the study indicated that the crude oil-utilizing bacteria which are part of the soil ecosystem could be exploited for natural and terrestrial remediation of crude oil polluted environments.

***Keywords:*** *Antimicrobial susceptibility, Bioaugmentation, Optimization, Plasmid curing*

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## 1. Introduction

The increase in petroleum exploration and production has brought with it an ever increasing rate of environmental pollution involving both terrestrial and aquatic habitat (Mandri and Lin, 2007). Oil spillage in an oil producing country is inevitable. The impact of these wastes in the Niger Delta ecosystems of Nigeria is an obvious environmental concern particularly with regards to the persistence and ecotoxicity of these wastes (Benka-Coker and Olumagin, 1995). Soil and ground water contamination by crude oil are becoming increasingly sensitive issues in Nigeria, since most of her potable water supply is derived from shallow and unconified aquifers (Akpe et al., 2013).

There are different types of physical and chemical methods for the remediation of oil contaminated soil such as burying, evaporation, dispersion, washing etc (Fowzia and Fakhruddin, 2018). However, these technologies are expensive and can lead to incomplete decomposition of contaminants. Therefore, it is important to develop an innovative, low cost and eco-

friendly method for the removal of hydrocarbon contamination from the soil. Bioremediation method is considered to be more economical and safer method for the treatment of hydrocarbon contaminated site. A wide variety of bacteria have the ability to degrade petroleum hydrocarbons and completely mineralize them (Fowzia and Fakhruddin, 2018). However, research has proved that plasmid borne bacteria are more efficient in pollution degradation than bacteria that do not bear plasmid. Many bacterial strains have genetic determinants of degradative abilities to pollutants. These determinants are often found in plasmids. These degradative abilities occur in high frequencies with much greater quantitative prominence after pollution. There was proposition that the development of degradative population in a polluted site can lead to gene transfer (vertical or horizontal gene transfer), transposons transfer, plasmids transfer and possible spontaneous mutation due to the presence of pollutants (Kulkarni and Kaliwal, 2015). Several literatures abound on the screening and growth optimization conditions of crude oil degradation using bacteria with limited reports on plasmid mechanisms and therefore necessitated the present study. The present study was designed to assess the role of plasmid mediated biodegradation of crude oil under optimal growth conditions.

## 2. Materials and Methods

### 2.1 Materials

#### 2.1.1 Procurement of Bonny light crude oil

Bonny light crude oil (API gravity= 32.15) was obtained from the Nigerian National Petroleum Corporation (NNPC) Port Harcourt Refinery, Alesa - Eleme, Rivers State, Nigeria was used in this research.

### 2.2 Experimental Design

#### 2.2.1 Sampling site

Soil samples were obtained from four sites of 15 year old hydrocarbon contamination at Awka, Aguleri, Onitsha and Ekwulobia in Anambra State. The Awka sampling site lies within latitude N06.22677º and Longitude E007.07602º with a mean elevation of 133 meters above sea level and located at Aroma Junction, Awka South Local Government Area of Anambra State.

The sampling point at Aguleri lies between latitude N6.33231° and longitude E6.87444° with mean elevation of 45 metres above sea level and located at Aguleri Junction in Anambra East Council Area. The sampling site at Onitsha lies within latitude N6.13378º and longitude E6.79393º with mean elevation of 43 meters above sea level and situated at Upper Iweka Axis of Onitsha South Local Government of Anambra State while the Ekwulobia area lies within latitude N5.99053º and longitude E7.17018º with a mean elevation of 88 meters, with Ekwulobia Motor Park in Aguata Local Government Area of Anambra State as the sample site.

### 2.3 Sampling Method

Soil samples were collected from depths of 0 - 10 cm at georeferenced point at the centre of the site and two other points 8 m away, and also, 3 subsamples 6 m away from each other were collected from each point (Nakamura *et al*., 2014). A total of 75 composite samples were collected from the 4 sampling sites. The sampling was performed with soil auger and transferred into a polyethylene bag. The polyethylene bag containing the composite sample was stored in a plastic bucket with dry ice, then, immediately sent to the Microbiology Laboratory of Nnamdi Azikiwe University, Awka, Anambra State and kept below 4 °C in a refrigerator for microbial and chemical analyses**.**

### 2.4 Isolation of Hydrocarbon Degrading Bacteria

The enrichment culture technique was used for the isolation of bacterial strains capable of utilizing crude oil as a sole source of carbon and energy as described by Gayathri *et al.* (2014). Crude oil mixed media were prepared by thoroughly mixing different concentrations of crude oil (1 %, 2 %, 5 % and 10 %) with 100 mL media (LB, MS and Nutrient broth) when the media were about to solidify (45 – 50 ºC). One gram of each soil sample was suspended in 9 mL of distilled water and kept at room temperature for 24 hours. On the next day, 250 µL of the supernatant was spread on crude oil containing Nutrient agar, LB and MS Petri-dishes, for the isolation of hydrocarbon degrading bacteria and incubated at room temperature. The Petri dishes were observed on the next day to till fourth day for the appearance of bacteria colonies (Hyina *et al*., 2003). The cultures were randomly selected with series of seven transfers. The purified bacterial strains were stored on agar slants and kept at 4 °C under refrigeration for further analysis.

### 2.5 Preliminary Screening of Isolate for Hydrocarbon Degradation

#### 2.5.1 Preparation of inoculum

Inoculum was prepared as described by Nwanyanwu *et al*. (2016). The test isolates were grown in nutrient broth medium contained in Erlenmeyer flasks (100 mL) at room temperature for 48 hr. Thereafter, the cells were harvested by centrifugation at 6000 rpm for 10 min and washed in sterile de-ionized water. The cell suspensions were standardized by adjusting the turbidity to optical density of 0.1 at absorbance of 540 nm and used throughout the study unless otherwise stated.

#### 2.5.2 Turbidometric test

The MS medium was also used in assessing the ability of the bacterial isolates to utilize hydrocarbon fraction which contained 4 % (v/v) of the hydrocarbon fraction. The medium was made out in 100 mL conical flask containing 50 mL of the MS medium and sterilized. The 60 bacterial isolates were inoculated individually into separate flasks. The flasks were incubated at room temperature on an orbital shaker at 120 rpm for 7 days. Cell growth measured as optical density at 600 nm (OD600) was used as a parameter for crude oil degradation capability (Nwanyanwu *et al*., 2016). Crude oil degrading bacteria that have high degrading potentials were selectively picked and used for further research.

### 2.6 Plasmid Curing

After screening test for presence or absence of plasmid, 8 out of 22 isolates were plasmid borne and they were subjected to plasmid curing. The plasmid curing was performed using the method described by Isiodu *et al.* (2016). Bacterial cells were grown in broth overnight. Five (5) mL of Nutrient broth supplemented with 0.1 mg/mL acridine orange was prepared. Zero point one millilitre (0.1 mL) of freshly prepared culture suspension of the test strains from $10^{-5}$ dilution was inoculated

into Nutrient broth containing the acridine orange, incubated at 37 ºC ( pH 7.6) for 4 days in dark and plated out on nutrient agar. Similarly, control cultures were prepared without acridine orange in Nutrient broth. Colonies that were able to grow on nutrient agar but not on modified solid mineral salt medium were isolated and considered cured.

**2.9.2 Biodegradation capability test of plasmid cured isolates in solid media**

To confirm the effectiveness of the plasmid curing, biodegradation ability test was done using the 8 isolates. Each of the isolates {plasmid free cells and plasmid borne cells (control) was grown in nutrient broth. One milliliter (1.0 mL) of the cell suspensions of each of the plasmid free cells and plasmid borne cells was transferred into 250 mL Erlenmeyer flasks containing 100 mL of sterile mineral salt medium, supplemented with 2 mL (2 % v/v) of Bonny-light crude oil. The flasks were then incubated in a shaker at 120 rpm at 30 ˚C for 3 days. Flask containing sterile mineral salt medium and 2 % of crude oil but without test organisms also served as control 2. The 3 day old cultures were serially diluted (Tenfold dilutions) and $10^{-5}$ dilution plated on MS media. The growths of the isolates in the media were classified as; good (+++), moderate (++), poor (+) and no growth (-) and these served as evidence of biodegradation.

**2.9.3 Antimicrobial susceptibility tests of plasmid of isolates after curing**

Four (4) bacterial isolates were selected for the antibiotic susceptibility test based on the antibiotic susceptibility test of the isolates in the pre-plasmid curing. The selected isolates were $C_3$, D1, $J_3$ and $L_2$ which were resistant to all the test antibiotics. Antibiotics susceptibility patterns of selected isolates were determined with seven commercially and widely used antibiotics to confirm the drug resistance of these isolates using disk diffusion method on Mueller-Hilton agar. A suspension of the test organisms was prepared by adjusting the turbidity, which was then adjusted to a 0.5 McFarland standard. A Mueller-Hinton agar plate was prepared using manufacturers instruction. With the help of a sterile cotton swab, a uniform lawn of bacterial growth was prepared on Muller-Hinton agar plates (pH 7.0). Before streaking, the swab was pressed against the wall of the tube containing the suspension to drain out the excess fluid. Antibiotic discs of Norfloxacin (10 mg), Chloramphenicol (30 mg), Amoxicillin (20 mg), Ampiclox (20 mg), Streptomycin (30 mg), Rifampicin (20 mg), and Nalidixic Acid (30 mg) were placed aseptically on the surface of the inoculated plates with a sterile forcep. The plates were then incubated at room temperature for 24 hr. After incubation, the plates were observed for the presence of zones of inhibition.

**2.10 Characterization and Identification of Crude Oil Degrading Organism**

The selected pure cultures were identified using Gram Staining, spore test, motility test, indole test, Voges-Proskauer-Methyl Red test, urease test, coagulase test, starch hydrolysis, citrate utilization test and hydrogen sulphide test, catalase test, oxidase test, nitrate reduction, sugar fermentation and growth on MacConkey agar.

**2.11 Optimization of Growth Condition**

**2.11.1 Temperature optimization**

The aim of this study was to obtain optimum temperatures for each of the test organisms for the bioremediation study. Among the environmental factors, temperature is one of the important factors controlling activity and survival of microorganisms as well as the rate of degradation. The temperatures considered for optimization study are 25 ºC, 30 ºC, 35 ºC, 40 ºC and 45 $^0$C.The cultures were maintained in five separate flasks at varying temperature. In this study a pH of 7.0 ± 0.2 was maintained, as it was maintained earlier at the time of isolation of microbial strains. Experiment was carried out in eleven 100 mL conical flasks containing 50 mL MSM media with 2 % of petroleum crude oil inoculated with log phase culture of each of the 8 plasmid borne and plasmid free bacterial isolates. The flasks were kept in shaker incubator at 120 rpm for 7 days at room temperature. At the end of incubation, samples were drawn and assayed for growth in terms of OD at 600 nm in a UV-Visible spectrophotometer (Shimadzu-Japan).Un-inoculated MSM was used as control. All the experiments were conducted in triplicates.

**2.11.2 pH optimization**

The choice of pH (pH 4, 5, 6, 7, 8, 9, 10,11and 12) was to establish the optimum pH for the bacterial growth and survival on hydrocarbons. The 8 plasmid borne bacterial cultures were prepared in three different sets containing 9 flasks in each set. Each was maintained at a different pH. The concentration of the broth and the carbon source was the same as in temperature optimization. The pH was adjusted by adding 0.1 N solution of NaOH and HCl. The flasks were sterilized, and kept in shaker incubator chamber at 120 rpm for 7 days at room temperature and each of the 8 bacterial cultures were grown at its optimal temperature obtained in the earlier study.

**2.11.3 Optimum concentration of crude oil**

The aim of this experiment was to assess the effect of different hydrocarbon concentrations on the growth of bacteria and to establish the most effective concentration for the screened hydrocarbon degraders. Different concentrations of the crude oil (v/v %) were used to find the optimum concentration of crude oil for the growth of the selected microbes. The concentrations studied were 0.5 %, 1 %, 2 %, 3 %, 5 % and 10 %.Each of the isolates cured of the plasmid was cultured in the mineral salt medium containing of the above concentrations of the crude oil and the medium incubated for 72 hr in a rotary shaker at room temperature. Plasmid borne strains of each of the isolates were used as controls. Cell growth measured as optical density at 600 nm was used as a parameter for crude oil degradation capability.

## Results and Discussion

**3.1 Preliminary Screening of Isolates for Hydrocarbon Degradation**

The preliminary screening test showed that the isolates exhibited variations in their growths in the growth medium containing crude oil and the optical density (A600 nm) ranged between 0.10 to 0.42 as shown in Table 1. Twenty-two isolates exhibited pronounced growths (OD600 nm > 0.30) viz: $A_1$, $A_2$, $B_1$, $C_1$, $C_3$, $D_1$, $E_1$, $E_3$, $F_2$, $F_5$, $G_2$, $H_2$, $H_4$, $I_3$, $I_5$, $I_6$, $J_3$, $K_1$, $K_4$, $L_2$, $M_1$ and $N_4$, indicated in table 1 and were selected for further

studies. Isolate designated as C3 had the highest growth with optical density of 0.42 while the isolate with least growth was designated as $K_5$ and had optical density of 0.10. Sixty hydrocarbon utilizing bacteria were isolated from crude oil supplemented mineral salt agar medium (Table 1). Since all the bacteria in the present study were isolated from a petroleum contaminated soil sample, they survived in the oil-supplemented media very easily as also reported by other authors (Rahman *et al.*, 2003). Table 1 indicated that 22 of the isolates were observed to have high pollutant degrading potentials (A600nm > 0.3) due to their crude oil utilization ability. Absorbance (A600 nm) of the cells grown in a medium with petroleum as a sole carbon source has been used as an index of PHC biodegradation potential (Binazadeh *et al.*, 2009; Celik *et al.*, 2008; Ciric *et al.*, 2010; Husain *et al.*, 2011). The other isolates showed variations in their growth on the substrate and the isolate with least growth has optical density of 0.11.Ciric *et al.* (2010) had differentiated growth of the alkane degraders based on A600nm using the following criteria: No growth = A600nm 0.00–0.019; +, minimal growth = A600nm 0.02–0.099; ++,moderate growth = A600nm 0.1–0.2; +++, maximum growth = A600nm > 0.2.Nwanyanwu *et al.* (2016) isolated *Micrococcus* sp. RS38 which showed impressive level of growth during screening in crude oil and other petroleum products where the organisms grew at equal optical densities of > 0.2 within 14 days of incubation. Vinothini *et al.* (2015) reported optical density of 0.55 by *Pseudomonas putida* which crude oil degradation ability was screened based on the growth efficiency on 2 % crude oil at the 7$^{th}$ day of incubation period.

**Table 1:** Preliminary screening of isolates for hydrocarbon degradation

| Isolate code | OD600 | Isolate code | OD600 | Isolate code | OD600 | Isolate code | OD600 |
|---|---|---|---|---|---|---|---|
| A1 | **0.35** | **E1** | **0.31** | G4 | 0.18 | J2 | 0.12 |
| **A2** | **0.37** | E2 | 0.28 | G5 | 0.11 | **J3** | **0.40** |
| A3 | 0.28 | **E3** | **0.35** | H1 | 0.15 | J4 | 0.28 |
| **B1** | **0.35** | E4 | 0.18 | **H 2** | **0.34** | J5 | 0.20 |
| B2 | 0.27 | E5 | 0.19 | H3 | 0.12 | **K1** | **0.33** |
| B3 | 0.29 | F1 | 0.31 | **H4** | **0.31** | K2 | 0.12 |
| B4 | 0.19 | **F2** | **0.36** | I1 | 0.25 | K3 | 0.14 |
| **C1** | **0.31** | F3 | 0.29 | I2 | 0.11 | **K4** | **0.30** |
| C2 | 0.27 | F4 | 0.15 | **I3** | **0.30** | K5 | 0.10 |
| **C3** | **0.42** | **F5** | **0.32** | I4 | 0.17 | L1 | 0.13 |
| **D1** | **0.38** | G1 | 0.16 | I5 | **0.31** | **L2** | **0.37** |
| D2 | 0.22 | **G2** | **0.37** | **I6** | **0.36** | L3 | 0.15 |
| D3 | 0.24 | G3 | 0.14 | J1 | 0.10 | L4 | 0.14 |
| **M1** | **0.35** | M2 | 0.21 | M3 | 0.17 | M4 | 0.23 |
| N1 | 0.28 | N2 | 0.19 | N3 | 0.15 | **N4** | **0.32** |

### 3.2 Biodegradation Capability Test for Plasmid Cured Isolate in Solid Media

Table 2 presented the growths of the 8 bacteria in the solid media before and after the removal of the plasmid DNA. However; the growths of the isolates were not the same after the removal of their plasmids. Isolates designated as $C_1$, $D_1$, $L_2$ and $J_3$ showed various degrees of growths after curing of their plasmids whereas isolates $G_2$, $H_4$, $K_4$ and $I_6$ were unable to grow after plasmid removal. In addition, isolates C1 and J3 recorded the highest growths on the solid media after the plasmid removal. Table 2 showed that plasmid cured mutants of *Pseudomonas aeruginosa* KAVK01, *Bacillus* cereus C12, *Ochrobacterium intermedium* E85b and *Bacillus subtilis* SDDlas were able to grow crude oil medium while plasmid cured strains of *Bacillus cereus* S024, *Bacillus subtillis* LK 4-5, *Enterobacter cloacae* GEBRI *and Bacillus licheniformis* 129 were unable to grow in crude oil supplemented medium. Table 2 also demonstrated that the non- plasmid cured isolates had greater growths than plasmid free ones. Unlike the findings of Kalaivani *et al*.(2012) which all the three plasmid cured isolates (*Pseudomonas aeruginosa*, *Pseudomonas putida* and *Bacillus subtilis*) lost the ability to grow in crude oil medium,4 out of 8 bacteria cured of plasmids in this study retained their ability to degrade crude oil although non-cured isolates recorded higher growths. Dam *et al*. (2012) reported that the plasmid cured culture of *Geobacillus stearothermophilus* PS11 strain could not grow in presence of crude oil or any of the solvents. The inability to grow in presence of crude oil or other solvents might be due to the removal or inactivation of gene(s) responsible for petroleum hydrocarbon degradation. Similarly, Kumar *et al*. (2012) also reported absence of growth by plasmid cured colony of strain *Geobacillus stearothermophilus*"AAP7919" which was grown on nutrient broth with anthracene as sole carbon source. The inability of the isolates to grow might be due to the removal or inactivation of gene(s) responsible for anthracene degradation from *Geobacillus stearothermophilus*"AAP7919"**.**Coral and Karagoz (2005) also reported that catabolic pathways, which encode different aromatic hydrocarbon degradation routes, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid.

**Table 2:** Biodegradation capability of plasmid cured isolates in solid media

| Isolate designates | Before curing | After curing |
|---|---|---|
| $C_3$ | + + + | + + |
| $D_1$ | + + + | + |
| $G_2$ | + + | - |
| $H_4$ | + + | - |
| $I_6$ | + + | - |
| $J_3$ | + + + | + + |
| $K_4$ | + + | - |
| $L_2$ | + + + | + |

**Key:** + + + = Heavy growth; + + = Moderate growth; + = Minimal growth; - = No growth

### 3.3 Antimicrobial Susceptibility Tests for Isolates before and after Plasmid Curing

The antibiotic susceptibility tests of the isolates were described in Table 3. It was observed that 4 plasmid borne cells resisted the antibiotics tested after pre-cured examination but these isolates were 95% sensitive to the same antibiotics after post cured test. Plasmid cured isolate designated as $C_3$ was sensitive to norfloxacin, chloramphenicol, amoxicillin, rifampicin, ampiclox and nalidixic acid but resistant to streptomycin. In the same vain, plasmid cured isolate ($D_1$) was sensitive to all the antibiotics investigated. Plasmid cured strain ($J_3$) was sensitive to chloramphenicol, streptomycin, ampiclox but resistant to amoxicillin and nalidixic acid while cured strain ($L_2$) was sensitive to all antibiotics. Antibiotic susceptibility test (Table 3) of some isolates showed that some cured strains of the bacteria lost its original antibiotic resistance. The results of this study suggested that the antibiotic resistance of wild type of *Pseudomonas aeruginosa* in all tested antibiotics were conferred by plasmid DNA with exception of streptomycin whose resistance seems to be encoded by genes of the bacterial chromosome. A similar finding had been reported from a plasmid borne determinant of *Pseudomonas* sp. isolated from cotton leaf (Saha *et al.*, 2001).This suggested that genes encoding resistance to antibiotics by bacteria that possess plasmids. Incidence of multiple antibiotics resistant bacteria especially those that possess plasmid, in this study is in agreement with the study of Akinyemi *et al*. (2006).

### 3.4 Identification of Crude Oil Degrading Organisms

The biochemical characteristics of the bacterial isolates shown in Table 4 indicated that isolates which were able to degrade crude oil belonged to genera *Bacillus, Pseudomanas* sp., *Ochrobacterium* sp. and *Enterobacter* sp. Several species of Gram positive bacteria carry multiple plasmids which serve as adaptive mechanism especially those belonging to the genera *Staphylococcus, Streptococcus, Lactobacillus, Bacillus* and *Corynebacterium* (Kunninalaiyan *et al*., 2001, Igwilo-Ezikpe *et al*., 2010)

**Table 3:** Antibiotics susceptibility tests of the isolates

zones of inhibition of isolates (mm) before and after curing

| | $C_3$ | | $D_1$ | | $J_3$ | | $L_2$ | |
|---|---|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After | Before | After |
| **Antibiotics** | | | | | | | | |
| 10mg NB | 0 | 12 | 0 | 8 | 0 | 8 | 0 | 13 |
| 30mg CH | 0 | 9 | 0 | 14 | 0 | 10 | 0 | 12 |
| 30mg ST | 0 | 0 | 0 | 7 | 0 | 8 | 0 | 9 |
| 20mg AML | 0 | 8 | 0 | 10 | 0 | 0 | 0 | 10 |
| 20mg APX | 0 | 10 | 0 | 8 | 0 | 9 | 0 | 8 |
| 20mg RD | 0 | 9 | 0 | 9 | 0 | 10 | 0 | 12 |
| 30mg NA | 0 | 7 | 0 | 8 | 0 | 0 | 7 | 8 |

**NB**: Norfloxacin (NB), Chloramphenicol (CH), Amoxicillin (AML), Ampiclox (APX), Streptomycin (ST), Rifampicin (RD), Nalidixic Acid (NA).
0 = no zone of inhibition

**Table 4:** Morphological and biochemical characteristics of isolates

| Property | Isolates Codes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $D_1$ | $C_3$ | $L_2$ | $J_3$ | $H_4$ | $G_2$ | $K_4$ | $I_6$ |
| Gram reaction | + | - | + | - | + | + | + | - |
| Shape | SR | SR | SR | SR | SR | SR | SR | SR |
| Arrangement | S | S | S | S | S | S | S | S |
| Spore Test | - | - | - | - | - | - | - | - |
| Catalase Test | + | + | + | + | + | + | + | + |
| Indole Test | - | - | - | - | - | - | - | - |
| Motility Test | + | + | + | + | + | + | + | + |
| Methyl Red | - | - | - | - | - | - | - | - |
| Voges-Proskauer | + | - | + | - | + | + | + | + |
| Citrate Test | - | + | - | - | - | - | - | + |
| Urease Test | - | - | - | - | - | - | - | - |
| Starch Hydrolysis | + | - | + | - | + | + | + | - |
| Growth on MacConkey | - | + | + | + | + | + | + | - |
| $N0_3$ Reduction | + | + | + | + | + | + | + | + |
| Coagulase | - | - | - | - | - | - | - | - |
| $H_2S$ | - | - | - | - | - | - | - | - |
| Oxidase | - | + | - | + | - | - | - | - |
| Acid from: | | | | | | | | |
| Mannitol | - | - | + | - | + | - | + | + |
| Glucose | + | - | + | - | + | + | + | - |
| Xylose | | - | - | - | | - | - | - |
| Lactose | + | - | + | - | + | + | + | + |
| Sucrose | + | - | + | - | + | + | + | + |
| Arabinose | - | - | + | - | + | + | + | + |
| Galactose | - | - | - | - | - | - | - | - |
| Saccharose | - | - | - | - | - | - | - | - |

Key: SR = Short rod; S = Single

### 3.5 Optimization of Growth Condition

#### 3.5.1 Temperature

The temperatures selected for optimization study were 25 ºC, 30 ºC, 35 ºC and 45 ºC (Figures 1 and 2) .However, temperature was observed to influence the growth of the eight bacteria selected for the hydrocarbon degradation study as shown in the Figure 1. The result indicated that plasmid borne *Pseudomonas aeruginosa* KAVK01, *Ochrobacterium intermedium* E85b, *Bacillus subtilis* SDDlas *and Bacillus subtilis* LK4-5 *had* optimum temperature at 30 °C while *Bacillus cereus* C12, *Bacillus licheniformis* 129, *Enterobacter cloacae* GEBRI *and Bacillus cereus* S024 had their optimum temperatures at 35 °C .The results further revealed that all the test organisms exhibited low growth at 25 °C and 45 °C.

Similarly, some plasmid cured isolates showed the same growth patterns as in plasmid borne cells but retarded growths were observed among plasmid cured cells of *Bacillus subtilis* LK4-5, *Bacillus licheniformis* 129, *Enterobacter cloacae* GEBRI *and Bacillus cereus* S024 across all temperatures (Figure 2). Comparatively, the growths of plasmid borne isolates were higher than the plasmid cured isolates across all temperatures while some cured cells had insignificant growths at all temperatures. Summarily, the most suitable temperatures based on the growth of the eight bacteria were 30 ºC and 35 ˚C and these temperatures were the incubation temperatures employed for further investigations in this research.

The Figures 1 and 2 depict slight variations in optimum temperatures among the isolates with no significant changes among the plasmid cured and borne cells. *Pseudomonas aeruginosa* KAVK01, *Ochrobacterium intermedium* E85b, *Enterobacter cloacae* GEBRI**,** *Bacillus subtilis* SDDlas *and Bacillus subtillis* LK4-5 *had optimum temperature* at 30 °*C while Bacillus cereus* EV-1, *Bacillus linchiniformis* 126 *and Bacillus cereus* S024 had their optimum temperatures at 35 °C. Si-Zhong *et al.* (2009) reported that the optimum temperature for biodegradation is usually 15 - 35 °C for aerobic processes and 25 - 45 ˚C for anaerobic processes. Temperature controls the bioavailability of low-solubility hydrocarbon and hence the nature and the extent of microbial metabolism (Margesin and Schinner, 2001). However, an observation of higher growths was made on plasmid borne bacterial isolates when compared with plasmid cured isolates at their various optimum temperatures.

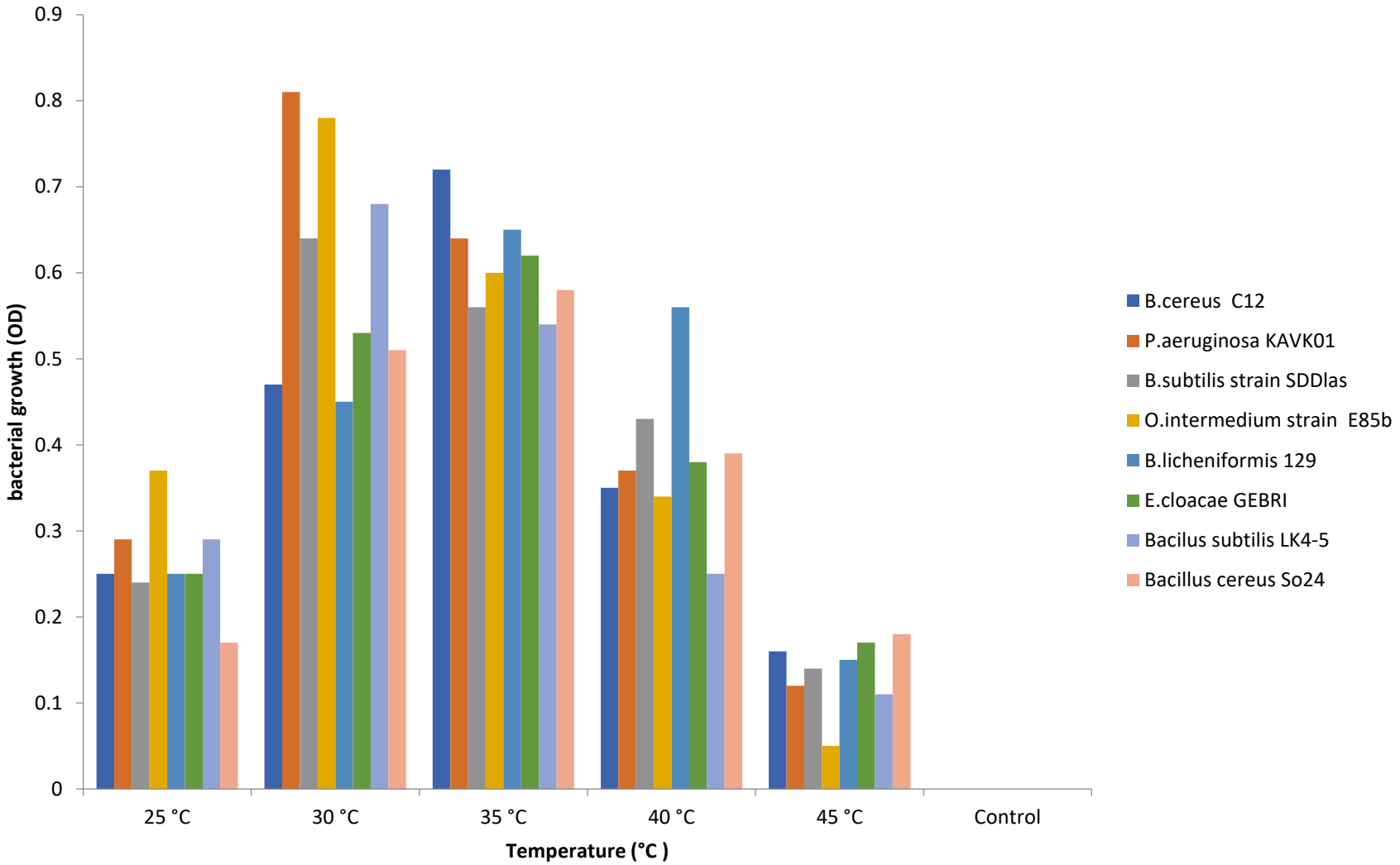


**Figure 1:** Growth of plasmid borne isolates at different incubation temperatures

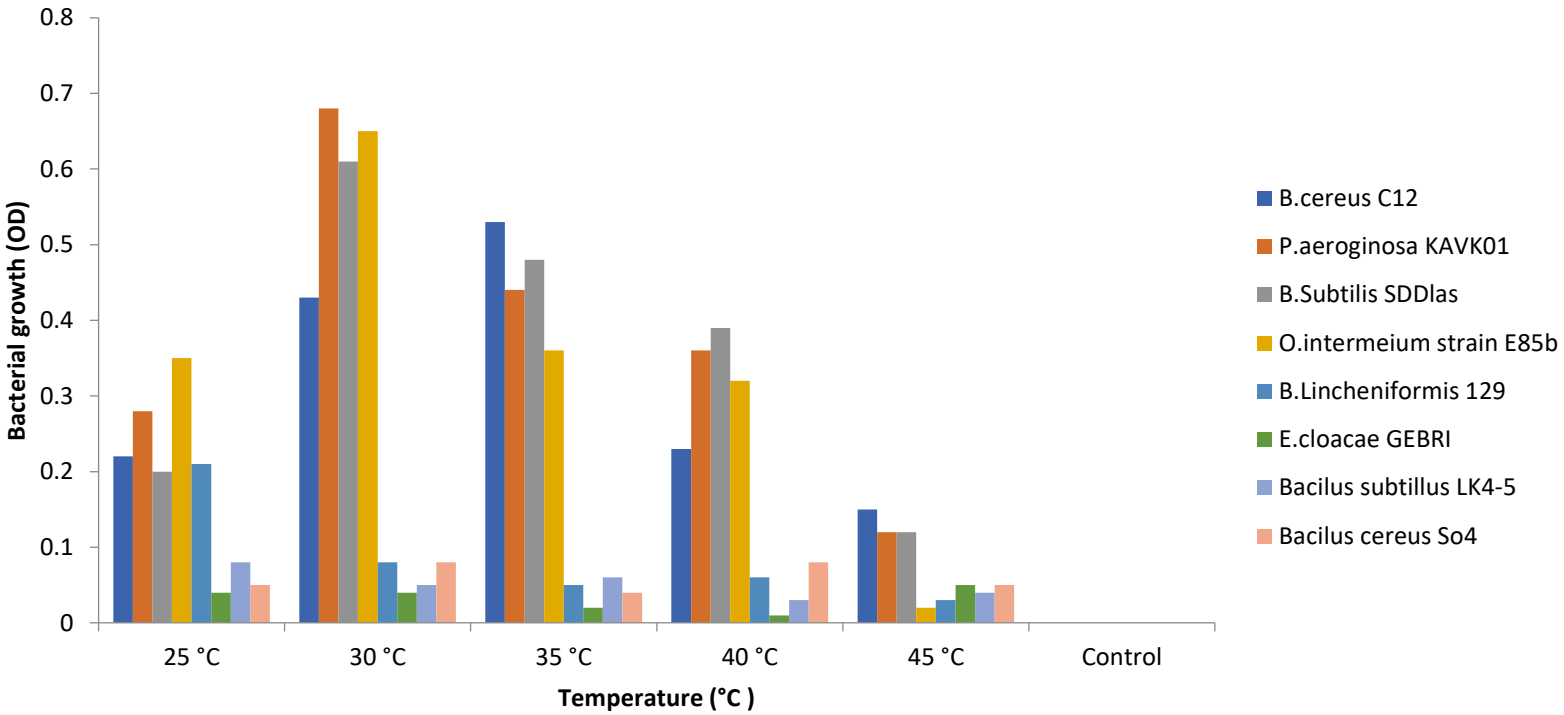


**Figure 2:** Growth of plasmid cured isolates at different incubation temperatures

### 3.5.2 pH Optimization

The results presented in Figure 3 indicated that plasmid borne isolates utilized and degraded crude oil at a wide range of pH from 6 to 10, but low growths and oil remediation occurred at high acidic (pH 4) and high alkaline medium (pH 12).However, plasmid cured cells of *Bacillus subtillis* LK4-5, *Bacillus lichiniformis* 129, *Bacillus cereus* SO24 *and Bacillus cereus* S024 were unable to utilize the crude oil at all pH studied (Figure 4).

Although there were slight differences in growths at pH 7.0 and pH 8.0 among plasmid borne isolates. Statistical analysis showed that all the isolates had significant growths at pH 7.0, so it was considered as optimum pH for oil bioremediation. There was no statistically significant difference in growths among plasmid borne cells at pH 7.0 and pH 8.0. Some plasmid cured cells had retarded growths at all pH. Conclusively, both plasmid borne and cured bacterial isolates had optimum pH at 7.0 and all the bioremediation studies in this work were done at this pH. The result as shown in Figures 3 and 4 established that all the test organisms had their best growth at pH of 7.0 and difference in pH between cured and wild type strains was significant However, plasmid borne bacteria grew and degraded crude oil at a wide range of pH from 6 to 10, while low growths and oil degradations occurred at acidic medium (pH 4) and high alkaline medium (pH 12) meaning that the isolates preferred alkaline to neutral values of pH than acidic conditions. The measurement of pH in soil could indicate the potential for microbial growth (Asira, 2013).Biodegradation can occur under a wide range of pH; however, a pH of 6.5 to 8.5 is generally optimal for biodegradation in most aquatic and terrestrial systems. Moisture influences the rate of contaminant metabolism because it influences the kind and amount of soluble materials that are available as well as the osmotic pressure and pH of terrestrial and aquatic systems (Cases and Lorenzo, 2005).

### 3.5.3 Optimization of crude oil concentration

The growth of the bacterial isolates as shown in Figure 5 were established by A540 nm measurement using the mineral salt media (MSM) which contained different degree of pollutions (0.5 %, 1 %, 2 %, 3 %, 5 % and 10 %) of crude oil as only carbon sources. Increase in the OD is an indication of growth of respiring cells. The result showed the ability of some test bacteria to grow in crude oil at varying concentrations, from as low as 0.5 % to as high as 10 % (v/v). The visual increases in turbidity corroborated with this observation when compared with the control which has no visible growth. Figure 5 revealed that all plasmid borne bacteria grew on all hydrocarbon concentrations and their growths were greater than that of plasmid cured organisms. However, plasmid cured cells of *Bacillus subtilis* LK4-5, *Bacillus lichiniformis* 129, *Enterobacter cloacae* GEBRI *and Bacillus cereus* S024 had diminished or no growth. A cursory observation on Figure 5 showed that the highest growths for all isolates on the crude oil occurred at 3 % (v/v) crude oil concentration within the 72 hours period of incubation. Therefore, 3 % (v/v) crude oil was considered as the optimum concentration for the biodegradation study. An obvious decline in growth and turbidity was seen at 5 % and 10 % (v/v). Results of the growths of isolates on varying crude oil pollution revealed a marked growth difference between plasmid borne and cured isolates. The growths of plasmid borne cells were significantly ($P < 0.05$) higher than that of cured cells. The test organisms had their maximum growths at 3 % (v/v) and sub-optimum at 2 % and 5 % (v/v) crude oil. The decline in growths above 5 % (v/v) crude oil concentration was also observed among the test organisms (Figure 5). Plasmid borne *Pseudomonas aeruginosa* was the best degrader of the hydrocarbon with the OD reading of 0.96 at 3 % (v/v) crude oil while the plasmid cured *Pseudomonas aeruginosa* had OD reading of 0.58.The growths of plasmid borne and cured isolates of other test organisms followed the same growth patterns as in *Pseudomonas aeruginosa*. So it was indicating that the genes involved in crude oil degradation were both plasmid and chromosomal encoded and presence of plasmid confers greater remediation ability. Plasmid-mediated degradation of dimethoate was observed in *Pseudomonas aeruginosa* and *Bacillus licheniformis;* Chlorpyrifos by *Micrococcus*, *Enterobacter* and *Pseudomonas* with plasmid borne genes for its degradation (Singh et al., 2003; Bhagobaty and Malik, 2008; Kulkarni and Kaliwal, 2015). Mandal *et al.* (2005) reported that *Bacillus licheniformis* strain isolated from the intestine of *Labeo rohita* by an enrichment technique showed capability of utilizing dimethoate as the sole source of carbon with the help of plasmid.

## Conclusion

The whole study revealed that the sampling sites are reservoirs of crude oil degrading bacteria. The isolates possess catabolic genes which are plasmid and chromosomally mediated. Neutral pH, 35 °C temperature and 3 % crude oil concentration were found to be optimum conditions. The degrading potentials of these isolates could be exploited in crude oil and hydrocarbons biodegradations.

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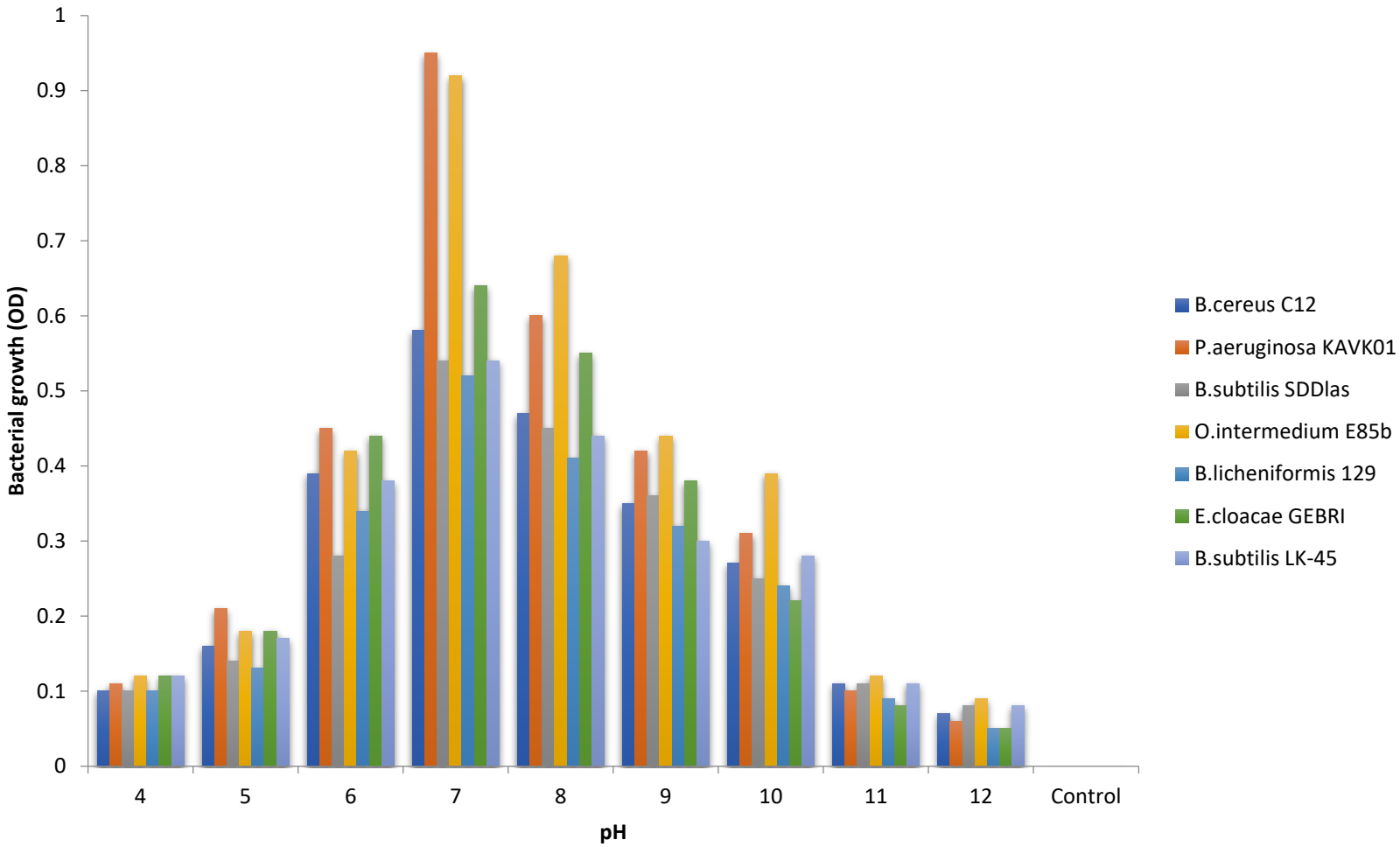


**Figure 3:** Growth of plasmid borne isolates at different incubation pH

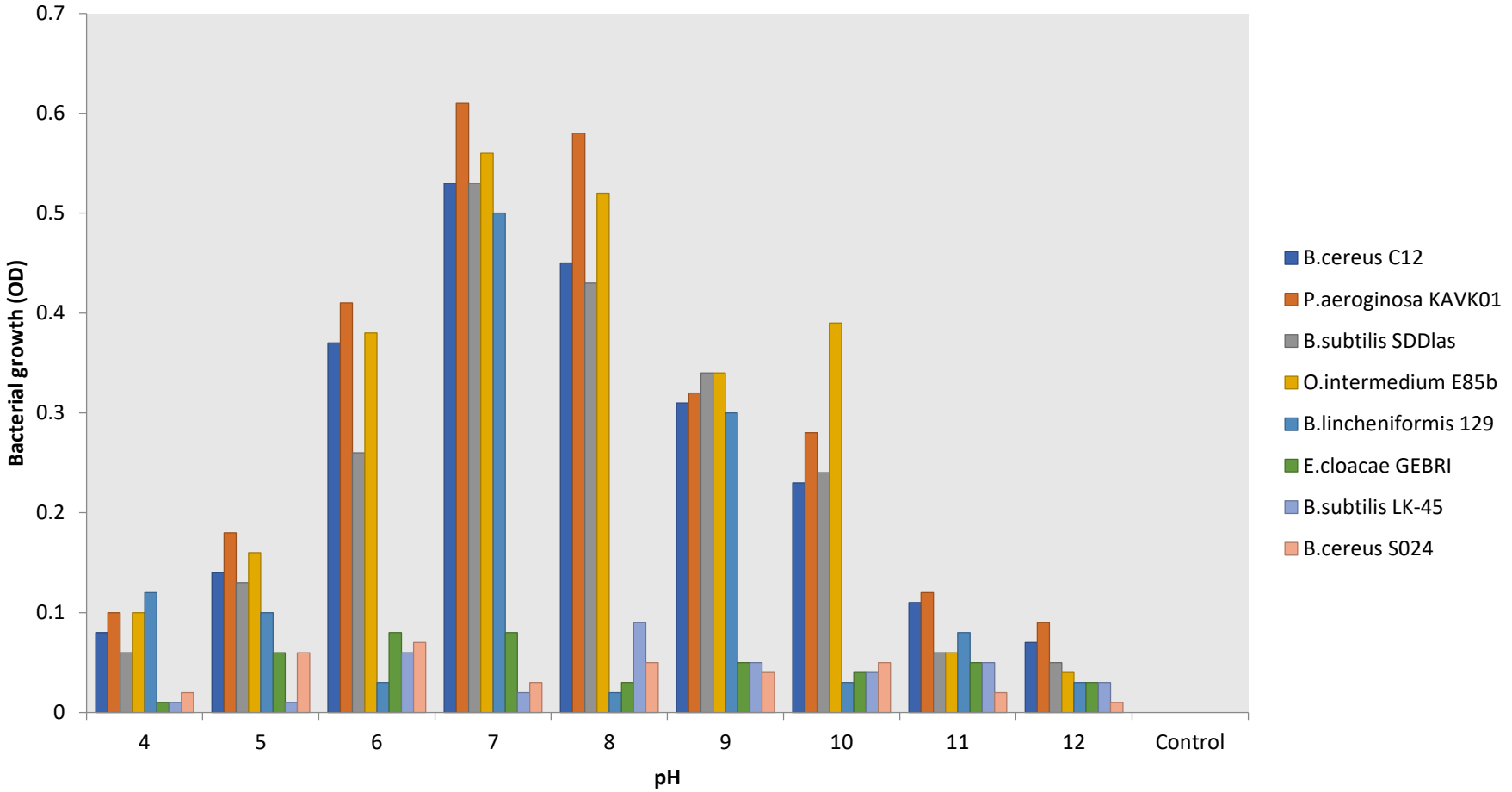


**Figure 4:** Growth of plasmid cured isolates at different incubation pH

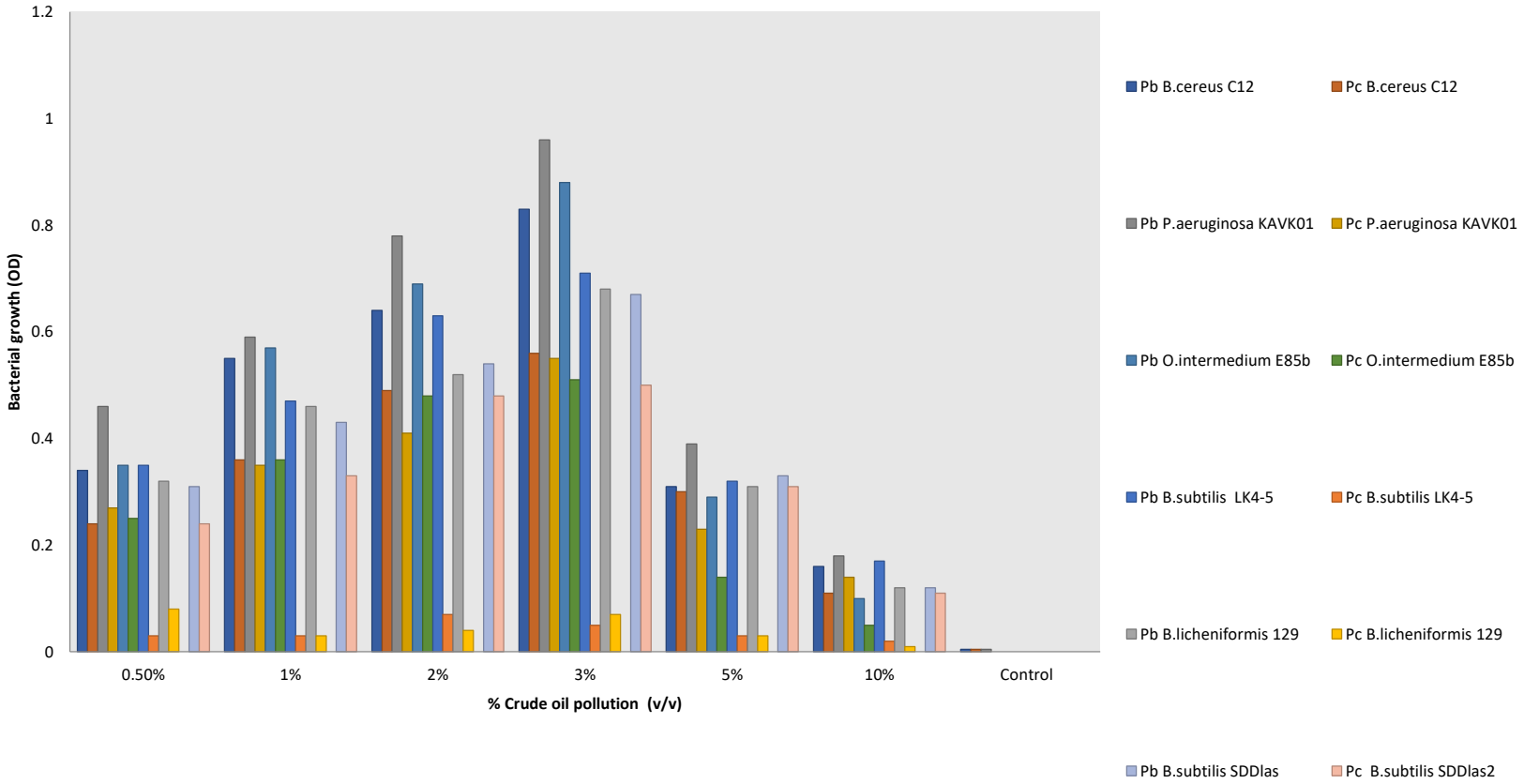


**Figure 5:** Optimization of concentrations of crude oil.
Note: plsb. = plasmid borne
Plsc. = plasmid cure

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