Journal of Advances in Biology & Biotechnology

15(2): 1-9, 2017; Article no.JABB.35414
ISSN: 2394-1081

Isolation, Screening and Characterization of Hydrocarbon-Utilizing Bacteria Isolated from Bitumen-Contaminated Surface Water in Agbabu, Ondo State

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TOO and JOO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors TOO and DOS managed the analyses and literature searches of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2017/35414
Editor(s):
(1) Andrzej Kowalski, Department of Biochemistry and Genetics, Institute of Biology, Jan Kochanowski University, Kielce, Poland.
(2) Michael BamitaleOsho, Department of Biological Sciences, McPherson University, Nigeria and Department of Microbiology, Faculty of Sciences, OlabisiOnabanjo University, Ago-Iwoye, Nigeria.
Reviewers:
(1) Arnaldo Sarti, Universidade Estadual Paulista, Brazil.
(2) Eliton da Silva Vasconcelos, Federal University of São Carlos, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history/21372

Original Research Article

Received 12th July 2017
Accepted 21st August 2017
Published 13th October 2017

ABSTRACT

Surface water samples from contaminated surface water in Agbabu, Odigbo Local government area of Ondo State were analyzed for hydrocarbon-utilizing bacteria. The mean total bacterial count and mean total hydrocarbon- degrading bacterial counts were determined using pour plate technique. The hydrocarbon-utilizing potentials of the isolates were further determined by screening them in minimal salt broth supplemented with 2% crude-oil over a period of 14 days. The growth of the isolates was monitored by measuring the absorbance ($OD_{600\ nm}$) and Total viable count (log_{10} CFU/ml). The effect of temperature, pH and crude-oil concentration on their degradative potentials was determined. The isolates were identified based on their cultural, morphological and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology. The Mean Total bacteria count was 7.70 (log_{10} CFU/ml) ranging from 6.25 to 8.77 (log_{10} CFU/ml) while the Mean Total hydrocarbon-utilizing bacteria was 7.14 (log_{10} CFU/ml) ranging from 4.77 to 7.64 (log_{10} CFU/ml). Six

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bacteria genera isolated from the samples collected include: *Staphylococcus, Micrococcus, Pseudomonas, Enterobacter, Bacillus* and *Alkaligens.* Five isolates namely; *Pseudomonas aeruginosa, Bacillus cereus, Bacillus lichenformis, Micrococcus luteus* and *Alkaligens faecalis* showed maximum utilization of crude-oil as sole carbon source and were selected for further studies. The result of varying crude-oil concentrations (1.0 -2.5%) on each of the selected hydrocarbon-utilizing bacteria showed that the isolates grew optimally at 2% crude-oil concentrations where two of the isolates *B. cereus* and *P. aeruginosa* had the highest growth rate of 0.77 and 0.75 respectively. However, their growth gradually reduced at increased crude-oil concentration (2.5%).The growths of the isolates were optimal at neutral to alkaline pH (7-9) while their growths were greatly reduced at acidic pH 3. The optimum temperature for the growth of the isolates ranged between 40°C-45°C.The result from this research affirms that an effective utilization of crude-oil would require simultaneous action of several metabolically versatile microorganisms under favorable environmental conditions such as pH, temperature and availability of nutrient.

Keywords: Bioremediation; biodegradation; crude-oil; temperature; pH.

1. INTRODUCTION

Petroleum is a complex mixture of varying molecular weight hydrocarbons and other organic compounds found beneath the earth's surface. It is formed from pyrolysis of hydrocarbon, in a variety of reaction, mostly endothermic at high temperature and pressure [1]. Petroleum constituent represent saturates, aromatics, resins and asphaltenes. It has been considered as the most prevalent pollutants, particularly in developing countries [2]. Wide scale production, transport, use and disposal of petroleum globally have made it to lead contaminants in both prevalence and quantity in the environment [3]. This contamination could be accidental spill by collision of oil tankers or by normal spill which is due to leakage or blowout of oilrig platforms, it can also occur by pipeline breakages. Environmental pollution arising from petroleum leakages in storage tanks, spillage during transportation of petroleum products, deliberate discharge of petroleum products and various industrial processes is hazardous to soil and water ecosystems [4]. There are several clean-up techniques for removing petroleum from the contaminated area. Among them, bioremediation methods are considered as low cost, highly efficient, environmental friendly alternative for remediation of crude oil contaminated sites [5,6]. Biodegradation by bacteria is considered as the most active process in petroleum degradation and bacteria are primary degraders of spilled oil [7,8] and this is specifically carried out largely by diverse bacterial population mostly *Pseudomonas* species [9,10]. They have the ability to make use of diverse substances such as petroleum and inorganic substances for energy and growth [11].

However, bioremediation of hydrocarbon is greatly influenced by factors such as pH value, temperature, moisture, oxygen supply, nutrient level and bacterial diversity. Temperature plays a significant role in controlling the nature and the extent of microbial hydrocarbon metabolism. Temperature affects the rate of biodegradation as well as the physical nature and chemical composition of hydrocarbons [12]. A temperature increase leads to an increase in diffusion rates of organic compounds notably by decrease of their viscosity [13]. Although microbial activity is generally reduced at low temperatures, many of the components in crude-oil can actually be degraded by psychrophilic and psychotropic microorganisms [14,15].

Hydrocarbon degradation is favored by near neutral pH values. Biodegradation is likely to be impaired where the pH has been shifted away from neutral by environmental conditions. In this study, hydrocarbon-degrading bacteria were isolated from bitumen-contaminated surface water and the effect of temperature, pH and crude oil concentration on their degradative abilities were determined.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Surface water samples were collected aseptically from 11 different Bitumen-contaminated sites in Agbabu, Odigbo Local government area of Ondo State. All samples were labeled and transported to the laboratory in ice packs for further microbiological analyses.

2.2 Isolation and Screening of Isolates

Hydrocarbon utilizing bacteria were isolated from surface water samples using minimal salt medium (MSM) supplemented with 1% crude oil

as a sole source of carbon. The composition of the MSM is as follows: K_2HPO_4 (1.8 g/L); NH_4Cl (4 g/L); $MgSO_4.7H_2O$ (0.2 g/L); NaCl (0.1 g/L); $Na_2SO_4.7H_2O$ (0.01 g/L); agar (20 g/L); carbon source (1%) and distilled water (1 L) with pH 7.2. 1 ml of each water sample was introduced in 250 ml Erlenmeyer flask containing 100 ml of MSM and 1ml crude oil. The medium was incubated at 37°C for seven days. After 7 days, 1.0 ml of sample was transferred into another 250 ml Erlenmeyer flask containing 100 ml fresh MSM and 1% crude oil and further incubated for another 7 days. At the end of 14^{th} day, samples were serially diluted. 0.1 ml from dilutions 10^{-6} and 10^{-7} was spread on MSM agar. The plates were incubated at 37°C for 72h. Pure cultures were obtained using streak techniques and stored at 4°C in agar slant for further use.

2.3 Identification of Isolates

Pure cultures were identified based on their cultural, morphological and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology [16].

2.4 Effect of Crude Oil Concentration on Hydrocarbon-Utilizing Potential of Isolates from Bitumen-Contaminated Surface Water in Agbabu

Five isolates were activated in peptone water and incubated overnight at 37°C. MSM containing 0.2 g of KCl, 6 g of Na_2HPO_4, 2.8 g of NaH_2PO_4, 0.1 g of $MgS0_4$ and 5 g of NaCl was prepared in Erlenmeyer flasks with different concentration of crude-oil (1%, 1.5%, 2% and 2.5%) and sterilized at 121°C for 15 minutes and cooled to 45°C. The isolates were inoculated into the medium and incubated for 14 days. Optical density was measured at 2 days interval at absorbance of 600 nm using a JENWAY Model-6705 spectrophotometer.

2.5 Effect of pH on Hydrocarbon-Utilizing Potential of Isolates from Bitumen-Contaminated Surface Water in Agbabu

One hundred milliliters of MSM containing .2 g of KCl, 6 g of Na_2HPO_4, 2.8 g of NaH_2PO_4, 0.1 g of $MgS0_4$ and 5 g of NaCl was prepared and the pH of the broth medium was adjusted to pH 3, pH 5, pH 7, pH 9 and pH 11 using acetate buffer for acidic pH and phosphate buffer for alkaline pH, 1% crude-oil was added and was sterilized at 121°C for 15 minutes. The isolates were activated on peptone water and 1 ml of the inoculums was introduced into the prepared MSM and incubated at 37°C for 14days. Bacterial growth was monitored at 2 days interval by measuring the optical density at 600 nm using a JENWAY Model–6705 spectrophotometer.

2.6 Effect of Temperature on Hydrocarbon-Utilizing Potential of Isolates from Bitumen-Contaminated Surface Water in Agbabu

One hundred milliliter of was prepared into 5 different Erlenmeyer flasks and 1% crude-oil was added and sterilized at 121°C for 15minutes. The isolates were activated on peptone water and 1ml of the inoculums was introduced into each of the prepared MSM. The inoculated flasks were incubated at 25°C, 35°C, 40°C and 45°C for 72 hr. Bacterial growth was monitored after 72hours by measuring the optical density at 600nm using a JENWAY Model – 6705 spectrophotometer.

2.7 Data Analysis

The data generated from this study was analyzed using Microsoft excel and Chi- square of the Statistical Procedure for Social Science version 22.0 (SPSS, Chicago, IL, USA). The values were considered significantly different at $p<0.05$.

3. RESULTS AND DISCUSSION

Bacteria that were able to grow and utilize crude-oil as a carbon and energy source were isolated from bitumen-contaminated surface water in Agbabu Ondo State. The Mean Total bacteria count (MTBC) was 7.70 (log_{10} CFU/ml) and it ranged from 6.25to 8.77(log_{10}CFU/ml) while the Mean Total hydrocarbon-utilizing bacteria count (MTHUBC) was 7.14 (log_{10} CFU/ml) and ranged from 4.77 to 7.64 (log_{10} CFU/ml) as shown in Table 1. There was variation in the Total bacteria count and Total hydrocarbon-utilizing bacteria count among the samples analyzed. Statistical analysis showed that there was significant difference in the hydrocarbon-utilizing bacteria (HUB) and non hydrocarbon-utilizing bacteria count (NHUB (Fig. 1). The higher count of HUB could be due to exposure of the bacteria isolates to hydrocarbon contaminated environment. This agrees with the work of (Rahman et al. [3] who reported that the population levels of hydrocarbon utilizers within the contaminated

environment appear to be a sensitive index of environmental exposure to hydrocarbons.

In this study, the frequency of distribution of the isolated organisms was also examined. *Pseudomonas aeruginosa* had the highest frequency occurrence of 20 (33%) while E. *aerogenes* had the least frequency of 3 (5%). The dominance of Gram-negative bacteria especially *Pseudomonas* at the location was not surprising as it has been documented by Adesanya et al. [17]. The ability to isolate high numbers of certain oil degrading microorganisms from an environment implies that these organisms are the active degraders in that environment [18,19].

The result of varying crude-oil concentrations (1.0 -2.5%) on each of the selected hydrocarbon-utilizing bacteria showed that the isolates grew optimally at 2% crude-oil concentrations where two of the isolates *B. cereus* and *P. aeruginosa* had the highest growth rate of 0.77 and 0.75, respectively. However, their growth gradually reduced at increased crude-oil concentration 2.5%. Chadli et al. [20] had reported that increase in concentration of crude-oil could lead to fast growth and reproduction of bacteria which could promote the biodegradation of crude-oil. However, too high concentration of hydrocarbons may also cause inhibition of biodegradation by nutrient or oxygen limitation or toxic effects.

Table 1. Mean total bacterial count and mean total hydrocarbon-degrading bacteria count (Log_{10} cfu/ml) of samples obtained from Agbabu, Ondo State

Samples	MTBC	MTHUBC
	Log_{10} Cfu/ml	Log_{10} Cfu/ml
AGW1	6.25	4.77
AGW2	7.58	5.40
AGW3	7.61	5.35
AGW4	8.04	6.40
AGW5	7.68	6.22
AGW6	7.55	5.35
AGW7	7.58	6.29
AGW8	7.75	4.64
AGW9	8.77	5.20
AGW10	8.26	5.52
AGW11	7.57	4.36
RANGE (MEAN)	6.25-8.77 (7.69)	4.36-6.40 (5.41)

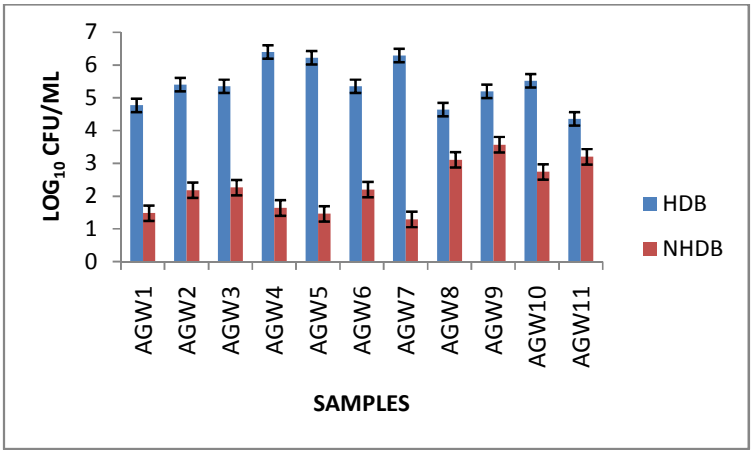

Fig. 1. Mean bacterial count (Log_{10} cfu/ml)
Values are the mean and standard errors of two replicates
Key: HDB- Hydrocarbon-utilizing bacteria, NHDB- Non Hydrocarbon-utilizing bacteria

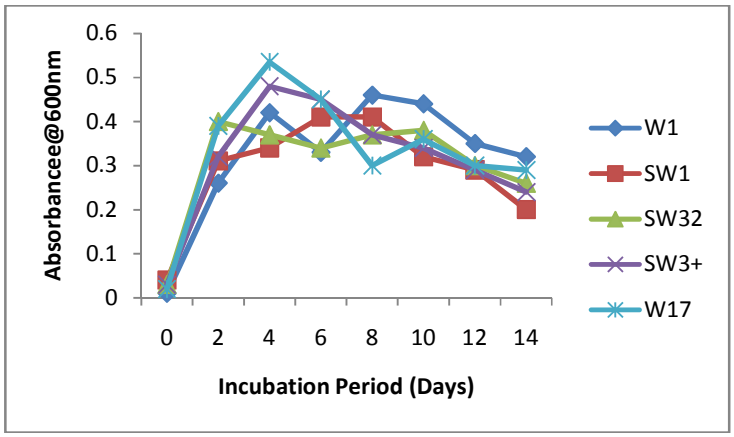

Fig. 2. Growth profile of bacterial isolates in minimal salt broth supplemented with 1% crude-oil over a period of 14 Days

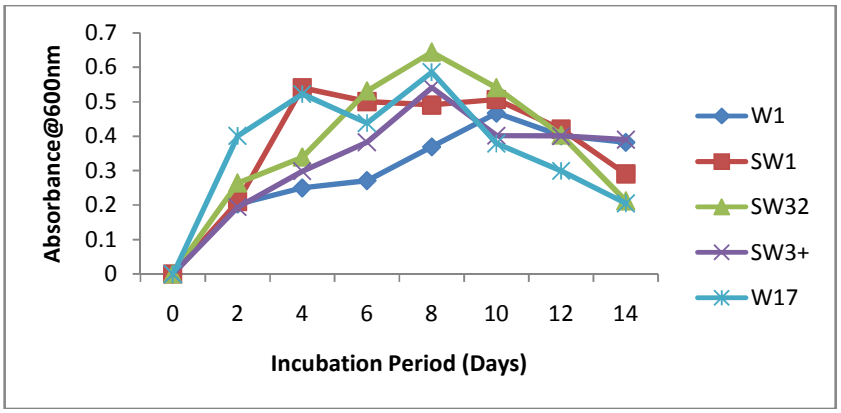

Fig. 3. Growth profile of bacterial isolates in minimal salt broth supplemented with 1.5% crude-oil over a period of 14 days

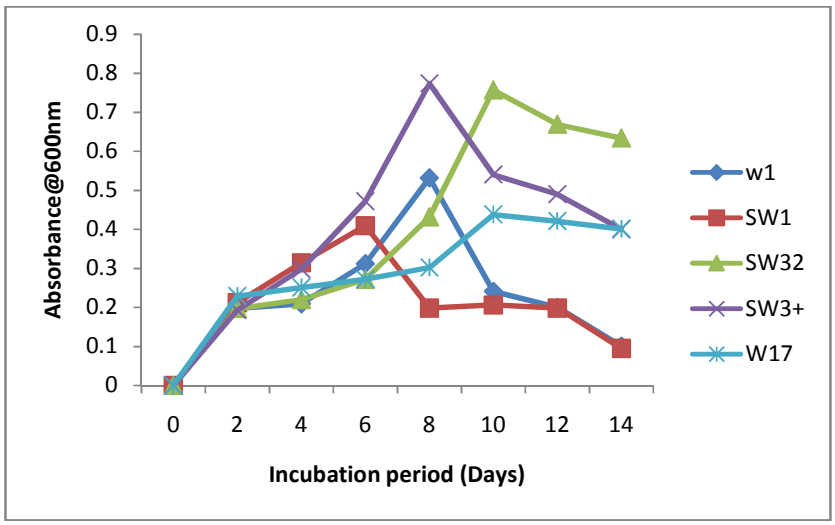

Fig. 4. Growth profile of bacterial isolates in minimal salt broth supplemented with 2% crude-oil over a period of 14 days

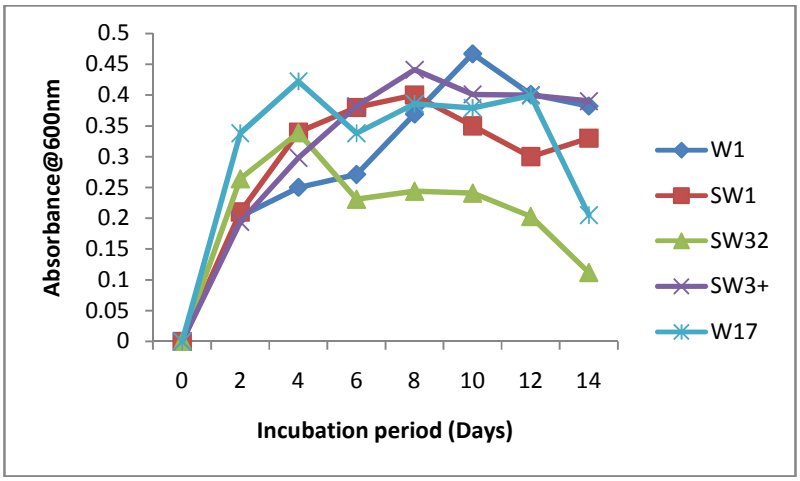

Fig. 5. Growth profile of bacterial isolates in minimal salt broth supplemented with 2.5% crude-oil over a period of 14 days

Key: W1= A. feacalis, SW1= Micrococcus luteus, SW32 =P. aeruginosa, SW3+ =Bacillus cereus, W17= B. lichenformis

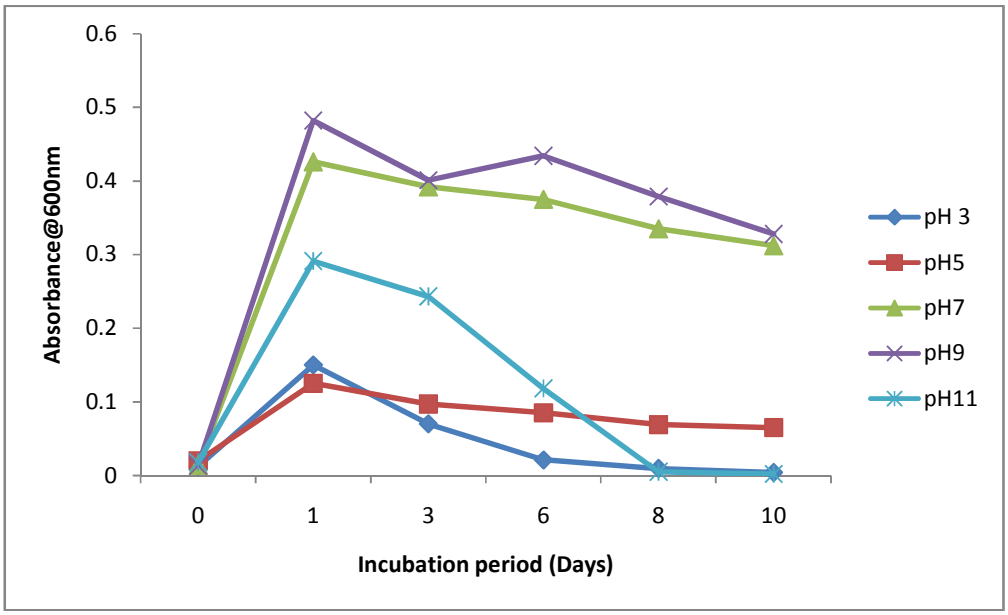

Fig. 6. Effect of pH on hydrocarbon-utilizing potential of *Bacillus cereus*

The effect of pH on the selected bacterial isolates was examined and it was observed that each of the isolates had optimum pH for growth. The growth of the isolates was optimal at neutral to alkaline pH (7 to 9) while their growths significantly reduced at acidic pH 3 range, indicating that the utilization of crude oil by the isolates was highest at the alkaline range. This agrees with the findings of Kim et al. [21] in Korea Refinery who studied the effect of pH on the rate of biodegradation of hydrocarbon and observed that degradation was optimum at pH 7.

The effect of temperature on the hydrocarbon-utilizing potentials of the selected isolates were also examined and it was observed that optimum temperature for the growth of the isolates ranged between 40°C and 45°C. In a research by Shallu Sihag et al. [22], it was observed that at the mesophilic and thermophilic temperature range, microbial enzyme activity increases which lead to increase in the rate of hydrocarbon degradation.

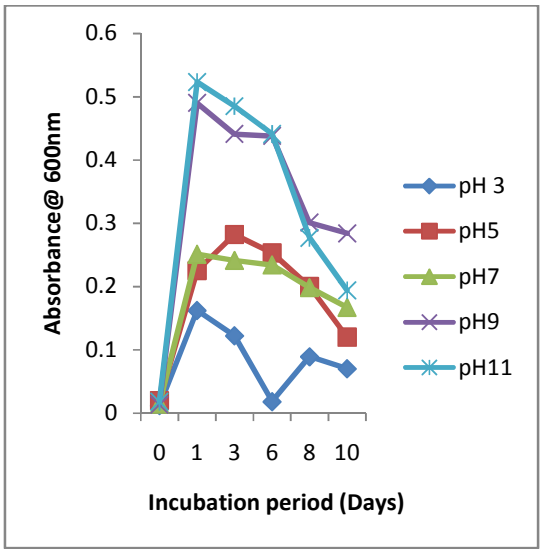

Fig. 7. Effect of pH on hydrocarbon-utilizing potential of *Bacillus lichenformis*

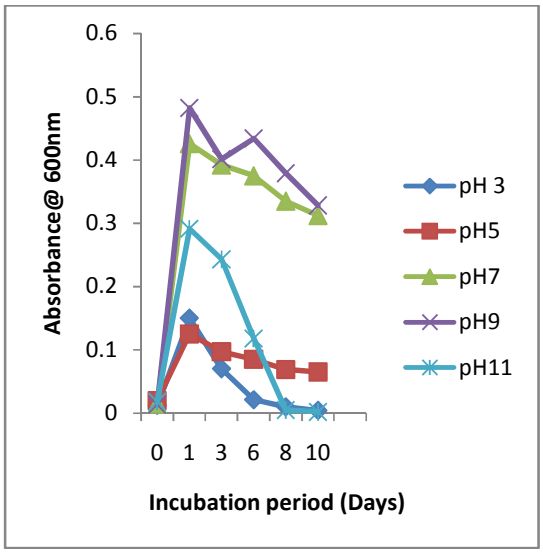

Fig. 8. Effect of pH on hydrocarbon-utilizing potential of *Micrococcus* sp

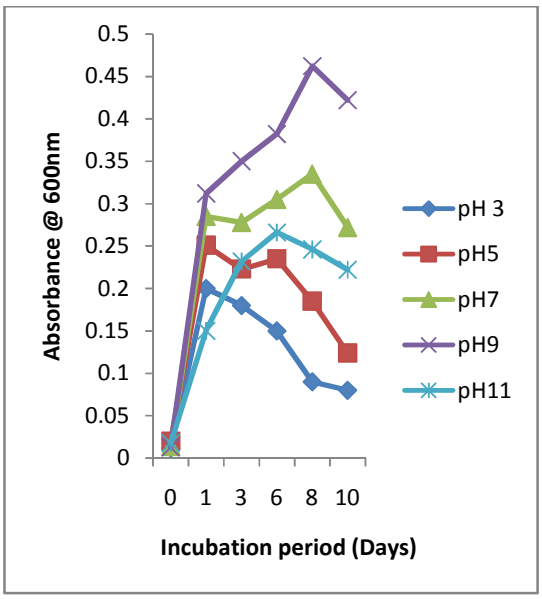

Fig. 9. Effect of pH on hydrocarbon-utilizing potential of *Acaligens faecalis*

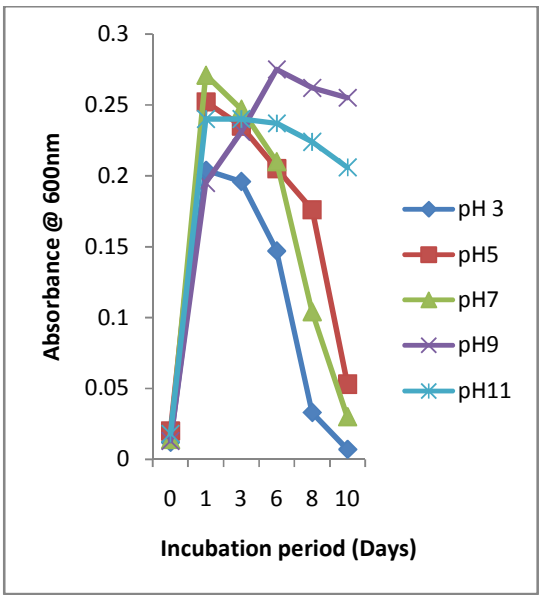

Fig. 10. Effect of pH on hydrocarbon-utilizing potential of *Pseudomonas aeruginosa*

Table 2. Percentage distribution of isolates from surface water samples in Agbabu, Ondo State

Isolates	Number of isolates	Frequency (%)
E. aerogenes	3	5
Staphylococcus aureus	7	12
P. aeruginosa	20	33
Acaligens sp.	4	7
Bacillus spp.	18	30
Micrococcus sp.	8	13

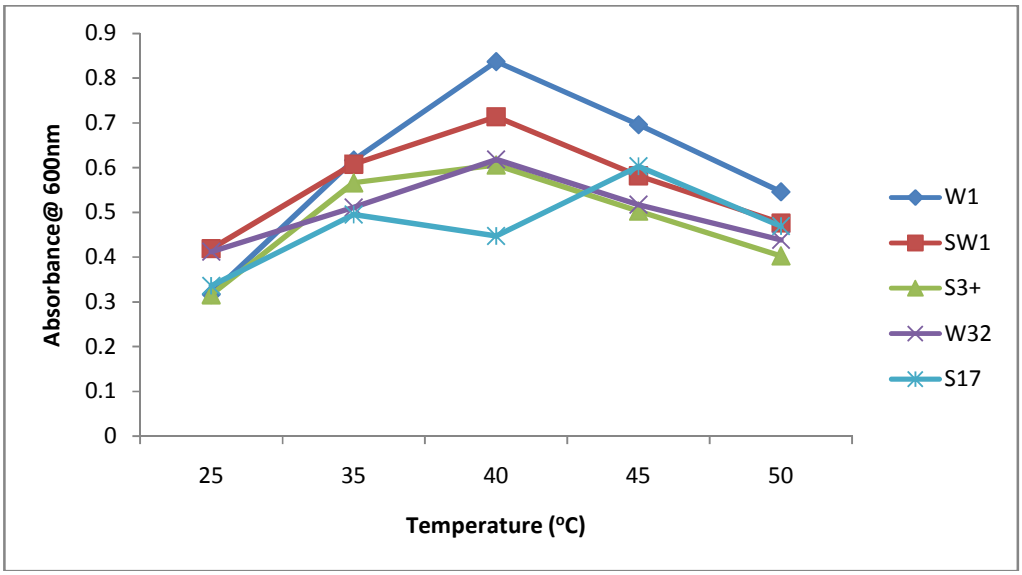

Fig. 11. Effect of temperature on hydrobarbon-utilizing potential of bacterial isolates
Key: W1= A. feacalis, SW1= Micrococcus luteus, SW32 =P. aeruginosa, SW3+ =Bacillus cereus, W17= B.lichenformis

4. CONCLUSION

The bacterial species isolated from bitumen-contaminated surface water in Agbabu can be harnessed in an attempt at developing strains that will be useful in environmental bioremediation of contaminated sites. However, effective utilization of crude-oil by these bacteria requires optimum environmental conditions such as pH, temperature and availability of nutrient.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/21372